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Cover: Detail of La Primavera (ca. 1475–1478) by Sandro Botticelli, used with permission of Uffizi Gallery, Florence, Italy.

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## The Cover

# Seasonal Variation in Host Susceptibility and Cycles of Certain Infectious Diseases

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Seasonal cycles of infectious diseases have been variously attributed to changes in atmospheric conditions, the prevalence or virulence of the pathogen, or the behavior of the host. Some observations about seasonality are difficult to reconcile with these explanations. These include the simultaneous appearance of outbreaks across widespread geographic regions of the same latitude; the detection of pathogens in the off-season without epidemic spread; and the consistency of seasonal changes, despite wide variations in weather and human behavior. In contrast, an increase in susceptibility of the host population, perhaps linked to the annual light/dark cycle and mediated by the pattern of melatonin secretion, might account for many heretofore unexplained features of infectious disease seasonality. Ample evidence indicates that photoperiod-driven physiologic changes are typical in mammalian species, including some in humans. If such physiologic changes underlie human resistance to infectious diseases for large portions of the year and the changes can be identified and modified, the therapeutic and preventive implications may be considerable.

From 1703 onward, the annual rise and fall of measles deaths in London was recorded in sufficient detail to allow for careful mathematical modeling in 1918 (1). Since then, surveillance for a variety of diseases has established that regular seasonal variation in incidence is the rule, rather than the exception, for acute infections. Seasonal variations should be distinguished from periodic large epidemics, as observed every 2 years for measles (2) or at less frequent and more irregular intervals for meningococcal meningitis (3) and rubella (4). This discussion will focus on the more robust annual cycle, which "locks in" large epidemics to the same time of year (3,4) and persists even after large epidemics have been eliminated by mass vaccination (2). The life cycles of pathogens spread by insect vectors or maintained in animal or environmental reservoirs add complexity because seasonal changes might influence not only the pathogen or human host but also the vector population and animal or environmental reservoir. Therefore, this discussion will focus on bacterial and viral pathogens maintained primarily by person-to-person spread.

The regular and predictable pattern of seasonal outbreaks dominates the epidemiology of many exclusively human pathogens (Figure 1). Different infections peak in each of the four seasons, but for each pathogen, the timing and characteristics of the annual outbreak are remarkably consistent from year to year. Other key observations have been made on the seasonality of infectious diseases, including the simultaneous onset of outbreaks in geographically remote areas and the persistence of pathogens in the off-season in the absence of epidemic spread (Table). In fact, latitude has a clear influence on the timing and magnitude of outbreaks of rotavirus infection (10), influenza (15), and poliomyelitis

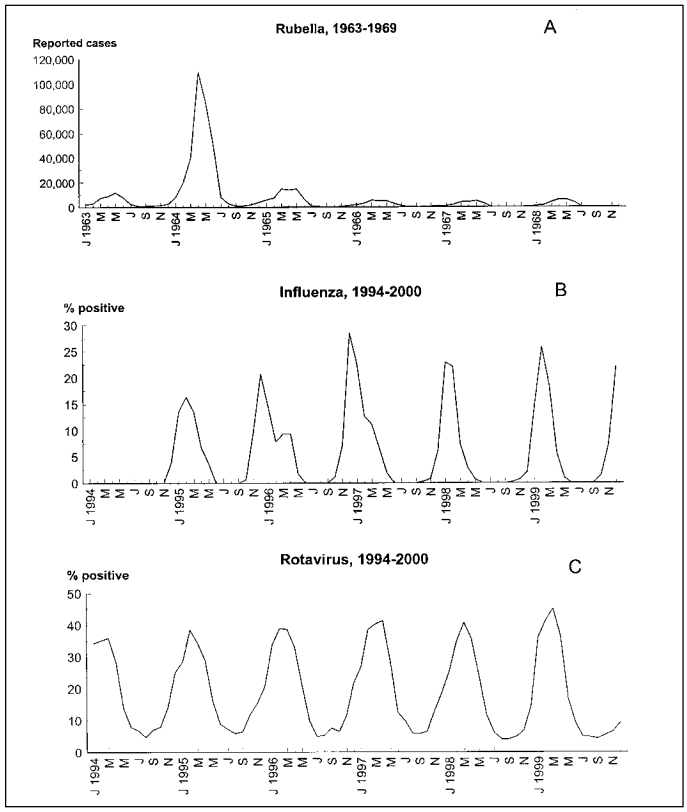


Figure 1. Seasonal variation in the occurrence of three human pathogens in the U.S. A: an annual cycle of rubella activity was maintained between larger epidemics, which occurred every 6 to 9 years. B: percentage of specimens testing positive for influenza viruses among specimens tested by World Health Organization and U.S. National Respiratory and Enteric Virus Surveillance System collaborating laboratories. C: a consistent pattern of rotavirus seasonality is evident in the U.S. National Respiratory and Enteric Virus Surveillance System. Adapted from references 4-6.

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Table. Observations on the seasonal occurrence of infectious diseases

| Observation | Examples |
|---|---|
| Pathogens peak at characteristic times in all seasons of the year | Winter: influenza, pneumococcus, rotavirus<br>Spring: RSV, measles<br>Summer: polio, other enteroviruses<br>Fall: parainfluenza virus type 1 |
| Timing and duration of peaks for each pathogen are similar from year to year | Measles: regular pattern since 1703 (1)<br>Influenza: annual peak varies by only 5 to 10 weeks in the United States (6) |
| Onset of epidemics often occurs simultaneously in areas that are geographically dispersed and have different weather conditions and diverse populations | Influenza: simultaneous outbreaks across North America, 16 European countries, and 6 Chinese provinces (7)<br>Pneumococcus: simultaneous outbreaks in seven surveillance areas (8) |
| Latitude is a critical determinant of timing and magnitude of peaks | An increasing magnitude of seasonal peaks as distance from the equator increases has been documented for polio (9) and rotavirus (10) and reported for influenza (11). |
| Pathogens can be detected in the off-season despite lower incidence of disease and virtual absence of epidemics | Meningococcus: no decrease in carriage in the off-season, despite absence of epidemic disease (12)<br>RSV: sporadic summer viral isolation but no epidemic spread (13)<br>Influenza: sporadic summer isolation, occasional clusters of disease without epidemic spread (14) |

RSV = respiratory syncytial virus. RSV peaks in the winter or spring in the United States, depending on location. For simplicity, it is listed here as a spring pathogen.

(Figure 2) (9). Reconciling these observations with the consistent seasonality of clinical illness is a continuing challenge.

## Explanations of Seasonality

Because seasonal cycles of infectious diseases are so universal and no single theory has proved satisfactory, explanations about their cause abound. More than one explanation or combination of explanations may be true. Explanations can be grouped into three types: pathogen appearance and disappearance, environmental changes, and host-behavior changes.

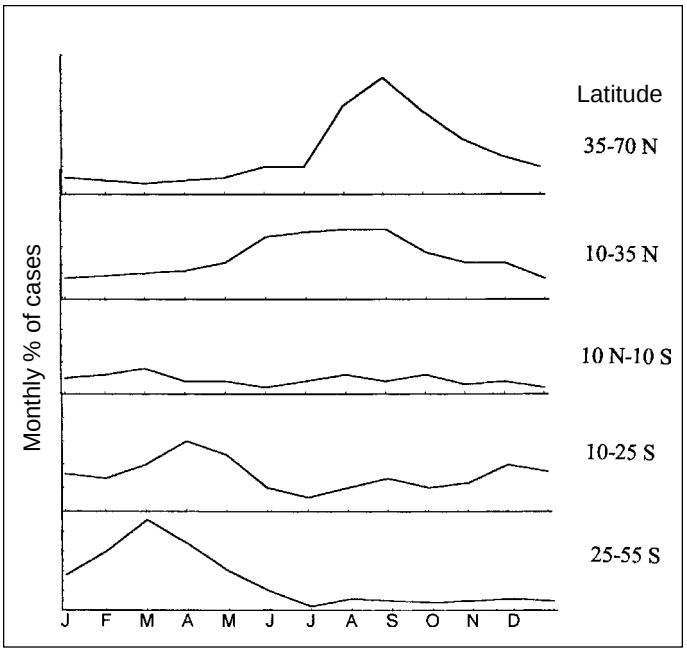


Figure 2. Seasonal variation in the incidence of poliomyelitis by latitude, 1956-57. As distance from the equator increases, a higher proportion of cases are evident in summer and fall months. Adapted from reference 9.

### Pathogen Appearance and Disappearance

Perhaps the most obvious explanation for the absence of disease during a period is that the pathogen is also absent during the period. However, the regular annual migration of epidemics of influenza, poliomyelitis, and rotavirus infection from northern latitudes across the equator to southern ones and back does not necessarily imply that the pathogens themselves migrate in this way.

Current theory holds that influenza is maintained only by direct spread in a series of chains of transmission from one ill person to another (16). Some evidence suggests that influenza viruses do spread geographically, particularly during pandemics, but whether geographic spread accounts for the patterns observed in annual outbreaks has been questioned (11,17,18). The simultaneous onset of geographically widespread outbreaks is difficult to reconcile with chains of person-to-person transmission. One hypothesis is that earlier "seeding" of the virus throughout the population must have occurred (17). During an 1826 influenza epidemic, one observer wrote, "...this epidemic affects a whole region in the space of a week, nay, a whole continent as large as North America, together with all the West Indies, in the course of a few weeks, while the inhabitants could not within so short a time have had any communication or intercourse whatever across such a vast extent of country" (11). A more recent hypothesis attributes geographic spread to the atmospheric dispersion of virus from Southeast Asia by trans-Pacific winds across the North American continent (18).

### Environmental Changes

Environmental changes, particularly changes in weather, are the explanations most often invoked for the seasonality of infectious diseases. Statistically significant correlations between epidemic cycles and cycles of temperature (19-22), humidity (21-23), rains (24), or winds (24) have been identified. However, correlations may be found with confounders as well as with causes.

In some cases, the association with weather is supported, but the biologic plausibility appears tenuous. Although the

seasonal incidence of poliomyelitis correlated quite well with the summer increase in relative humidity in Boston and Houston from 1942 to 1951 (23), the explanation that aerosolized poliovirus survives for a longer time at higher relative humidity is difficult to reconcile with the fecal-oral route of poliovirus transmission.

In other cases, the correlations are supported by biologic plausibility but are not consistently observed. In sub-Saharan Africa, the onset of meningococcal epidemics closely followed the season of dry winds and ended with the onset of the rains (25). It has been proposed that drying of mucosal surfaces increases the probability of bacteremic spread and that the rains moisten the mucosa or decrease the spread of the organism by dust. However, in Oregon and other areas, meningococcal disease peaks during the rainy season (26). Similarly, a significant correlation between the onset of the invasive pneumococcal disease season and a drop in mean daily temperatures below 24°C in Houston (19) was not confirmed in seven other areas with more widely varying weather patterns (8). Respiratory syncytial virus epidemics occur in the colder months of winter and spring in the United States (13) but paradoxically are significantly correlated with the hotter months in Singapore and Hong Kong (21,22).

### Host-Behavior Changes

Seasonal changes in poliomyelitis, measles, and other seasonal infectious diseases have been attributed to changes in the behavior of the host. Public swimming pools were a source of great concern during the polio epidemics of the 1950s, and summer peaks in polio and other enteroviruses were attributed to swimming (23,27,28). Subsequent studies discounted the importance of swimming in the spread of enterovirus infections (28).

Crowding of susceptible persons is one of the most common explanations for seasonal infectious diseases, and it certainly has biologic plausibility. The seasonal patterns of measles in England and Wales have been attributed to the timing of school holidays (29,30). Although such explanations are plausible, one must also ask why influenza outbreaks do not occur in crowded international conventions during summer, and why measles outbreaks are not common at summer camps. As one authority noted regarding meningococcal seasonality, "The story that African epidemics are caused by people crowding together at night during the dry season is a medical myth which is difficult to kill. Villagers sleep inside at the height of the rainy season at least as frequently as during the cold part of the dry season..." (24).

Comprehensive explanations of seasonal changes in infectious diseases should identify the means by which similar pathogens peak at different seasons (with characteristic timing and duration) and explain the prompt regionwide epidemics in geographically dispersed populations, the variation in epidemic patterns by latitude, and the persistence of the pathogen in the off-season without epidemic disease (Table).

## The Proposed Hypothesis

Regular annual variations in the incidence of many infectious diseases may be due to changes in susceptibility of the human host to the particular pathogen. Like the seasonal physiologic cycles of many mammalian species, these changes in susceptibility may be timed to the light/dark cycle, typically mediated by changes in the duration of the daily melatonin pulse. The changes in susceptibility may be distinct for different pathogens and may cover a broad range of possibilities, including (but not limited to) changes in the characteristic of mucosal surfaces, the expression of epithelial receptors, the leukocyte numbers or responsiveness, or other features of specific or nonspecific immunity.

This hypothesis would predict that pathogens do not physically migrate across the equator and that nationwide epidemics do not necessarily result from chains of person-to-person transmission. Rather, the pathogens may be present in the population year-round, and epidemics occur when the susceptibility of the population increases enough to sustain them. Perhaps the most significant prediction is that people are relatively resistant to disease if exposed in the off-season and that the specific physiologic process leading to seasonal resistance should be identifiable and perhaps modifiable.

## Seasonal Changes in Host Physiology

Many mammalian species undergo seasonal physiologic changes. The best characterized are changes in reproductive organs and other tissues seen in animals that are seasonal breeders. Humans are not seasonal breeders, but fertility has seasonal variations. Seasonal variations have been documented in other physiologic processes and immunologic features (31,32).

Producing offspring in a season during which food is unavailable and the environment is unsuitable for the young is an evolutionary dead-end for some species, leading to carefully regulated breeding seasons for many rodents (33), sheep (34), other ungulates (35), monkeys (36), and primates (37). Seasonal physiologic changes involve not just behavior but also the secretion of sex hormones and the size and function of reproductive organs. In controlled laboratory conditions, the duration of the light/dark cycle is the key parameter governing these seasonal changes, which can be completely replicated by artificial manipulation of the photoperiod. Photoperiod is most commonly used rather than temperature, humidity, food availability, or other seasonally varying parameters, presumably because its invariant nature best prevents accidental breeding at the wrong time of year. Under constant photoperiod, the physiologic changes can also be reproduced by controlling the duration of the daily melatonin pulse.

Seasonal physiologic changes have also been documented in processes not typically associated with breeding but potentially related to susceptibility to infectious agents. For example, even under constant conditions, red deer have distinct seasonal changes in digestive features (35), mice have seasonal changes in seizure threshold (38), and dairy cattle have seasonal changes in the fat and protein content of their milk (39). In recent years, seasonal changes in immunologic features have been documented. For example, Siberian hamsters exposed to short-day photoperiod demonstrate increased natural killer-cell activity and lymphocyte blastogenesis but decreased phagocytosis and oxidative burst activity by granulocytes (40); deer mice treated with melatonin in constant photoperiod exhibit increased lymphocyte response to mitogen stimulation (41).

A series of studies documented that the death rate in mice experimentally exposed to pneumococcal infection varied with the time of day (42-44). Survival patterns were altered by modifying environmental lighting conditions, rather than

feeding or activity, and susceptibility appeared related to the daily cycle of cortisone, although the specific physiologic feature responsible for increased susceptibility was not identified. Since these findings, understanding of the role of melatonin and its control of circadean and seasonal rhythms has increased greatly, but further studies of the influence of photoperiod on experimental pneumococcal infections in mice appear not to have been pursued.

Seasonal physiologic changes are not as well characterized for humans as for other mammals, but mounting data suggest that changes in photoperiod and the melatonin pulse may also influence human physiology (32). Blind people, who lack the capability for light to cue their biologic clocks, are often plagued by free-running circadian rhythms. A recent study demonstrated that these free-running rhythms can be entrained to a normal cycle by daily administration of melatonin (45). Although humans are sexually active year-round, a seasonal distribution in conceptions has consistently been demonstrated, and a variation in the ovulation rate has been postulated as the cause (31). Seasonal affective disorder, a well-characterized depression associated with short days and specific genetic defects (46), is treatable with extra hours of exposure to broad-spectrum light (47). Seasonal variations in heart attacks (48), breast cancer (49), and other seemingly noninfectious conditions have also been reported.

Recent research has focused on seasonal changes in immunologic values in humans. Specific melatonin receptors coupled with G-protein have been identified on lymphocytes (50). As in rodents, seasonal variations in lymphocyte mitogenic responses and in the quantity of circulating lymphocytes, neutrophils, CD4 and CD8 cells, and IL-6 have been reported (51-53). Some values, such as lymphocyte aryl hydrocarbon hydroxylase activity, peak in summer (54), while others, such as number of circulating B cells, peak in winter (52). Although statistically significant, the functional significance of these variations has not yet been established.

## Testing the Hypothesis

The above observations lend some biologic plausibility to the proposed hypothesis, but direct testing is needed. Several observations support the prediction that the host is less susceptible to infection or disease in the off-season.

In a double-blind placebo-controlled trial conducted in the Soviet Union during different seasons, nonimmune volunteers were given attenuated live influenza vaccine intranasally (55). Febrile reactions attributable to vaccine (calculated by subtracting the proportion of participants with reactions in the placebo group from the proportion in the vaccine group) were observed in 6.7% of 360 volunteers inoculated in Leningrad in January, compared with 0.8% of 197 inoculated in June ($p = 0.003$). Fourfold rises in antibody titer were seen in 31% to 40% in Krasnodar in January, depending on the vaccine strain, compared with 4.3% to 4.8% given the same strains in May and October (all $p < 0.001$). Similar trends with less significant differences were seen in three other cities.

Some years earlier, in a series of experiments on the transmission of influenza virus from infected to susceptible mice, <1% of mice exposed from July to October were infected, compared with 22% of those exposed in December or January ($p < 0.001$) (56). One year later, the investigators repeated the experiment with a different strain of mice, now kept under constant temperature and humidity, and observed that 34% were infected in May to October, compared with 58% in November to April ($p < 0.001$). The photoperiod conditions in these experiments were not noted.

It is not clear whether attempts were made to replicate these provocative experiments or if the potential importance of the observations was fully appreciated. The animal experiments may be relatively easy to confirm or refute, and the many live attenuated vaccines currently tested or used should provide ample material to evaluate the effects of season on immunogenicity or reactogenicity. The season of administration influences seroconversion rates to oral polio vaccine (57,58) and protection against polio (59), but much of this seasonal variation may be attributable to competition by other enteroviruses during summer (57). Vaccine-associated paralytic polio among vaccine contacts reflects the seasonal pattern of natural polio (60).

## Conclusion

Photoperiod-driven changes in host physiology might explain certain enigmatic observations about seasonality, but some observations remain unexplained. For example, the west-east movement of rotavirus is not easily attributable to host susceptibility changes timed to the light/dark cycle (5). The increase in hospitalizations coincident with warm weather and El Nino points to temperature rather than photoperiod as a key influence on some diarrheal disease pathogens (20). The sudden appearance and worldwide spread of a new pandemic strain of influenza virus also argues more for chains of transmission than for a crop of outbreaks from virus already present in the population.

Epidemiologists have long puzzled over why seasonal infectious disease outbreaks occur when they do. Perhaps the more important question is why they do not occur when they do not. Is the human population already relatively resistant for 6 to 9 months each year? If the absence of epidemics of summer influenza or winter polio is attributable to climate or weather, we may have little power to influence them. On the other hand, if these annual troughs are due to increased host resistance, opportunities abound for studying and modifying these changes. Such opportunities might include reviews of existing databases, careful evaluation of "experiments of nature," and studies in laboratory animals.

Databases surely exist that might shed light on this hypothesis. Clinical trials of live attenuated vaccines during the usual seasonal peak and seasonal trough for that particular disease could be reviewed for seasonal differences in reactogenicity and immunogenicity. Experiments of nature, in which groups adapted to summer come into contact with groups adapted to winter (as in a convention or a cruise ship with passengers from both Southern Hemisphere and Northern Hemisphere countries) and are exposed to a seasonal pathogen (such as influenza or an enterovirus), could be analyzed for differences in attack rate or clinical severity. Laboratory animals housed in photoperiod-controlled rooms could be exposed to seasonal pathogens and evaluated to see if photoperiod or melatonin modifies clinical and physiologic responses to infection. If differences are documented, the specific physiologic feature governing susceptibility changes could be isolated and identified.

It is time to have a closer look at these possible seasonal changes in host susceptibility and if they are confirmed,

identify and modify the physiologic changes underlying annual cycles of infectious diseases.

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Dr. Dowell is acting Associate Director for Global Health, National Center for Infectious Diseases, CDC. His research focuses on the epidemiology of infectious diseases.

### References

1. Brownlee J. An investigation into the periodicity of measles epidemics in London from 1703 to the present day by the method of the periodogram. Philosophical Transactions of the Royal Society of London 1918;B 208:225-50.
2. Anderson R, Grenfell BT, May RM. Oscillatory fluctuations in the incidence of infectious disease and the impact of vaccination: time series analysis. J Hyg Camb 1984;93:587-608.
3. Riedo F, Plikaytis B, Broome C. Epidemiology and prevention of meningococcal disease. Pediatr Infect Dis J 1995;14:643-57.
4. Witte J, Karchmer A, Case M, Herrmann KL, Abrutyn E, Kassanoff I, et al. Epidemiology of rubella. Am J Dis Child 1969;118:107-11.
5. Torok TJ, Kilgore PE, Clarke MJ, Holman RC, Bresee JS, Glass RI. Visualizing geographic and temporal trends in rotavirus activity in the United States, 1991 to 1996. National Respiratory and Enteric Virus Surveillance System Collaborating Laboratories. Pediatr Infect Dis J 1997;16:941-6.
6. Centers for Disease Control and Prevention. Update: Influenza activity—United States, 1999-2000 season. MMWR Morb Mortal Wkly Rep 2000;49:173-7.
7. Centers for Disease Control and Prevention. Update: Influenza activity—United States and worldwide, 1995-96 season, and composition of the 1996-97 influenza vaccine. MMWR Morb Mortal Wkly Rep 1996;45:326-9.
8. Dowell S, Whitney C, Wright C, Schuchat A. Seasonal changes in invasive pneumococcal disease. Emerg Infect Dis. In press 2001.
9. Paccaud M. World trends in poliomyelitis morbidity and mortality, 1951-1975. World Health Stat Quart 1979;32:198-224.
10. Cook S, Glass R, LeBaron C, Ho M-S. Global seasonality of rotavirus infections. Bull World Health Organ 1990;68:171-7.
11. Hope-Simpson R, Golubev D. A new concept of the epidemic process of influenza A virus. Epidemiol Infect 1987;99:5-54.
12. Blakebrough I, Greenwood B, Whittle H, Bradley A, Gilles H. The epidemiology of infections due to *Neisseria meningitidis* and *Neisseria lactamica* in a northern Nigerian community. J Infect Dis 1982;146:626-37.
13. Centers for Disease Control and Prevention. Update: respiratory syncytial virus activity—United States, 1998-1999 Season. MMWR Morb Mortal Wkly Rep 1999;48:1104-15.
14. Kohn M, Farley T, Sundin D, Tapia R, McFarland L, Arden N. Three summertime outbreaks of influenza type A. J Infect Dis 1995;172:246-9.
15. Cox NJ, Fukuda K. Influenza. Infect Dis Clin N Am 1998;12:27-38.
16. Cox N, Subbarao K. Influenza. Lancet 1999;354:1277-82.
17. Langmuir A, Schoenbaum S. The epidemiology of influenza. Hosp Pract 1976;11:49-56.
18. Hammond G, Raddatz R, Gelskey D. Impact of atmospheric dispersion and transport of viral aerosols on the epidemiology of influenza. Rev Infect Dis 1989;11:494-7.
19. Kim P, Musher D, Glezen W, Rodriguez-Barradas M, Nahm W, Wright C. Association of invasive pneumococcal disease with season, atmospheric conditions, air pollution, and the isolation of respiratory viruses. Clin Infect Dis 1996;22:100-6.
20. Checkley W, Epstein L, Gilman R, Figueroa D, Cama RI, Patz JA. Effects of El Niño and ambient temperature on hospital admissions for diarrheal diseases in Peruvian children. Lancet 2000;355:442-50.
21. Chew F, Doraisingham S, Ling A, Kumarasinghe G, Lee B. Seasonal trends of viral respiratory tract infections in the tropics. Epidemiol Infect 1998;121:121-8.
22. Sung R, Murray H, Chan R, Davies D, French G. Seasonal patterns of respiratory syncytial virus infection in Hong Kong: a preliminary report. J Infect Dis 1987;156:527-8.
23. Nathanson N, Martin J. The epidemiology of poliomyelitis: enigmas surrounding its appearance, epidemicity, and disappearance. Am J Epidemiol 1979;110:672-92.
24. Greenwood B. The epidemiology of acute bacterial meningitis in tropical Africa. Bacterial Meningitis. London: Academic Press; 1987. p. 61-91.
25. Greenwood B, Blakebrough I, Bradley A, Wali S, Whittle H. Meningococcal disease and season in sub-Saharan Africa. Lancet 1984;i:1339-42.
26. Diermayer M, Hedberg K, Hoesly F, Fischer M, Perkins B, Reeves M, et al. Epidemic serogroup B meningococcal disease in Oregon: the evolving epidemiology of the ET-5 strain. JAMA 1999;281:1493-7.
27. D'Alessio D, Minor T, Allen C, Tsiatis A, Nelson D. A study of the proportions of swimmers among well controls and children with enterovirus-like illness shedding or not shedding an enterovirus. Am J Epidemiol 1981;113:533-41.
28. Hawley H, Morin D, Geraghty M, Tomkow J, Phillips C. Coxsackievirus B epidemic at a boys' summer camp: isolation of virus from swimming water. JAMA 1973;226:33-6.
29. Hamer W. Epidemic disease in England—the evidence of variability and persistency of type. Lancet 1906;11:733-9.
30. Fine P, Clarkson J. Measles in England and Wales - I: an analysis of factors underlying seasonal patterns. Int J Epidemiol 1982;11:5-14.
31. Rojansky N, Brzezinski A, Schenker J. Seasonality in human reproduction: an update. Hum Reprod 1992;7:735-45.
32. Wehr T, Moul D, Barbato G, Giesen HA, Seidel JA, Barker C, et al. Conservation of photoperiod-responsive mechanisms in humans. Am J Physiol 1993;265:R846-57.
33. Carlson L, Zimmermann A, Lynch G. Geographic differences for delay of sexual maturation in *Peromyscus leukopus*: effects of photoperiod, pinealectomy, and melatonin. Biology of Reproduction 1989;41:1004-13.
34. Lincoln G. Reproductive seasonality and maturation throughout the complete life-cycle in the mouflon ram (*ovis musimon*). Anim Reprod Sci 1998;53:87-105.
35. Rhind S, McMillen S, Duff E, Hirst D, Wright S. Seasonality of meal patterns and hormonal correlates in red deer. Physiol Behav 1998;65:295-302.
36. Herndon J, Bein M, Nordmeyer D, Turner J. Seasonal testicular function in male rhesus monkeys. Horm Behav 1996;30:266-71.
37. Chan P, Hutz R, Dukelow W. Nonhuman primate in vitro fertilization: seasonality, cumulus cells, cyclic nucleotides, ribonucleic acid, and viability assays. Fertil Steril 1982;38:609-15.
38. Loscher W, Fiedler M. The role of technical, biological and pharmacological factors in the laboratory evaluation of anticonvulsant drugs. VI. Seasonal influences on maximal electroshock and pentylentetrazol seizure thresholds. Epilepsy Res 1996;25:3-10.
39. Sargeant J, Shoukri M, Martin S, Leslie K, Lissemore K. Investigating potential risk factors for seasonal variation: an example using graphical and spectral analysis methods based on the production of milk components in dairy cattle. Prev Vet Med 1998;36:167-78.
40. Yellon S, Fagoaga O, Nehlsen-Cannarella S. Influence of photoperiod on immune cell functions in the male Siberian hamster. Am J Physiol 1999;276:R97-102.
41. Demas G, Nelson R. Exogenous melatonin enhances cell-mediated, but not humoral, immune function in adult male deer mice (*Peromyscus maniculatus*). J Biol Rhythms 1998;13:245-52.
42. Feigin RD, San Joaquin VH, Haymond MW, Wyatt RG. Daily periodicity of susceptibility of mice to pneumococcal infection. Nature 1969;224:379-80.
43. Shackelford PG, Feigin RD. Periodicity of susceptibility to pneumococcal infection: influence of light and adrenocortical secretions. Science 1973;182:285-7.

44. Wongwiwat M, Sukapanit S, Triyanond C, Sawyer WD. Circadian rhythm of the resistance of mice to acute pneumococcal infection. Infect Immun 1972;5:442-8.
45. Sack R, Brandes R, Kendall A, Lewy A. Entrainment of free-running circadian rhythms by melatonin in blind people. N Engl J Med 2000;343:1070-7.
46. Sher L, Goldman D, Ozaki N, Rosenthal N. The role of genetic factors in the etiology of seasonal affective disorder. J Affect Disord 1999;53:203-10.
47. Eastman C, Young M, Fogg L, Liu L, Meaden P. Bright light treatment of winter depression: a placebo-controlled trial. Arch Gen Psychiatry 1998;55:883-9.
48. Pell J, Cobbe S. Seasonal variations in coronary heart disease. QJM 1999;92:689-96.
49. Ownby H, Frederick J, Mortensen R, Ownby D, Russo J. Seasonal variations in tumor size at diagnosis and immunological responses in human breast cancer. Invasion Metastasis 1986;6:246-56.
50. Calvo J, Rafil-El-Idrissi M, Pozo D, Guerrero J. Immunomodulatory role of melatonin: specific binding sites in human and rodent lymphoid cells. J Pineal Res 1995;18:119-26.
51. Boctor F, Charmy R, Cooper E. Seasonal differences in the rhythmicity of human male and female lymphocyte blastogenic responses. Immunol Invest 1989;18:775-84.
52. Maes M, Stevens W, Scharpe S, Bosmans E, De Meyer F, D'Hondt P, et al. Seasonal variation in peripheral blood leukocyte subsets and in serum interleukin-6, and soluble interleukin-2 and -6 receptor concentrations in normal volunteers. Experientia 1994;50:821-9.
53. Nelson R, Drazen D. Melatonin mediates seasonal adjustments in immune function. Reprod Nutr Dev 1999;39:383-98.
54. Paigen B, Ward E, Reilly A, Houten L, Gurtoo HL, Minowada J, et al. Seasonal variation of aryl hydrocarbon hydroxylase activity in human lymphocytes. Cancer Res 1981;41:2757-61.
55. Shadrin A, Marinich I, Taros L. Experimental and epidemiological estimation of seasonal and climato-geographical features of non-specific resistance of the organism to influenza. J Hyg Epidemiol Microbiol Immunol 1977;21:155-61.
56. Schulman J, Kilbourne E. Experimental transmission of influenza virus infection in mice. II. Some factors affecting the incidence of transmitted infections. J Exp Med 1963;118:267-75.
57. Swartz T, Skalska P, Gerichter C. Routine administration of oral polio vaccine in a subtropical area. Factors possibly influencing sero-conversion rates. J Hyg Camb 1972;70:719-26.
58. World Health Organization Collaborative Study Group on Oral Poliovirus V. Factors affecting the immunogenicity of oral poliovirus vaccine: a prospective evaluation in Brazil and the Gambia. J Infect Dis 1995;171:1097-106.
59. Deming M, Linkins R, Jaitch K, Hull H. The clinical efficacy of trivalent oral polio vaccine in the Gambia by season of vaccine administration. J Infect Dis 1997;175 s1:s254-7.
60. Schonberger L, McGowan JJ, Gregg M. Vaccine-associated poliomyelitis in the United States, 1961-1972. Am J Epidemiol 1976;104:202-11.

# *Cryptococcus neoformans* Infection in Organ Transplant Recipients: Variables Influencing Clinical Characteristics and Outcome

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Unique clinical characteristics and other variables influencing the outcome of *Cryptococcus neoformans* infection in organ transplant recipients have not been well defined. From a review of published reports, we found that *C. neoformans* infection was documented in 2.8% of organ transplant recipients (overall death rate 42%). The type of primary immunosuppressive agent used in transplantation influenced the predominant clinical manifestation of cryptococcosis. Patients receiving tacrolimus were significantly less likely to have central nervous system involvement (78% versus 11%, p =0.001) and more likely to have skin, soft-tissue, and osteoarticular involvement (66% versus 21%, p = 0.006) than patients receiving nontacrolimus-based immunosuppression. Renal failure at admission was the only independently significant predictor of death in these patients (odds ratio 16.4, 95% CI 1.9–143, p = 0.004). Hypotheses based on these data may elucidate the pathogenesis and may ultimately guide the management of *C. neoformans* infection in organ transplant recipients.

Invasive fungal infections have been reported in 5% to 59% of organ transplant recipients (1-4). Infections due to *Cryptococcus neoformans*, while less common than those due to *Candida* and mycelial fungi, are also an important posttransplant complication. The incidence of invasive candidiasis has declined in subsets of organ transplant recipients (e.g., liver transplant patients) as a result of fluconazole use and technologic advances in surgery (5). However, the risk factors and pathogenesis of *C. neoformans* infection in the transplant setting are poorly understood, and fluconazole prophylaxis is generally not used in the late posttransplant period when cryptococcal infections usually occur. Thus, the incidence and impact of cryptococcal infection in organ transplant recipients are unlikely to diminish in the foreseeable future. Indeed, as incidence of *C. neoformans* infection in HIV-infected patients has declined, organ transplant recipients have become the group of immunocompromised patients at highest risk for cryptococcosis. The overall death rate in transplant recipients with cryptococcal infection has been 20% to 100% (6-9). While the predictors of outcome in patients with *C. neoformans* have been well documented in nontransplant settings (10-12), predictors in transplant recipients are largely unknown. The unique neurotropism and predilection of *C. neoformans* to cause central nervous system (CNS) infections are well recognized; CNS has been the most common site for cryptococcal infections. However, 67% of our liver transplant recipients with cryptococcosis who received tacrolimus as primary immunosuppression had cutaneous or osteoarticular lesions; 17% had meningitis (8). Small sample size and lack of comparison with patients on other immunosuppressive regimens, however, precluded meaningful interpretation of these data.

Given the limited number of transplant recipients with *C. neoformans* infection at individual institutions, accumulating a sufficiently large sample was difficult, so we turned to reports and analyses of cases for valuable data. This review summarizes unique epidemiologic and clinical characteristics of *C. neoformans* in transplant recipients, as well as variables influencing the outcome of cryptococcal infections after transplantation.

## Methods

Cases of *C. neoformans* infection in transplant recipients were identified with a MEDLINE search through 1998 by cross-referencing the keywords "*Cryptococcus neoformans*" and "transplantation" or "transplant." Reference lists of original articles and textbooks were reviewed for additional cases. A patient was considered infected if *C. neoformans* was cultured from a clinical specimen in the presence of signs or symptoms of cryptococcus infection. The onset of infection after transplantation was determined on the basis of detailed case studies; summarized data providing only a mean or range for the group of transplant recipients were excluded. Cryptococcal infection was considered early-onset if it occurred within 12 months and late-onset if it occurred >12 months after transplantation. Predictors or risk factors for death were assessed only in detailed cases for which the variables to be analyzed were explicitly stated.

### Statistical Analysis

Patient demographic data were entered into the database PROPHET Statistics Version 5.0 (BBN Systems and

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Technologies, Cambridge, MA). The $\chi^3$ or the Fisher exact test was used to compare categorical variables. Continuous variables (e.g., time of onset) were compared by using the Student *t* test or the Mann-Whitney U test. Multiple comparisons were done by analysis of variance and the Kruskal-Wallis test. A multiple regression model was used to examine the risk factors for death.

## Results

A total of 178 cases of *C. neoformans* infection in organ transplant recipients were identified (1,6-9,13-56). Of these, 96 cases were individually detailed, and 82 were summarized in reports containing 2 to 22 cases. Of 178 cases, 145, 20, and 10 were in renal, liver, and heart transplant recipients, respectively. Three cases were reported in lung transplant recipients, and none were described in bowel or pancreas transplant recipients. Patients were 12 to 67 years of age (median 44 years); 78% were male. The mean incidence of *C. neoformans* infection was 2.8 per 100 transplants (0.3 to 5.3 per 100). The overall incidence was 2.4% in liver, 2.0% in lung, 3.0% in heart, and 2.8% in renal transplant recipients.

Of 127 transplant recipients who could be evaluated, 100 (79%) had azathioprine as the primary immunosuppressive agent, 9 (7%) had tacrolimus, 11 (9%) had cyclosporine, and 7 (6%) had cyclosporine and azathioprine. Of these 127 patients, 78 were also receiving prednisone in various dosages, 5 were not receiving prednisone, and data on prednisone use were unavailable for 44 patients. The incidence of cryptococcosis was 4.5 per 100 transplants in patients who received tacrolimus, 2.4 per 100 transplants in patients who received cyclosporine, and 3.4 per 100 transplants in patients who received azathioprine. These rates did not differ significantly. Rejection episodes preceding cryptococcal infection were documented in 17 (25%) of 67 patients; rejection had occurred a median of 7 months (from 5 days to 49 months) before onset of infection. Eleven (18%) of 62 patients had received augmented immunosuppression (predominantly corticosteroids) within 6 months of onset of cryptococcosis; two patients had received antilymphocyte preparations or OKT3 monoclonal antibodies for the treatment of allograft rejection.

### Time to Onset

Cryptococcosis occurred a median of 1.6 years (from 2 days to 12 years) after transplantation. Overall, 14 (15%) of 94 cases occurred within 3 months, 10 (11%) of 94 in 3 to 6 months, 15 (16%) of 94 in 6 to 12 months, and 55 (59%) of 94 >12 months after transplantation.

The time to onset varied significantly for different types of organ transplant recipients. The median time to onset after transplantation was 35 months for kidney, 25 months for heart, 8.8 months for liver, and 3 months for lung transplant recipients (p = 0.001). Overall, cryptococcosis developed in 100% of the lung, 75% of the liver, 33% of the heart, and 30% of the kidney transplant recipients within 12 months of transplantation (p = 0.002) (Table 1).

*C. neoformans* infection tended to occur later in patients who received azathioprine than in patients who received tacrolimus or cyclosporine (p = 0.16). The median time to onset was 11.4 months after transplantation in patients who received cyclosporine, 9.2 months in patients who received tacrolimus, and 27 months in patients who received only azathioprine-based immunosuppression (p = 0.16). Patients from the northeastern United States were more likely to have early-onset cryptococcosis (i.e., infection within 12 months of transplantation) than other patients (67% versus 31%, p = 0.004). Age, cytomegalovirus infection, or prior rejection episodes did not correlate with early- versus late-onset cryptococcal infection (Table 1).

Table 1. Variables associated with early and late-onset *Cryptococcus neoformans* infection in organ transplant recipients

| Variable (no. of patients for whom data available) | Early onset (within 12 months) (%) | Late onset (>12 months) (%) | p value |
|---|---|---|---|
| Mean age in yrs | 42.2 | 44.3 | NS[a] |
| Type of transplant | | | 0.001 |
| Liver (20) | 75 | 25 | |
| Kidney (54) | 28 | 72 | |
| Heart (9) | 33 | 67 | |
| Lung (2) | 100 | 0 | |
| Cytomegalovirus (CMV) infection (2) | 50 | 50 | NS |
| No CMV infection (6) | 67 | 33 | |
| Prior rejection (16) | 50 | 50 | NS |
| No prior rejection (45) | 36 | 64 | |
| U.S. region | | | 0.004[b] |
| Northeast (24) | 67 | 33 | |
| West (19) | 32 | 68 | |
| Midwest (9) | 22 | 78 | |
| South (20) | 40 | 60 | |
| Other countries | | | |
| Europe (7) | 28 | 71 | |
| Asia (3) | 33 | 67 | |
| Site involved | | | |
| Lung (26) | 42 | 58 | NS |
| Central nervous system (57) | 40 | 60 | |
| Skin/osteoarticular (23) | 30 | 70 | |
| Death rate (84) | 34 | 41 | |

[a]NS = not significant, p >0.05.
[b]Northeastern United States versus all other regions.

### Clinical Manifestations

Of 159 patients, 87 (55%) had *C. neoformans* infection at the CNS site only; 20 (13%) had skin, soft tissue, or osteoarticular infection only; and 10 (6%) had pulmonary infection only. One patient each had prostate gland infection, myositis, chorioretinitis, and isolated renal allograft involvement due to *C. neoformans* (14,15,19,32). In 38 (24%) of the 159 patients, more than one site of infection was documented: CNS in 115 (72%) of 159; pulmonary in 39 (25%) of 159; and skin, soft tissue, or osteoarticular involvement in 34 (21%) of 159 patients.

Patients receiving tacrolimus were significantly less likely to have CNS involvement than patients receiving nontacrolimus-based immunosuppression (78% versus 11%, p = 0.013). Skin, soft-tissue, or osteoarticular involvement was significantly more likely to occur with a tacrolimus- (66%) than with a nontacrolimus-based immunosuppressive regimen (21%, p = 0.006). When patients who received tacrolimus were compared with those who received cyclosporine, CNS involvement (1 [11%] of 9 versus 12 [67%] of 18, p = 0.01) was significantly lower, and skin, soft-tissue, or osteoarticular involvement was significantly higher with tacrolimus than with cyclosporine immunosuppressive therapy (6 [67%] of 9 versus 4 [22%] of 18, p = 0.04).

Positive blood cultures for *C. neoformans* were documented in 15 (38%) of 39 transplant recipients for whom blood cultures were performed. However, 32 (91%) of 35 patients for whom serum cryptococcal antigen was performed had a positive serum cryptococcal antigen of 1:2 to 1:8192 (median 1:256). Leukocytosis was largely absent, the mean peripheral leukocyte count of the patients in this review was 6,560/mm$^3$ (range 2,000 to 12,000/mm$^{3)}$. Sixty-eight (74%) of 91 patients were febrile.

### CNS Infection

Of 125 patients with CNS involvement (6,7,9,13,14,16,20,22,23,26,30,31,37-39,42,43,45-47,49-51,53,57), 122 (98%) had meningitis. Space-occupying lesions (contrast enhancing mass lesions) due to *C. neoformans* were present in three patients (7,23). Thirty-nine (62%) of 63 patients with CNS cryptococcosis had headache, 30 (48%) of 62 had confusion or lethargy, and 2 (1%) of 25 had coma on admission. Serum cryptococcal antigen was positive in 18 (86%) of 21 patients with CNS infection (median titer 1:256; range 1:4 to 1:4096). However, 100% of 37 patients had a positive CSF cryptococcal antigen (median titer 1:256; range 1:4 to 1:32,768). CSF cultures yielded *C. neoformans* in 76 (93%) of 82 patients, and India ink preparation was positive in 36 (77%) of 47 patients with CNS infection (Table 2).

Table 2. Cerebrospinal fluid (CSF) characteristics in organ transplant recipients with central nervous system *Cryptococcus neoformans* infection

| Variable (no. of patients whom data available) | Value[a] |
|---|---|
| Opening pressure, mm $H_20$ (17) | 330 (140-700) |
| Leukocytes, mm$^3$ (27) | 33 (0-485) |
| Protein, mg/dL (27) | 74 (16-715) |
| Glucose, mg/dL (27) | 36 (4 – 113) |
| No. with positive India ink | 80% (38/47) |
| No. with positive CSF cryptococcal antigen | 100% (27/27) |
| Titer, median (range) | 1:512 (1:4-1:32,768) |
| No. with positive CSF culture | 93% (76/82) |
| No. with positive serum cryptococcal antigen | 88% (14/16) |
| Titer, median (range) | 1:128 (1:4-1:4096) |

[a]Median and range unless otherwise stated.
[b]Numbers of patients for whom data were available.

### Pulmonary Infection

Unilateral, nodular, or cavitary infiltrates were the most frequent radiographic signs of pulmonary cryptococcosis (1,7,9,13,23,26,29,37-40,46,49,50,54-56). Pleural effusions were documented in 4 of 42 patients. Serum cryptococcal antigen was detectable in 100% of 12 patients with pulmonary lesions (titers of 1:4 to 1:8192).

### Skin, Soft Tissue, or Osteoarticular Infection

Seventy-two percent of patients with cutaneous cryptococcosis (6,9,13,16,17,21,22,25,27-29,35-37,40,44, 46,49,54-56,58) had cellulitis; *C. neoformans* was cultured from an aspirate or biopsy in all these cases. Other signs included papular or nodular lesions. Septic arthritis and osteomyelitis were documented in five cases. Nineteen (90%) of 21 patients with skin or osteoarticular cryptococcal infections had positive serum cryptococcal antigen.

### Death Rate

The overall death rate among organ transplant recipients with cryptococcal infection was 72 (42%) of 172. The death rate was 8 (40%) of 20 for liver, 57 (41%) of 139 for kidney, 6 (60%) of 10 for heart, and 1 (33%) of 3 for lung transplant recipients. Death rates did not differ between patients on tacrolimus and patients on other primary immunosuppressive regimens (33% versus 38%, p >0.05). CNS infection (p = 0.04), renal failure (defined as serum creatinine >1.5 mg/dL on admission, p = 0.005), and abnormal mental status (p = 0.03) were significant predictors of death in univariate analysis (Table 3). In logistic regression analysis (with the above variables in the model), only renal failure on admission was predictive of death (odds ratio 16.4; 95% CI 1.9 to 143; p = 0.004). The death rate was 25 (48%) of 52 in patients receiving amphotericin B deoxycholate, 29 (38%) of 77 in patients receiving amphotericin B plus 5 flucytosine, and 3 (21%) of 14 in patients receiving fluconazole (p = 0.16). Fluconazole, however, was less likely to be used in patients with CNS infection; 5% of patients with CNS compared with 23% of those with extraneural infection had received fluconazole (p = 0.01).

Forty-nine (49%) of 101 patients with CNS cryptococcal infection died. Of 79 patients with CNS infection who received an antifungal agent, 22 had received amphotericin B alone, 52 had received amphotericin B plus 5-flucytosine, and 5 had received fluconazole. Death rates did not differ between patients with CNS infection who received amphotericin B alone (59%) and patients with CNS infection who received amphotericin B plus flucytosine (44%). Abnormal mental

Table 3. Variables associated with death in organ transplant recipients with *Cryptococcus neoformans* infection

| Variable (no of patients for whom data available) | Death (%) | Survival (%) | p value |
|---|---|---|---|
| Mean age in yrs | 43.6 | 43.4 | NS[a] |
| Prior rejection (17) | 35 | 65 | NS |
| No rejection (50) | 28 | 72 | |
| Rejection within 6 months of onset of cryptococcosis (3) | 33 | 67 | NS |
| Increased immunosuppression (11) | 46 | 54 | NS |
| Fever (39) | 31 | 69 | NS |
| No fever (21) | 33 | 67 | |
| Renal failure (37) | 43 | 57 | 0.005 |
| No renal failure (18) | 6 | 94 | |
| Mental status | | | |
| Abnormal (22) | 54 | 45 | 0.03 |
| Normal (53) | 28 | 72 | |
| Treatment | | | NS |
| AmB[b] (52) | 48 | 52 | (0.16) |
| AmB + 5 FC[c] (77) | 38 | 62 | |
| Fluconazole (14) | 21 | 79 | |
| Site involved | | | 0.04[d] |
| Central nervous system (101) | 49 | 51 | |
| Pulmonary (32) | 22 | 78 | |
| Skin/osteoarticular (28) | 21 | 79 | |
| Type of transplant | | | NS |
| Liver (20) | 40 | 60 | |
| Kidney (139) | 41 | 59 | |
| Heart (10) | 60 | 40 | |
| Lung (3) | 33 | 67 | |

[a]NS = not significant, p >0.05.
[b]AmB = amphotericin B deoxycholate.
[c]FC = flucytosine.
[d]p value represents the difference for CNS versus other sites.

status and absence of headache (p = 0.07) correlated with poor outcome in patients with CNS cryptococcal infection (Table 4). Presence of fever, CSF pleocytosis, positive blood cultures, and CSF cryptococcal antigen titer did not correlate with outcome (Table 4).

Table 4. Variables associated with death in patients with central nervous system *Cryptococcus neoformans* infection

| Variable (no. of patients for whom data available) | Death (%) | Survival (%) | p value |
|---|---|---|---|
| Mean age in yrs | 40.6 | 42.4 | NS[a] |
| Fever (29) | 34 (10/29) | 66 (10/29) | NS |
| No fever (7) | 43 (3/7) | 57 (4/7) | |
| Headache (20) | 25 (5/20) | 75 (15/20) | NS |
| No headache (21) | 52 (11/21) | 48 (10/21) | (0.09) |
| Abnormal mental status (20) | 55 (11/20) | 45 (9/20) | NS |
| Normal mental status (26) | 31 (8/26) | 69 (18/26) | |
| White blood cell >20/mm$^3$ (20) | 40 (8/20) | 60 (12/20) | NS |
| White blood cell <20/mm$^3$ (13) | 62 (8/13) | 38 (5/13) | |
| Cryptococcal antigen titer ≥1,024 (10) | 20 (2/10) | 80 (8/10) | NS |
| Cryptococcal antigen titer <1,024 (17) | 35 (6/17) | 65 (11/17) | |
| Positive blood culture (8) | 13 (1/8) | 87 (7/8) | NS |
| Negative blood culture (16) | 50 (8/16) | 50 (8/16) | |
| Renal failure (22) | 54 (12/22) | 46 (10/22) | 0.011 |
| No renal failure (12) | 8 (1/12) | 92 (11/12) | |
| Therapy | | | NS |
| AmB[b] alone (55) | 47 (26/55) | 53 (29/55) | |
| AmB + 5 FC[c] (32) | 50 (16/32) | 50 (16/32) | |
| Fluconazole (5) | 40 (2/5) | 60 (3/5) | |

[a]NS = not significant, p >0.05.
[b]AmB = Amphotericin B deoxycholate.
[c]FC = flucytosine.

## Discussion

*C. neoformans* infection was documented in 2.8% of the organ transplant recipients, with an overall death rate of 42%. A number of findings in our study have previously not been fully appreciated in the context of cryptococcal infections after transplantation. For example, the type of primary immunosuppression after organ transplantation may influence the predominant clinical manifestation. Patients receiving tacrolimus were less likely to have CNS involvement and more likely to have skin, soft tissue, or osteoarticular involvement due to *C. neoformans* than patients who received nontacrolimus-based immunosuppression. Furthermore, both tacrolimus and cyclosporine were less likely to be associated with CNS involvement and more likely to be associated with cutaneous infection than azathioprine.

A number of biologic plausibilities exist for this observation. Tacrolimus is a natural macrolide antifungal product (59,60). Although its immunosuppressive effect outweighs its antifungal action in vivo, tacrolimus is toxic to *C. neoformans* in vitro by inhibition of calcineurin (59-61). Furthermore, tacrolimus suppresses the growth of *C. neoformans* at 37°C but not at 24°C, which suggests that the target of tacrolimus, calcineurin, is required at higher body temperatures (59,61). Thus, temperature-dependent inhibition of cryptococci by tacrolimus may prevent CNS infection but allow growth of fungus at cooler body sites, e.g., skin, soft tissue, and bone. Cyclosporine also possesses in vitro antifungal activity by inhibition of calcineurin (60,61). However, cyclosporine does not effectively penetrate the CNS, while tacrolimus crosses the blood-brain barrier (61,62). Thus, the relative rarity of meningitis compared with extraneural manifestations of cryptococcosis in patients receiving tacrolimus may merely be due to high cerebrospinal fluid levels of tacrolimus.

Strains of *C. neoformans* known to be selectively dermatotropic and rhinotropic have been demostrated in animal models (63,64). In addition, *C. neoformans* serotype D is more likely to be associated with cutaneous lesions (65). However, the precise reason for dermatotropism or the propensity of these strains to occur in transplant recipients receiving calcineurin-inhibiting agents (e.g., cyclosporine and tacrolimus) has not been elucidated.

The immunosuppressive agents (cyclosporine, tacrolimus, and rapamycin) have in vitro activity against fungi, including *C. neoformans* (59,61,66,67). The antifungal activity of cyclosporine and tacrolimus is mediated by fungal homologs of calcineurin and that of rapamycin through complexes with TOR kinase (61,66). Mutations in calcineurin A and B genes have been shown to confer resistance to cyclosporine and tacrolimus and in FKBP12 gene, to tacrolimus and rapamycin in vitro (66). In addition, TOR I mutants of cryptococci have been identified that are resistant only to rapamycin (66). Despite high seroprevalence of cryptococcal antibodies in early childhood (68), cryptococcal infection is rare in transplant recipients. These data suggest that the immunosuppressive agents currently used may be conferring some degree of protection against *Cryptococcus*. Whether *C. neoformans* infections in patients receiving these immunosuppressive agents represent breakthrough infections due to resistant mutants, however, remains to be determined.

Although the susceptibility of transplant recipients to *C. neoformans* is well recognized, it is not known whether cryptococcal infection in these patients is newly acquired or a reactivation of latent infection. That cryptococcal disease may be due to a reactivation of latent infection is suggested by the following observations in the nontransplant setting: 1) autopsy studies have documented pulmonary granulomas containing *C. neoformans* in patients who had no history of *C. neoformans* infection (69); 2) molecular typing in African patients residing in Europe indicated that cryptococcosis resulted from a reactivation of latent infection (70); 3) serologic evidence of *C. neoformans* infection was documented in most children in New York City in early childhood, even though symptomatic infections were rare (68).

We previously reported that transplant recipients from the northeastern United States were more likely to have cryptococcosis than transplant recipients from other regions of the United States (8). This review shows that cryptococcal infections in patients from the Northeast developed significantly earlier after transplantation than in other patients. Although, there is incontrovertible evidence of primary acquisition of cryptococcosis in isolated case reports (71), our data suggest that *C. neoformans* may have a predilection for certain geographic areas and that most cryptococcal infections in transplant recipients may result from a reactivation of latent infection.

Epidemiologic studies of *C. neoformans* have been hampered by lack of sensitive and specific immunologic tests to evaluate the prevalence of latent infection. New

immunoblotting assays (68,72), however, have unique implications not only for discerning whether cryptococcal infections result from reactivation or primary acquisition but also for identifying patients at high risk for reactivation or patients never exposed (who may therefore be vulnerable to primary infection).

The relative rarity of cryptococcal infections in pediatric organ transplant recipients has been noted (55). However, the precise reason for this is not known. If cryptococcosis represents reactivation of latent infection in a transplant setting and primary cryptococcal infection is acquired asymptomatically in childhood, it is plausible that pediatric transplant recipients may not yet have acquired the infection. *C. neoformans* infection is also strikingly rare in bone marrow transplant recipients, possibly because fluconazole prophylaxis is used widely for candidiasis or because thymic regeneration in bone marrow transplant recipients may render T cells more efficacious against cryptococci than T cells present in solid organ transplant recipients (Heitman J, pers. comm.).

Although various clinical manifestations have been described, molluscum contagiosum-like lesions are characteristic of cutaneous cryptococcosis in HIV-infected patients. In the transplant setting, cutaneous cryptococcal infection most frequently mimicked (and was clinically indistinguishable from) bacterial cellulitis. A unique propensity for the extremities to be the site of cutaneous cryptococcosis in transplant recipients was noted in this review; 94% of the patients with cutaneous *C. neoformans* infections had lesions on upper or lower extremities. Cutaneous cryptococcosis, however, represents disseminated infection and should be treated with systemic antifungal agents.

Elevated CSF pressure without evidence of obstructive hydrocephalus, believed to result from basilar meningitis and impaired reabsorption of CSF across arachnoid villi, has recently been recognized as an important complication of cryptococcal meningitis (73). HIV studies have shown that high baseline opening pressure in patients with cryptococcal meningitis correlated inversely and independently with survival. CSF opening pressure was recorded infrequently in organ transplant recipients. However, all 17 patients in whom such a measurement was conducted had intracranial pressure $\geq$140 mm of $H_2O$; the death rate in these patients was 8 (47%) of 17. These data underscore the need for assessing intracranial pressure in all patients with cryptococcal meningitis, including organ transplant recipients.

Overall, 72 (42%) of 172 of the transplant recipients with *C. neoformans* infection died. Preexistent renal failure was an independently significant predictor of death in transplant recipients with cryptococcosis. Renal failure has been proposed to increase the risk for cryptococcosis (62). Uremia decreased lymphocyte transformation and chemiluminescence by splenic cells in *C. neoformans*-infected mice (74).

This review summarizes the overall impact and highlights the key features of *C. neoformans* infection in organ transplant recipients. These include the effect of primary immunosuppressive agents on the clinical manifestations of cryptococcosis; geographic diversity in the incidence and onset of infection posttransplantion; and variables influencing outcome, specifically in the transplant setting. More importantly, however, we have identified a number of outstanding questions with implications relevant to elucidating the pathogenesis of *C. neoformans* infection. These questions involve the biologic basis of tissue tropism, reasons for the predominance of dermatotropic strains in recipients of tacrolimus, the role or virulence of immunosuppressive-agent resistant *C. neoformans* mutants in the transplant setting, and the relative rarity of cryptococcal infections in pediatric and bone marrow transplant recipients. We caution that a retrospective study may carry unknown bias. In this regard, our data may be considered hypotheses generating.

Dr. Husain is an infectious diseases fellow at the University of Pittsburgh Medical Center. His research interests include infections in immunocompromised hosts, in particular fungal infections in organ transplant recipients.

## References


1. Kanj SS, Welty-Wolf K, Madden J, Tapson V, Baz MA, Davis D, et al. Fungal infections in lung and heart-lung transplant recipients, report of 9 cases and review of the literature. Medicine 1996;75:142-56.
2. Paterson DL, Singh N. Invasive aspergillosis in transplant recipients. Medicine 1999;78:123-32.
3. Kusne S, Furukawa H, Abu-Elmagd K, Irish W, Rakela J, Fung J, et al. Infectious complications after small bowel transplantation in adults: an update. Transplant Proceed 1996;5:2761-2.
4. Benedetti E, Gruessner A, Troppmann C, Papalois BE, Sutherland DER, Dunn DL, et al. Intra-abdominal fungal infections after pancreatic transplantation: incidence, treatment, and outcome. J Am Coll Surg 1996;183:307-16.
5. Singh N. Antifungal prophylaxis in organ transplant recipients: seeking clarity amidst controversy. Clin Infect Dis 2000;31:545-53.
6. Chugh KS, Sakhuja V, Jain S, Singh V, Tarafdar A, Joshi K, et al. Fungal infections in renal allograft recipients. Transplant Proc 1992;24:1940-2.
7. Jabbour N, Reyes J, Kusne S, Martin M, Fung J. Cryptococcal meningitis after liver transplantation. Transplantation 1996;61:146-67.
8. Singh N, Gayowski T, Wagener MM, Marino IR. Clinical spectrum of invasive cryptococcosis in liver transplant recipients receiving tacrolimus. Clin Transplant 1997;11:66-70.
9. Carlson KC, Mehlmauer M, Evans S, Chandrasoma P. Cryptococcal cellulitis in renal transplant recipients. J Am Acad Dermatol 1987;17:469-72.
10. Saag MS, Powderly WG, Cloud GA, Robinson P, Grieco MH, Sharkey PK, et al. Comparison of amphotericin with fluconazole in the treatment of acute AIDS-associated cryptococcal meningitis. N Engl J Med 1992;326:83-9.
11. Zuger A, Louie E, Holzman RS, Simberkoff MS, Rahal JJ. Cryptococcal disease in patients with the acquired immunodeficiency syndrome. Diagnostic features and outcome of treatment. Ann Intern Med 1986;104:234-40.
12. Chuck SL, Sande MA. Infection with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. N Engl J Med 1989;321:794-9.
13. Shaariah W, Morad Z, Suleiman AB. Cryptococcosis in renal transplant recipients. Transplant Proc 1992;24:1898-9.
14. Biswas J, Gopal L, Sharma T, Parikh S, Madhavan HN, Badrinath SS. Recurrent cryptococcal choroiditis in a renal transplant patient. Retina 1998;18:273-6.
15. Scully RE, Mark EJ, McNeely WF, McNeely BU. Case records of the Massachusetts General Hospital. Case 7-1994. N Engl J Med 1994;330:490-6.
16. Parisi A, Sacchi P, Filice G. Treatment of cryptococcal meningitis in liver transplantation. Infection 1998;26:314-5.
17. Kaben U. Cryptoccosis of the skin. Hautarzt 1989;40:31-3.
18. Conti D, Tolkoff-Rubin NE, Baker GP Jr, Doran M, Cosimi AB, DelMonico F, et al. Successful treatment of invasive fungal infection with fluconazole in organ transplant recipients. Transplantation 1989;48:692-5.

19. O'Neil KM, Ormsby AH, Prayson RA. Cryptococcal myositis: a case report and review of the literature. Pathology 1998;30:316-7.
20. John GT, Mathew M, Snehaltha E, Anandi V, Date A, Jacob CK, et al. Cryptococcosis in renal allograft recipients. Transplantation 1994;58:855-6.
21. Sinott JT, Holt DA. Cryptococcal pyarthrosis complicating gouty arthritis. South Med J 1989;82:1555-6.
22. Leff RD, Smith EJ, Aldo-Benson MA, Arnoff GR. Cryptococcal arthritis after renal transplantation. South Med J 1981;74:1290.
23. Britt RH, Enzmann DR, Remington JS. Intracranial infection in cardiac transplant recipients. Ann Neurol 1981;9:107-19.
24. Dauber JH, Paradis IL, Dummer JS. Infectious complication in pulmonary alograft recipients. Clin Chest Med 1990;11:291-308.
25. Hall JC, Brewer JH, Crouch TT, Watson KR. Cryptoccal cellulitis with multiple sites of involvement. J Am Acad Dermatol 1987;17:329-32.
26. Jennings III HS, Bradsher RW, McGee ZA, Johnson HK, Alford RH. Acute cryptococcal cellulitis in renal transplant recipients. South Med J 1981;74:1150-3.
27. Shrader SK, Watts JC, Dancik JA, Band JD. Disseminated cryptococcosis presenting as cellulitis with necrotizing vasculitis. J Clin Microbiol 1986;24:860-2.
28. Anderson DJ, Schmidt C, Goodman J, Pomeroy C. Cryptococcal disease presenting as cellulitis. Clin Infect Dis 1992;14:666-72.
29. Gloster HM Jr, Swerlick RA, Solomon AR. Cryptococcal cellulitis in a diabetic, kidney transplant patient. J Am Acad Dermatol 1994;30:1025-6.
30. Hoston JR, Pedley TA. The neurological complications of cardiac transplantation. Brain 1976;99:673-94.
31. Kapoor A, Flenchner SM, O'Malley K, Paolone D, File TM Jr, Cutrona AF. Cryptococcal meningitis in renal transplant patients associated with environmental exposure. Transplant Infect Dis 1999;1:213-7.
32. Ooi HS, Chen BTM, Cheng HL, Khoo OT, Chan KT. Survival of a patient transplanted with a kidney infected with *Cryptococcus neoformans*. Transplantation 1971;11:428-9.
33. Beine JP, Lontie M, Vandenpitte J. Cryptococcal meningoencephalitis and 5-fluorocytosine. BMJ 1971;2:107.
34. Agut H. Puzzles concerning the pathogenicity of human herpesvirus-6 [Editorial]. N Engl J Med 1994;329:203-4.
35. Mayers DL, Martone WJ, Mandell GL. Cutaneous cryptococcosis mimicking Gram-positive cellulitis in a renal transplant patient. South Med J 1981;74:1032-3.
36. Lye WC, Chin NK, Lee YS. Disseminated cryptococcosis presenting with a pleural effusion in a kidney transplant recipient: early diagnosis by pleural biopsy and successful treatment with oral fluconazole. Nephron 1993;65:646.
37. Gallis HA, Berman RA, Cate TR, Hamilton JD, Caullie Gunnells J, Stickel DL. Fungal infection following renal transplantation. Arch Intern Med 1975;135:1163-72.
38. Mishima T, Kobayashi Y, Ohkubo M, Marumo F, Yoshimura H, Uchida H, et al. A case of renal transplant recipient complicated with cryptococcosis and amphotericin B induced acute tubular necrosis. Jpn Circ J 1977;41:1009-13.
39. Bach MC, Sahyoun A, Adler JL, Schlesinger RM, Breman J, Madras P, et al. High incidence of fungus infections in renal transplantation patients treated with antilymphocyte and conventional immunosuppression. Transplant Proc 1973;5:549-53.
40. Tipple J, Haywood H, Lee HM, Duma RJ. Cryptoccosis in renal transplant patients. Proc Clin Dial Transplant Forum 1976;6:13-9.
41. Murphy JF, McDonald FD, Dawson M, Reite A, Turcotte J, Fekety R Jr. Factors affecting the frequency of infection in renal transplant recipients. Arch Intern Med 1976;136:670-7.
42. Duston M, McHenry MC, Braun WE, Fieker DH, Gavan TL, Novick AC. Cryptococcal meningitis causing fever of unknown origin in renal transplant recipients. Report of two cases initially diagnosed by urine cultures. Transplantation 1981;32:334-6.
43. Krajewski S. Cryptococcal meningoencephalitis as a resultof long-term immunosuppression after kidney transplantation. Neuropathol Pol 1982;20:495-503.
44. Marcus JR, Hussong JW, Gonzalez C, Dumanian A. Risk factors in necrotizing fascitis: a case involving *Cryptococcus neoformans*. Ann Plast Surg 1998;40:80-3.
45. Tilney N, Kohler TR, Strom TB. Cerebralmeningitis in immunosuppressed recipients of renal allografts. Ann Surg 1982;195:104-9.
46. Watson AJ, Whelton A, Russell RP. Cure of cryptococcemia and preservation of graft function in a renal transplant recipient. Arch Intern Med 1984;144:1877-8.
47. Schroter GPJ, Temple DR, Hunsbert BS, Weill R III, Starzl TE. Cryptoccosis after renal transplantation: report of ten cases. Surgery 1976;79:268-77.
48. Plunkett JM, Turner BI, Tallent MB, Johnson K. Cryptococcal septicemia associated with urologic instrumentation in a renal allograft recipient. J Urol 1981;125:241-2.
49. Nampoory MRN, Khan ZU, Johny KV, Constandi JN, Gupta RK, Al-Muzairi I, et al. Invasive fungal infections in renal transplant recipients. J Infect 1996;33:95-101.
50. Horrevorts AM, Huysmans FTM, Koopman RJJ, Meis JFGM. Cellulitis as first clinical presentation of disseminated cryptococcosis in renal transplant recipients. Scand J Infect Dis 1994;26:623-6.
51. Hellman RN, Hinrichs J, Sicard G, Hoover R, Golden P, Hoffsten P. Cryptococcal pyelonephritis and disseminated cryptococcosis in a renal transplant recipient. Arch Intern Med 1981;141:128-30.
52. Page B, Thervet E, Legendre C, Kreis H. Cryptococcosis after renal transplantation. Transplant Proc 1999;27:1732.
53. van den Elshout FJJ, Huysmans FThM, Muytjens HL, Koene RAP. *Cryptococcus neoformans* meningitis following renal transplantation. Neth J Med 1987;31:183-90.
54. Conces DJ, Vix VA, Tarver RD. Pleural cryptococcosis. J Thorac Imag 1990;5:84-6.
55. Singh N, Rihs JD, Gayowski T, Yu VL. Cutaneous cryptococcosis mimicking bacterial cellulitis in a liver transplant recipient: case report and review in solid organ transplant recipients. J Clin Transplant 1994;8:365-8.
56. Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. N Engl J Med 1999;340:1981-8.
57. Valero G, Graybill JR. Successful treatment of cryptococcal meningitis with amhotericin B colloidal dispersion: report of four cases. Antimicrob Agent Chemother 1995;39:2588-90.
58. Granier F, Kanitakis J, Hermier C, Zhu YY, Thivolet J. Localized cutaneous cryptococcosis successfully trated with ketoconazole. J Am Acad Dermatol 1987;16:243-9.
59. Odom A, Del Poeta M, Perfect J, Heitman J. The immunosuppressant FK506 and its nonimmunosuppressive analog L-685, 818 are toxic to *Cryptococcus neoformans* by inhibition of a common target protein. Antimicrob Agent Chemother 1997;41:156-61.
60. Cardenas ME, Muir RS, Breuder T, Heitman J. Targets of immunophilin-immunosuppressant complexes are distinct highly conserved regions of calcineurin A. EMBO J 1995;14:2772-83.
61. Cruz MC, Del Poeta M, Wang P, Wenger R, Zenke G, Quesniaux VFJ, et al. Immunosuppressive and nonimmunosuppressive cyclosporine analogs are toxic to the opportunistic fungal pathogen *Cryptococcus neoformans* via cyclophilin-dependent inhibition of calcineurin. Antimicrob Agent Chemother 2000;44:143-9.
62. Perfect JR. Cryptococcosis. Infect Dis Clin North Am 1989;3:77-102.
63. Van Custem J, Fransen J, Van Gerven E, Janssen PAJ. Experimental cryptococcosis: dissemination of *Cryptococcus neoformans* and dermatropism in guinea pigs. Mykosen 1986;29:561-5.
64. Dixon DM, Polak A. *In vivo* and *in vitro* studies with atypical, rhinotropic isolate of Cryptococcus neoformans. Mycopathologia 1986;96:33-40.
65. Dromer F, Mathoulin S, Dupont B, Letenneur L, Ronin O. Individual and environmental factors associated with infection due to *Cryptococcus neoformans* serotype D. Clin Infect Dis 1996;23:91-6.

66. Cruz MC, Cavallo LM, Gorlach JM, Cox G, Perfect JR, Cardenas ME, et al. Rapamycin antifungal action is mediated via conserved complexes with FKBP12 and TOR kinase homologs in *Cryptococcus neoformans*. Mol Cell Biol 1999;19:4101-12.
67. Lorens MC, Heitman J. TOR mutations confer rapamycin resistance by preventing interaction with FKBP12-rapamycin. J Biol Chemother 2000;270:27531-7.
68. Goldman DL, Khine H, Abadi J, Lindenberg DJ, Pirofski L, Niang R, et al. Serologic evidence for *Cryptococcus neoformans* infection in early childhood. Pediatrics. In press 2001.
69. Haugen RK, Baker RD. The pulmonary lesions in cryptococcosis with special reference to subpleural nodules. Am J Clin Pathol 1954;37:1381-90.
70. Garcia-Hermoso D, Janbon G, Dromer F. Epidemiological evidence for dormant *Cryptococcus neoformans* infection. J Clin Microbiol 1999;37:3204-9.
71. Nosanchuk JD, Shoham S, Fries BC, Shapiro DS, Levitz SM, Casadevall A. Evidence of zoonotic transmission of *Cryptococcus neoformans* from a pet cockatoo to an immunocompromised patient. Ann Intern Med 2000;132:205-8.
72. Chen L-C, Goldman DL, Doering T, Pirofski L, Casadevall A. Antibody response to *Cryptococcus neoformans* proteins in rodents and humans. Infect Immun 1999;67:2218-24.
73. Graybill JR, Sobel J, Saag M, van der Horst C, Powderly W, Cloud G, et al. Diagnosis and management of increased intracranial pressure in patients with AIDS and Cryptococcal meningitis. Clin Infect Dis 2000;30:47-54.
74. Fromtling RA, Fromtling AM, Staib S, Muller S. Effect of uremia on lymphocyte transformation and chemiluminescence by spleen cells of normal and *Cryptococcus neoformans*-infected mice. Infect Immun 1981;32:1073-8.

# PulseNet: The Molecular Subtyping Network for Foodborne Bacterial Disease Surveillance, United States

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PulseNet, the national molecular subtyping network for foodborne disease surveillance, was established by the Centers for Disease Control and Prevention and several state health department laboratories to facilitate subtyping bacterial foodborne pathogens for epidemiologic purposes. PulseNet, which began in 1996 with 10 laboratories typing a single pathogen (*Escherichia coli* O157:H7), now includes 46 state and 2 local public health laboratories and the food safety laboratories of the U.S. Food and Drug Administration and the U.S. Department of Agriculture. Four foodborne pathogens (*E. coli* O157:H7; nontyphoidal *Salmonella* serotypes, *Listeria monocytogenes* and *Shigella*) are being subtyped, and other bacterial, viral, and parasitic organisms will be added soon.

Molecular subtyping of bacterial isolates by characterization of proteins or nucleic acids has been successfully applied to aid epidemiologic investigations of foodborne disease outbreaks since the initial use of plasmid fingerprinting nearly 20 years ago (1,2). Since that time, several methods for identifying restriction fragment length polymorphisms on chromosomal DNA have been developed, and molecular subtyping has become an essential component of epidemiologic investigations of infectious diseases (3-10).

This widespread use of molecular typing has resulted in a plethora of techniques and protocols for subtyping even the same species of bacteria (11). Because each laboratory uses its own protocols for molecular typing and designations of patterns, the results cannot be compared with those of another laboratory, even if both laboratories have used essentially the same methods. This lack of comparability has greatly diminished the power of molecular subtyping methods.

In 1993, during the investigation of an *Escherichia coli* O157:H7 outbreak caused by contaminated hamburgers served in a fast-food restaurant chain in the western United States, Barrett et al. in our laboratory applied pulsed-field gel electrophoresis (PFGE) to characterize clinical and food isolates of *E. coli* O157:H7 and demonstrated its utility in outbreak investigations (12). Subsequently, our laboratory received numerous requests from state health departments for subtyping *E. coli* O157:H7. The demand soon overwhelmed our testing capacity, and delays in subtyping isolates meant that results were mostly useful only for laboratory confirmation of conclusions from epidemiologic investigations. We reasoned that decentralization of subtyping activities and transfer of standardized molecular subtyping methodology to public health laboratories should enable more timely subtyping of clinical and food isolates. One result would be information useful to epidemiologists while they were investigating outbreaks. In addition, routine subtyping of isolates of foodborne pathogenic bacteria received by public health laboratories should lead to identification of outbreaks not readily recognizable by other means. Use of standardized subtyping methods would allow isolates to be compared from different parts of the country, enabling recognition of nationwide outbreaks attributable to a common source of infection, particularly those in which cases are geographically separated.

In 1995, the Centers for Disease Control and Prevention (CDC), with the assistance of the Association of Public Health Laboratories (APHL), selected the state public health laboratories in Massachusetts, Minnesota, Washington, and Texas as area laboratories for a national molecular subtyping network for foodborne bacterial disease surveillance. This network later became known as PulseNet (13). Standardized PFGE typing and pattern analysis technology would be transferred to the area laboratories, which would assume responsibility for subtyping foodborne pathogenic bacteria from their states and providing subtyping service to neighboring states that requested assistance. At about the same time, CDC and five state health departments, as part of a response to emerging infectious disease threats (14), implemented an active foodborne disease surveillance program called FoodNet (15). The objectives of FoodNet were to accurately estimate the burden of foodborne disease in the United States, investigate the sources of infection in outbreaks and sporadic cases, and build public health infrastructure for dealing with emerging foodborne disease issues. In 1996, FoodNet included Minnesota, Oregon,

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Connecticut, and Georgia and selected counties in California. Participants in FoodNet recognized the advantages offered by PulseNet, and the public health laboratories in Oregon and Georgia began participating in PulseNet. The first 5-day workshop on standardized methods for PFGE for foodborne pathogenic bacteria was held in January 1996. By early 2000, PulseNet included 46 state public health laboratories, the public health laboratories in New York City and Los Angeles County, California, the U.S. Department of Agriculture's Food Safety and Inspection Service Laboratory (USDA-FSIS) and the U.S. Food and Drug Administration laboratories in the Center for Food Safety and Applied Nutrition (FDA-CFSAN) and Center for Veterinary Medicine (Figure 1). In addition, six provincial Canadian laboratories joined PulseNet in 1999-2000; their participation is coordinated through the National Laboratory for Enteric Pathogens, Canadian Science Centre for Human and Animal Health Winnipeg, Manitoba.

As PulseNet's capacity expands, the need for epidemiologic assessment of new information expands in parallel because timely evaluation of clusters identified by the network is critical and warranted. PulseNet's laboratory evaluation of isolates from clusters or outbreaks identified through routine epidemiologic surveillance has already demonstrated its value in early recognition of outbreaks and rapid identification of their sources. A welcome consequence is engendering close collaboration between epidemiologists and microbiologists throughout the public health system.

## Standard Protocols

During 1996 and early 1997, we evaluated the standard protocol for *E. coli* O157:H7 at participating PulseNet laboratories. The original protocol, similar to the one used by Barrett et al. (12), required 3 to 4 days of testing; it involved an overnight incubation for cell lysis and another for restriction of chromosomal DNA. A set of 64 *E. coli* O157:H7 strains was compiled to evaluate the reproducibility of DNA fingerprint patterns in different laboratories. This set was sent to participating laboratories, which were asked to type strains by using the standardized protocol and return the raw electronic images of PFGE patterns to a common CDC database for study. Data analysis showed that when the standardized protocol is strictly followed by participating laboratories, results are highly reproducible and DNA patterns generated at different laboratories can be compared (Table 1). Also included in this set were duplicates of nine isolates to assess intralaboratory reproducibility of PFGE patterns; the testing laboratories were unaware of the duplicate strains until results were analyzed and reported. For six of nine sets, all laboratories generated patterns that were exact matches within each set. For each of the three remaining sets of duplicates, one of seven laboratories did not generate an exact match but matched the duplicates at 95%-97% similarity.

## Standardized Equipment for Participating Laboratories

PulseNet laboratories use CHEF-DRII, CHEF-DRIII, or CHEF-Mapper (Bio-Rad Laboratories, Hercules, CA) for PFGE of restricted bacterial DNA. Although all three instruments can run PulseNet protocols, CHEF-Mapper allows greater flexibility in development of electrophoretic separation conditions and nonlinear ramping. After electrophoresis, the gels are stained with ethidium bromide, and PFGE patterns are digitized in a TIFF format (uncompressed .tif file) by using a Gel-Doc 1000 (replaced by Gel-Doc 2000; Bio-Rad Laboratories) or other image acquisition equipment capable of 768 x 640 pixels or higher resolution. Molecular Analyst Fingerprinting Plus with Data Sharing Tools (MAFP-DST; Bio-Rad Laboratories; sold as GelCompar in Europe) is the software program used by PulseNet laboratories for analysis of PFGE patterns. MAFP-DST is being replaced with BioNumerics software (Applied

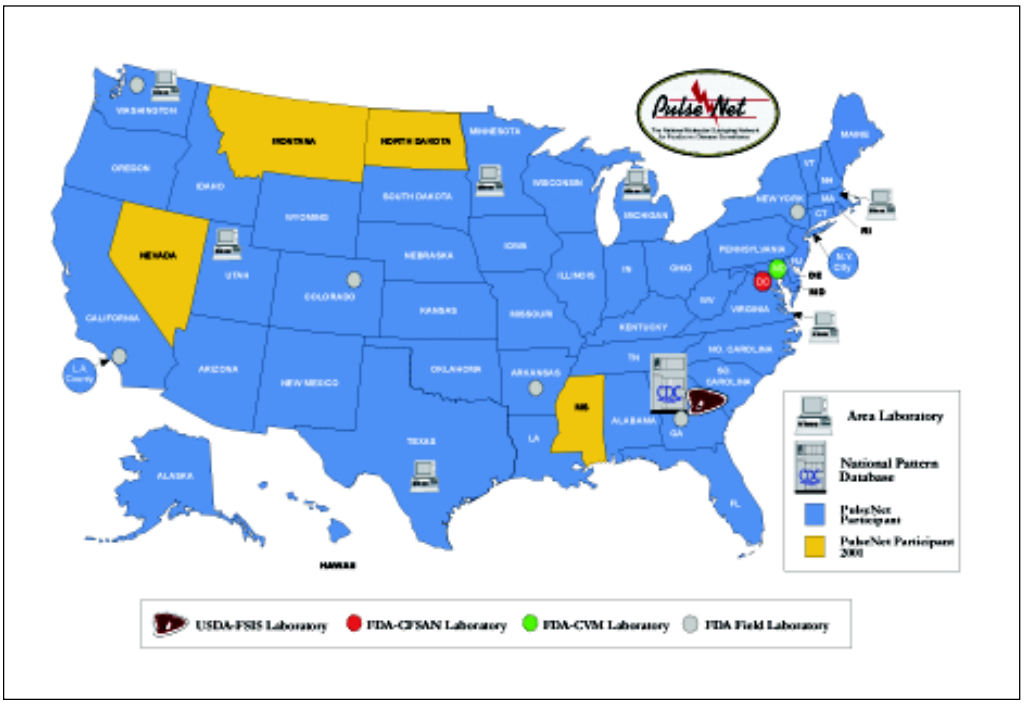


Figure 1. Locations of PulseNet laboratories in the United States. PulseNet participant states are currently participating. States labeled PulseNet participants 2001 are expected to complete the requirements for entry by December 2001. The area laboratories provide surge capacity and technical support to neighboring states. FDA-CFSAN: U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition Laboratory; FDA-CVM: U.S. Food and Drug Administration, Center for Veterinary Medicine Laboratory; USDA-FSIS: U.S. Department of Agriculture, Food Safety and Inspection Service Laboratory.

Table 1. Interlaboratory reproducibility of pulsed-field gel electrophoresis patterns of 64 *Escherichia coli* isolates by eight laboratories following the PulseNet standardized protocol

| Result | Laboratory (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Matched expected pattern | 64/64[a] (100) | 63/63 (100) | 59/63 (93.7) | 62/64 (96.9) | 62/64 (96.9) | 62/64 (96.9) | 61/64 (95.3) | 61/64 (95.3) |
| ≤ 1-band difference from expected pattern | 64/64 (100) | 63/63 (100) | 62/63 (98.4) | 64/64 (100) | 63/64 (98.4) | 64/64 (100) | 64/64 (100) | 64/64 (100) |

[a]Values represent no. of patterns fitting in the specified category/no. of isolates tested.

Maths, Kortrijk, Belgium); the change-over will be completed in 2001. Each PulseNet laboratory has all the above equipment and has the capability to normalize the patterns, compare them with other patterns, and maintain local databases of PFGE patterns for each bacterial pathogen of interest.

## National Database of PFGE Patterns and Associated Epidemiologic Information

A national database of PFGE patterns is being assembled for foodborne bacterial pathogens. These databases reside on a PulseNet server at CDC. For each bacterial pathogen, the normalized PFGE pattern is associated with a pattern database and a database of epidemiologic and clinical information for isolates. One isolate may be associated with more than one PFGE pattern in the database because PulseNet protocols may call for the use of more than one restriction enzyme to achieve appropriate discrimination between epidemiologically unrelated isolates. The *E. coli* O157:H7 database is functional; databases for nontyphoidal *Salmonella* serotypes and *Listeria monocytogenes* are under construction.

Seven PulseNet laboratories (four state public health laboratories, FDA-CFSAN, USDA-FSIS, and CDC) have direct access to the PulseNet database server through the Internet, enabling them to submit normalized PFGE patterns and associated epidemiologic information. (The DST version of the Molecular Analyst software creates a special "bundle" file for comparison with the national database.) Laboratories query the national database for identical matches or closely related patterns (>95% related under specified conditions). If identical or close matches to the submitted patterns are found, the submitting laboratory can access epidemiologic information associated with those patterns from the text database. When a PulseNet participating laboratory logs on to the PulseNet server, it will display a "recent match" message if two or more laboratories submit identical or closely related patterns within a specified time. This alert provides an early warning to PulseNet laboratories about possible multisite foodborne disease outbreaks.

PulseNet laboratories that do not yet have direct online access to the PulseNet server may still electronically submit raw TIFF images and normalized PFGE patterns (bundle files) to the PulseNet database administration team by e-mail or through file transfer protocols (ftp). The team compares the submitted patterns with those in the national database and e-mails the results to the submitting laboratory as quickly as possible. We expect that direct access to the PulseNet server will be available to all participating laboratories that have satisfactorily completed certification requirements by June 30, 2001.

## Developing Standardized Protocols

Standardized protocols for foodborne bacterial pathogens were developed in priority order based on the ability of PFGE to discriminate among strains of the organism and the epidemiologic utility of the resulting data. Standardized PFGE protocols have been developed for *E. coli* O157:H7, *Salmonella enterica* serotype Typhimurium, *L. monocytogenes*, and *Shigella* species. The *S.* Typhimurium protocol is applicable to most other nontyphoidal *Salmonella* serotypes, including *S.* Enteritidis. However, neither PFGE nor other molecular subtyping methods provide acceptable discrimination among strains of this highly clonal serotype. Standard PFGE protocols for *Campylobacter jejuni, C. coli*, and *Clostridium perfringens* (7) are being developed and validated. Although *C. jejuni* and *C. coli* infections are common, developing a standardized PFGE protocol for these organisms was not a high priority because they infrequently cause outbreaks. On the other hand, although outbreaks of *C. perfringens* infections are seldom widespread, state and local public health laboratories requested a standardized subtyping protocol to assist with local outbreak investigations. All PulseNet protocols are 1-day procedures based on the PFGE protocol developed by the Washington State Public Health Laboratory in response to the need for more rapid techniques (16). All new protocols and modifications of existing protocols are evaluated initially at the developing laboratory, followed by a second evaluation at CDC, alpha-testing at one or two PulseNet laboratories, and beta-testing at several PulseNet laboratories before they are adopted as official PulseNet protocols. Evaluation criteria include reproducibility of patterns, appropriateness of the strain used as the reference standard, and robustness of the procedure. Once a protocol is officially adopted, no changes can be made except by a petition to CDC's PulseNet Task Force, discussion of the proposed changes, and adoption of the proposal by PulseNet laboratories. The PulseNet Task Force at CDC is composed of personnel who carry out PulseNet-related activities. The Task Force members develop and evaluate protocols, provide technical support for participating laboratories, organize and conduct training workshops, administer the certification program and proficiency testing program, and maintain the national databases of PFGE patterns for the bacteria under surveillance in PulseNet.

## Quality Control and Assurance Program

A quality assurance program has been instituted for PulseNet to ensure the integrity of results obtained with the standardized PFGE techniques. This program requires strict adherence to the standardized PFGE protocols (17). In addition, the quality assurance program consists of standards for training, analytical procedures, documentation, and

equipment; standard operating procedures; an initial certification set of isolates for each organism; and an ongoing proficiency testing program. The standards detail the minimum requirements a laboratory must meet for training personnel, analytical procedures, documentation, equipment calibration and maintenance, proficiency testing, and review of results. The laboratory standard operating procedures describe procedures for record keeping, equipment maintenance, gel image acquisition, data analysis, and administrative policies. The certification sets consist of isolates with known patterns, which are sent to each laboratory. Laboratories type the isolates by the standardized protocol and send the gel images to the PulseNet National Database Administration Team for review. This team, part of the PulseNet Task Force, is responsible for maintaining and updating the PFGE pattern databases for foodborne disease-causing bacteria. Team members review new patterns submitted to the databases and verify matches. In addition, this team evaluates the certification data submitted by PulseNet laboratories. Laboratories with DST version of the MAFP software also analyze their gel images and send the results (bundle files) to the PulseNet National Database Administration Team for review. This team checks gel images and bundle files against the master certification set to ensure that the laboratory has obtained the correct patterns. Successful completion of the certification set allows PulseNet-affiliated laboratories to compare results with the National Database. As part of the proficiency testing program, laboratories will be sent a combination of isolates and TIFF files on a semiannual basis both to test the laboratory's ability to perform the standardized protocol correctly and to ensure that data analysis is consistent from laboratory to laboratory.

A quality assurance and control manual, being developed, will describe standardized training, laboratory and administrative procedures, and policies. A proficiency testing manual, also in preparation, is designed to maintain the reproducibility of patterns and consistency in analysis of patterns that make PulseNet a valuable ally for epidemiologists.

Laboratories joining PulseNet are sent the standardized PFGE protocols and certification sets appropriate to the organism(s) being tested. Appropriate training is scheduled and follow-up is provided by means of the certification sets and the regularly scheduled proficiency testing program. An annual meeting enables microbiologists from participating PulseNet laboratories to discuss new protocols and software upgrades and exchange information on problems and solutions.

## Standardized Nomenclature for PFGE Patterns

A major problem in comparing and interpreting molecular subtyping information from different laboratories has been the lack of a universal naming system for PFGE patterns. In response, we have developed a standardized nomenclature system for designating PFGE patterns in PulseNet. Each unique pattern in the database is represented by a 10-character code as follows:

XXXYYY.0000

The first three characters in the code represent the bacterial pathogen, the next three characters denote the enzyme used for DNA restriction, and the last four characters represent the pattern designation. For example, in the pattern designation EXHA26.0026, EXH represents *E. coli* O157:H7, A26 represents restriction endonuclease *Avr*II, and 0026 is the pattern number. Because the pattern numbers are assigned sequentially to unique patterns, no evolutionary, phylogenetic, or clonal relationships should be implied from the order of pattern numbers.

A priority order has been developed for inclusion of foodborne bacterial pathogens in PulseNet (Table 2). The prioritization takes into account the availability of an acceptable molecular subtyping method for a pathogen, severity of disease caused by that pathogen, propensity for the pathogen to cause outbreaks, and the potential for recognizing outbreaks and taking preventive action by routine subtyping.

Table 2. Priority order for inclusion of foodborne bacterial pathogens in PulseNet

| Pathogen | Expected year of inclusion |
|---|---|
| *Escherichia coli* O157:H7 | 1997 |
| Nontyphoidal *Salmonella* serotypes | 1998 |
| *Listeria monocytogenes* | 1999 |
| *Shigella sonnei* | 1999 |
| *Clostridium perfringens* | 2001 |
| *Campylobacter jejuni* | 2001 |
| *Vibrio parahaemolyticus* | 2001 |
| *V. cholerae* | 2001 |
| *Clostridium botulinum* | 2002 |
| Other pathogenic *E. coli* | 2002 |
| *Yersinia enterocolitica* | 2003 |

## Role of PulseNet in Outbreak Investigations

PulseNet plays several roles in detecting, investigating, and controlling outbreaks. Identification by PulseNet of an increase in a specific subtype of a pathogen may be an early indication of an outbreak. PFGE patterns submitted to the national database by participating laboratories may link apparently unrelated cases that are geographically dispersed. Once a cluster is detected through PulseNet, an epidemiologic investigation is initiated to determine if there is a common source. This epidemiologic investigation may be guided by the PFGE subtypes identified through PulseNet. PulseNet can identify outlier cases in other areas and define the geographic scope of the outbreak. If a common food source is identified and the pathogen is isolated from that food, subtyping helps confirm it as the outbreak strain. Finally, once control measures are instituted, PulseNet can help confirm that the outbreak is over by showing a substantial decrease in circulation of the outbreak strain in the affected communities. The following examples illustrate these PulseNet functions.

In 1996, epidemiologists at the Washington State and Seattle-King County health departments traced an outbreak of *E. coli* O157:H7 infections in four states and one Canadian province to commercial unpasteurized apple juice. Of 70 persons identified as part of this outbreak, 25 required hospitalization, 14 had hemolytic uremic syndrome, and one died. DNA fingerprinting by PFGE at the Washington State Public Health Laboratory, a PulseNet area laboratory, showed that isolates from patients and the apple juice were the same strain. Prompt recognition of the apple juice as the source of this outbreak resulted in rapid recall of the widely distributed product (18).

In 1997, the Colorado State Public Health Laboratory, which had just initiated PFGE typing of *E. coli* O157:H7, identified a cluster of 14 ill persons whose *E. coli* O157:H7 isolates had matching PFGE patterns. About the same time, the USDA laboratory isolated an *E. coli* O157:H7 strain from a ground beef patty from the same package as a patty eaten by an ill person. DNA fingerprinting by PFGE on the human isolate from Colorado and the food isolate from USDA-FSIS were generated by the PulseNet standardized protocol. The PFGE patterns were transmitted electronically to CDC via the Internet, where they were found to be indistinguishable. This outbreak pattern was then transmitted to PulseNet sites and compared with patterns from >300 other recent *E. coli* O157:H7 isolates. No matching patterns were found other than one case in Kentucky, providing strong evidence that the outbreak was not nationwide.

In May 1998, PulseNet facilitated the investigation of two clusters of *E. coli* O157:H7 infections in the northeastern United States. Timely fingerprinting of *E. coli* O157:H7 isolates by the Massachusetts Area Laboratory for PulseNet and other PulseNet laboratories in that region revealed two simultaneous clusters of *E. coli* O157:H7 infections (32 isolates in four of five states with one PFGE pattern and 25 isolates in all of five states with a second PFGE pattern), one of which could be traced to two supermarkets that received ground beef from the same distributor. Without assistance from PulseNet, epidemiologists would have found it difficult to identify cases associated with each cluster.

Also in May 1998, the state public health departments in both Illinois and Pennsylvania informed CDC about increases in *Salmonella* Agona infections. Serotype-specific surveillance data from other states quickly confirmed that 10 states had increases in *S*. Agona infections. A national outbreak of *S*. Agona was occurring, with no obvious source. Subsequently, the outbreak was traced to contaminated ready-to-eat toasted oats cereal product from a food-processing facility in Minnesota (19). PulseNet laboratories helped in this investigation by distinguishing cases that were associated with the outbreak from those that were not. In addition, timely PFGE typing of *S*. Agona by PulseNet laboratories helped identify outbreak-associated cases in states where the contaminated product was not initially thought to have been distributed. PFGE subtyping of *S*. Agona isolates was important in confirming the successful control of the outbreak. Not only did the number of reported isolates return to baseline, but also the outbreak strain disappeared. By the time this investigation was completed, PulseNet laboratories had typed >1,000 isolates of *S*. Agona. Four hundred nine cases (one fatal) in 23 states were linked to this outbreak (CDC, unpub. data).

From October 20 to November 9, 1998, health officials in Connecticut, New York, Ohio, and Tennessee reported increases in *Listeria* infections in their states (20). PFGE typing by PulseNet laboratories showed that several case isolates from different states had indistinguishable DNA fingerprints. On further investigation, 101 *Listeria* infections (including 15 perinatal infections) with bacteria having the same or highly similar DNA fingerprints were identified in 22 states. Fifteen deaths and six miscarriages or stillbirths were reported among patients who were infected with the outbreak strain. This outbreak was traced to contaminated hot dogs and sandwich meat produced at a single large meat-processing plant in Michigan (21). After the company voluntarily recalled the implicated lots of product and suspended production, the outbreak rapidly ended.

## Surveillance for Foodborne Outbreaks

Twenty years ago, most foodborne outbreaks were local problems that typically resulted from improper food-handling practices. Outbreaks were often associated with individual restaurants or social events and often came to the attention of local public health officials through calls from affected persons. These persons, who may have known others who had become ill after eating a shared meal or visiting the same restaurant, provided health officials with much of the information needed to begin an investigation.

Today, foodborne disease outbreaks commonly involve widely distributed food products that are contaminated before distribution, resulting in cases that are spread over several states or countries. It is less common for ill persons to know others who were ill or to be able to identify a likely source of their infection. For these reasons, it is becoming increasingly important to be able to identify potential common exposures through DNA fingerprinting of patient isolates.

For foodborne outbreak surveillance to be effective, isolates must be subtyped routinely and the data analyzed promptly at the local level. Clusters can often be detected locally that could not have been identified by traditional epidemiologic methods alone. This is especially true of infections with common pathogens such as *S.* Typhimurium, which occur so frequently that clusters may be hidden among sporadic cases. For *S*. Typhimurium isolates received by the microbiology laboratory at the Minnesota Department of Health from August 14 to September 14, 1995, temporal distribution did not suggest any obvious clustering, but the distribution of PFGE subtypes suggested multiple common sources with continuing exposures (Figure 2). Epidemiologic investigation ultimately linked three of the subtypes to local restaurants, where exposure to *S*. Typhimurium occurred throughout the month (Jeffrey B. Bender, pers. comm.). Without subtyping data, it would have been very difficult to associate cases with exposures occurring over such a prolonged period.

In September 1998, the Minnesota state public health laboratory informed other PulseNet laboratories that it was investigating two clusters of *Shigella sonnei* infections associated with restaurants in Minnesota and asked if other states had observed increases in *S. sonnei* infections or *S. sonnei* isolates with the outbreak PFGE pattern. The Los Angeles County public health department immediately responded that it was also investigating restaurant-associated outbreaks of *S. sonnei* and that the PFGE pattern of their outbreak strain was very similar to the Minnesota pattern. Epidemiologic and laboratory investigations ultimately determined that outbreaks in Massachusetts, Florida, and two Canadian provinces were linked to the Minnesota and Los Angeles outbreaks. With the assistance of the FDA's outbreak trace-back and coordination group, parsley imported from Mexico was identified as the common vehicle (22). Mexican and U.S. authorities inspected the parsley farm and recommended changes in growing and harvesting practices to prevent recurrence of the problem. Rapid sharing of PFGE subtyping data through PulseNet played a critical role in linking these apparently unrelated outbreaks and identifying a common vehicle.

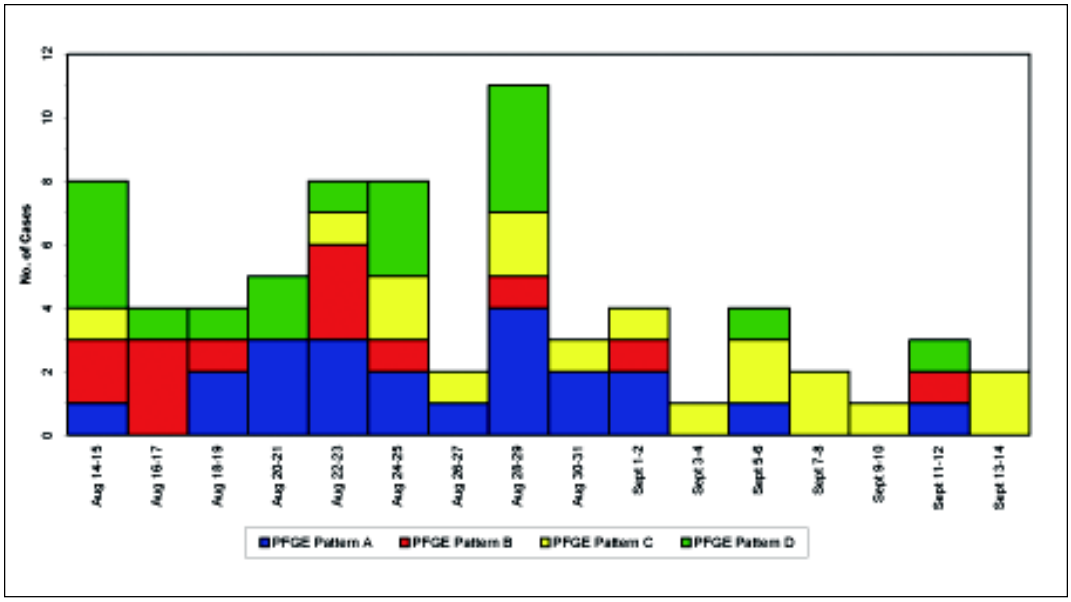


Figure 2. Distribution by date and pulsed-field gel electrophoresis subtype of *Salmonella* ser. Typhimurium isolates received by the Minnesota Department of Health, August 14-September 14, 1995. Data provided by Jeffrey Bender and John Besser, Minnesota Department of Health.

The use of molecular subtyping as part of routine surveillance has benefits beyond outbreak detection. Temporal clustering of unrelated cases is not uncommon, and without molecular subtyping, valuable public health resources can be wasted investigating pseudo-outbreaks. In June and July 1994, an outbreak of *E. coli* O157:H7 infections was suspected when the New Jersey Department of Health and Senior Services received reports of 48 culture-confirmed cases; only four were reported during the same period in 1993 (23). PFGE subtyping found most isolates to have unique patterns, indicating that a large outbreak was unlikely. The probable reason for the sudden increase in case reports was the concomitant increase in the number of laboratories culturing stools for *E. coli* O157:H7 (Figure 3).

Although PulseNet has proven invaluable in detecting foodborne disease outbreaks and facilitating their investigation, molecular subtyping is an adjunct to epidemiologic investigation and not a replacement for it. The observation that isolates from two or more persons have indistinguishable PFGE patterns should not be considered proof that the persons had a common exposure, merely that the isolates in question share a common ancestry. Moreover, outbreaks can be caused by more than one subtype, so that differences in PFGE pattern alone cannot prove that isolates did not have a common source (24,25).

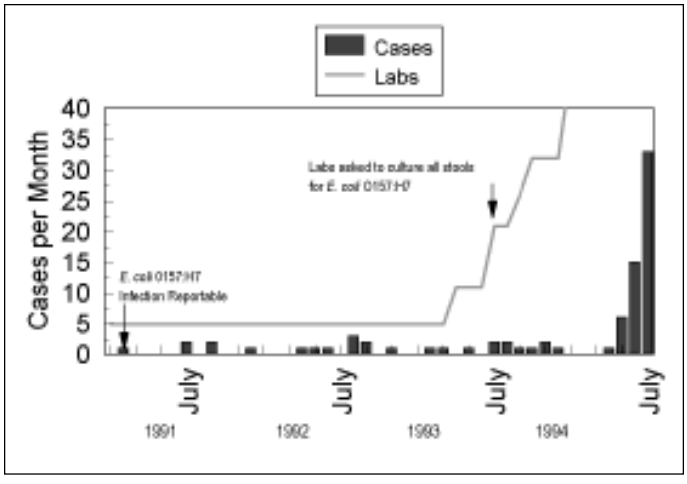


Figure 3. Reported cases of culture-confirmed *Escherichia coli* O157:H7 infection and percentage of surveyed laboratories routinely testing all stool specimens for *E. coli* O157:H7, New Jersey, January 1991 through July 1994 (10).

## Requirements for Effective Functioning

Although the area laboratories are set up to assist neighboring state public health laboratories that are not PulseNet participants, every state must have PFGE subtyping capacity for optimum performance of the network. A dramatic indication of this was provided during the 1997 ground beef-associated *E. coli* O157:H7 outbreak in Colorado. When the outbreak pattern was posted on PulseNet ListServ, most laboratories that were network participants responded within 48 hours that they had no matching PFGE patterns from recent *E. coli* O157:H7 isolates. In contrast, it took more than 2 months to identify a case in Kentucky (not a PulseNet participant state in 1997) that was related to the Colorado outbreak. The Association of Public Health Laboratories has determined that PulseNet participation is a core capacity for all state and territorial public health departments in the United States.

For the network to work efficiently in detecting foodborne disease outbreaks through routine surveillance, PulseNet laboratories must perform, at a minimum, routine PFGE subtyping of *E. coli* O157:H7 and *L. monocytogenes* as soon as isolates are received. In addition, they must perform PFGE subtyping of other foodborne pathogenic bacteria (*Campylobacter jejuni* and *C. coli*, *Salmonella* serotypes, *Shigella* spp., *Bacillus cereus*, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *Clostridium botulinum*, *C. perfringens*, *Yersinia enterocolitica*) rapidly when the number of isolates received by the laboratory exceeds the expected number for that period. Unfortunately, microbiologists at state and local public health laboratories often have responsibilities for all pathogenic bacteria and may not be able to type incoming isolates of foodborne pathogenic bacteria in a timely manner. In addition, like public health surveillance in general, PulseNet

depends on physicians requesting culture of patient specimens if a bacterial infection is suspected and the clinical diagnostic laboratory promptly forwarding isolates to the public health laboratory for typing.

PulseNet relies on the cooperation of all participants in typing foodborne pathogenic bacteria by strict adherence to the standard protocol. Without such a total commitment, PFGE patterns from different PulseNet laboratories could not be compared to ascertain which cases are associated with a specific outbreak. The importance of this was underscored by a recent experience. One PulseNet laboratory had decided to change the PulseNet protocol for *S.* Typhimurium and was using a variation of the standard protocol. A cluster of *S*. Typhimurium cases was detected in that state, and PFGE analysis confirmed that many of the isolates were indistinguishable. However, when attempts were made to determine if an increase in *S*. Typhimurium infections in neighboring states were related to the cluster, the PFGE patterns could not be compared. This caused a delay of several days in the investigation because isolates from the first state had to be re-typed by the standardized protocol.

## Cost-Benefit Analysis

Elbasha et al. recently compared the costs and benefits of PulseNet's molecular subtyping-based surveillance system, using as an example the Colorado state public health laboratory's investigation of the 1997 *E. coli* O157:H7 outbreak in which contaminated frozen hamburger patties were implicated (26). If 15 cases were averted by the recall of potentially contaminated ground beef, the PulseNet system in Colorado would have recovered all costs of start-up and 5 years of operation. These authors point out that the system becomes even more cost-effective if one takes into account resources that would have been wasted to investigate apparent increases in sporadic cases of *E. coli* O157:H7 infections.

## The Growth and Future of PulseNet

Within a very short time, PulseNet has grown beyond expectations and has convincingly demonstrated its effectiveness as a tool for foodborne disease surveillance. It began with one pathogen (*E. coli* O157:H7) and 10 participating laboratories in 1996 that submitted 191 PFGE patterns to the PulseNet database during that year. In 1999, four pathogens (*E. coli* O157:H7, *Salmonella*, *Shigella*, and *Listeria monocytogenes*) were tracked through PulseNet and >9,500 patterns were submitted to the PulseNet database. State and local public health laboratories contributed >78% of the patterns to the PulseNet database (Figure 4). As the FDA are increasing the number of laboratories that perform PFGE subtyping using PulseNet protocols, and the USDA are setting up their own PulseNet-compatible local networks, their contributions to the PulseNet database will no doubt substantially increase. In addition, the representation of PFGE patterns in the PulseNet databases will increase for pathogenic bacteria isolated from foods.

As more public health laboratories at the local and state levels join PulseNet, the role of the area laboratories is changing. The area laboratories provide training and consultation to neighboring PulseNet laboratories, coordinate multistate outbreak investigations when requested, and

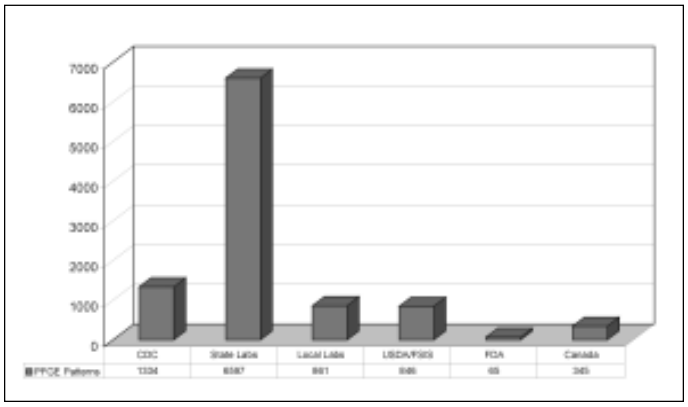


Figure 4. Sources of pulsed-field gel electrophoresis patterns submitted to PulseNet in 1999.

provide surge capacity for neighboring PulseNet laboratories. Three additional area laboratories, in Michigan, Utah, and Virginia, were designated in 2000, bringing the total number to seven.

Canada is already an active participant, and international expansion of the network with partners in Mexico, South America, and Europe is anticipated. The long-term vision for PulseNet is a global network of public health laboratories working with food regulatory agencies and industry to improve food safety worldwide.

Finally, we recognize that the methods currently used for subtyping and data analysis will not always be state-of-the-art. We are working to develop, evaluate, and validate DNA sequencing-based subtyping methods for foodborne pathogens. These methods will be gradually implemented in the network, and compatibility will be maintained with existing PFGE data. We are also working with software developers to implement new versions of pattern analysis software and DNA sequence comparison software to improve pattern matching, automate pattern normalization and sequence alignment, and reduce subjectivity in subtype comparisons.

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Dr. Swaminathan is chief of the Foodborne and Diarrheal Diseases Laboratory Section, CDC, and the principal architect of PulseNet.

### References

1. Holmberg SD, Wachsmuth IK, Hickman-Brenner FW, Cohen ML. Comparison of plasmid profile analysis, phage typing, and antimicrobial susceptibility testing in characterizing *Salmonella typhimurium* isolates from outbreaks. J Clin Microbiol 1984;19:100-4.

2. Holmberg SD, Wachsmuth K. Plasmid and chromosomal DNA analyses in the epidemiology of bacterial diseases. In: Swaminathan B, Prakash G, editors. Nucleic acid and monoclonal antibody probes: applications in diagnostic microbiology. New York: Marcel Dekker; 1989. p. 105-29.
3. Ackers ML, Mahon BE, Leahy E, Goode B, Damrow T, Hayes PS, et al. An outbreak of *Escherichia coli* O157:H7 infections associated with leaf lettuce consumption. J Infect Dis 1998;177:1588-93.
4. Barrett TJ. Molecular fingerprinting of foodborne pathogenic bacteria: An introduction to methods, uses and problems. In: Tortorello ML, Gendel SM, editors. Food microbiological analysis: new technologies. New York: Marcel Dekker; 1997. p. 249-64.
5. Graves LM, Swaminathan B, Hunter SB. Subtyping *Listeria monocytogenes*. In: Ryser EM, Marth EH, editors. *Listeria*, listeriosis and food safety. New York: Marcel Dekker; 1999. p. 279-98.
6. Jimenez A, Barros-Velazquez J, Rodriguez J, Villa TG. Restriction endonuclease analysis, DNA relatedness and phenotypic characterization of *Campylobacter jejuni* and *Campylobacter coli* isolates invovled in food-borne disease. J Appl Microbiol 1997;82:713-21.
7. Maslanka SE, Kerr JG, Williams G, Barbaree JM, Carson LA, Miller JM, et al. Molecular subtyping of *Clostridium perfringens* by pulsed-field gel electrophoresis to facilitate food-borne-disease outbreak investigations. J Clin Microbiol 1999;37:2209-14.
8. Threlfall EJ, Hampton MD, Ward LR, Rowe B. Application of pulsed-field gel electrophoresis to an international outbreak of *Salmonella agona*. Emerg Infect Dis 1996;2:130-2.
9. Threlfall EJ, Ward LR, Hampton MD, Ridley AM, Rowe B, Roberts D, et al. Molecular fingerprinting defines a strain of *Salmonella enterica* serotype Anatum responsible for an international outbreak associated with formula-dried milk. Epidemiol Infect 1998;121:289-93.
10. Wachsmuth K. Molecular epidemiology of bacteria infections: Examples of methodology and of investigations of outbreaks. Rev Infect Dis 1986;8:682-92.
11. Swaminathan B, Matar GM. Molecular typing methods. In: Persing DH, Smith TF, Tenover FC, White TJ, editors. Diagnostic molecular microbiology. Washington: American Society for Microbiology; 1993. p. 26-50.
12. Barrett TJ, Lior H, Green JH, Khakhria R, Wells JG, Bell BP, et al. Laboratory investigation of a multi-state food-borne outbreak of *Escherichia coli* O157:H7 by using pulsed-field gel electrophoresis and phage typing. J Clin Microbiol 1994;32:3013-7.
13. Stephenson J. New approaches for detecting and curtailing foodborne microbial infections. JAMA 1997;277:1337-40.
14. Centers for Disease Control and Prevention. Addressing emerging infectious disease threats: a prevention strategy for the United States. Atlanta: U.S. Department of Health and Human Services; 1994.
15. Angulo FJ, Voetsch AC, Vugia D, Hadler JL, Farley M, Hedberg C, et al. Determining the burden of human illness from food borne diseases. CDC's emerging infectious disease program Food Borne Diseases Active Surveillance Network (FoodNet). Vet Clin North Am Food Anim Pract 1998;14:165-72.
16. Gautom RK. Rapid pulsed-field gel electrophoresis protocol for *E. coli* O157:H7 and other Gram-negative organisms in one day. J Clin Microbiol 1997;35:2977-80.
17. Centers for Disease Control and Prevention. Standardized molecular subtyping of foodborne bacterial pathogens by pulsed-field gel electrophoresis: a manual. Atlanta: National Center for Infectious Diseases; 1996 (updated 2000).
18. Cody SH, Glynn MK, Farrar JA, Cairns KL, Griffin PM, Kobayashi J, et al. An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice. Ann Intern Med 1999;130:202-9.
19. Centers for Disease Control and Prevention. Multistate outbreak of *Salmonella* serotype Agona infections linked to toasted oats cereal—United States, April-May, 1998. MMWR Morb Mortal Wkly Rep 1998;47:462-4.
20. Centers for Disease Control and Prevention. Multistate outbreak of listeriosis—United States, 1998. MMWR Morb Mortal Wkly Rep 1998;47:1085-6.
21. Centers for Disease Control and Prevention. Update: Multistate outbreak of listeriosis—United States, 1998-1999. MMWR Morb Mortal Wkly Rep 1999;47:1117-8.
22. Centers for Disease Control and Prevention. Outbreaks of *Shigella sonnei* infection associated with eating fresh parsley—United States and Canada, July-August, 1998. MMWR Morb Mortal Wkly Rep 1999;48:285-9.
23. Mead P, Finelli L, Lambert-Fair MA, Champ D, Townes J, Hutwagner L, et al. Risk factors for sporadic infection with *Escherichia coli* O157:H7. Arch Intern Med 1997;157:204-8.
24. Keene W, Hedberg K, Herriott DE, Hancock DD, McKay RW, Barrett TJ, et al. A prolonged outbreak of *Escherichia coli* O157:H7 infections caused by commercially distributed milk. J Infect Dis 1997;176:815-8.
25. Jackson LA, Keene WE, McAnulty JM, Alexander ER, Diermayer M, Davis MA, et al. Where's the beef? The role of cross-contamination in four chain restaurant-associated outbreaks of *Escherichia coli* O157:H7 in the Pacific Northwest. Ann Intern Med 2000;160:2380-5.
26. Elbasha EH, Fitzsimmons TD, Meltzer MI. Costs and benefits of a subtype-specific surveillance system for identifying *Escherichia coli* O157:H7 outbreaks. Emerg Infect Dis 200;6:293-7.

# Spoligotype Database of *Mycobacterium tuberculosis*: Biogeographic Distribution of Shared Types and Epidemiologic and Phylogenetic Perspectives

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We give an update on the worldwide spoligotype database, which now contains 3,319 spoligotype patterns of *Mycobacterium tuberculosis* in 47 countries, with 259 shared types, i.e., identical spoligotypes shared by two or more patient isolates. The 259 shared types contained a total of 2,779 (84%) of all the isolates. Seven major genetic groups represented 37% of all clustered isolates. Two types (119 and 137) were found almost exclusively in the USA and accounted for 9% of clustered isolates. The remaining 1,517 isolates were scattered into 252 different spoligotypes. This database constitutes a tool for pattern comparison of *M. tuberculosis* clinical isolates for global epidemiologic studies and phylogenetic purposes.

In 1997, 8 million new cases of tuberculosis (TB) were reported worldwide; 3.5 million cases were considered highly contagious (1). With Africa and some countries having up to 20% of their populations infected with HIV, AIDS will have a major impact on TB in coming years (2). Emergence of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* is also of great epidemiologic concern (3). In this context, molecular fingerprinting of *M. tuberculosis* complex isolates is a powerful tool that permits detection of transcontinental spread of TB (4) and outbreaks (5). Our laboratory has described a preliminary spoligotyping database that suggested the biogeographic specificity of some of the spoligotypes from the Caribbean (6). The initial aim of this work was twofold. First, such an inventory was mandatory to detect and estimate the relative importance of TB of foreign origin in the French Caribbean. Although the incidence of TB in Martinique and Guadeloupe is comparable with that in metropolitan France (approximately 10/100,000 new cases each year), this region is part of an area of Latin America and the Caribbean with high TB prevalence. Second, we used spoligotyping results to infer potential phylogenetic relationships of *M. tuberculosis* strains in the Caribbean region and the history of TB by using molecular markers. An updated database could also be helpful in developing new statistical approaches in the field of population genetics of circulating *M. tuberculosis* clinical isolates.

By systematically analyzing published spoligotypes, we have now collected 3,319 spoligotyping patterns of various origins in a single database, essentially from Europe and the USA (Table 1). This database includes 259 shared types containing 2 to 476 patterns (because of the size of this database, a graphic of it appears online only, at http://www.cdc.gov/ncidod/EID/vol7no3/sola_data.htm). The main database also includes 540 "orphan patterns" (clinical isolates showing a unique spoligotype), for a current total of 799 distinct spoligotype patterns. This article describes the nomenclature and phylogenetic reconstruction of these 259 shared types.

## The Database

Spoligotyping based on the variability of the Direct Repeat (DR) locus and analysis of a variable number of tandem DNA repeats (VNTR) of *M. tuberculosis* were performed according to the original protocols (7,8). For the construction of the database, spoligotyping results were entered into Excel spreadsheet files in chronological order, according to the availability of results from published articles and our own investigations. The database was searched regularly for new shared types, i.e., identical spoligotypes shared by two or more patient isolates. For phylogenetic reconstruction, the spoligotyping results were entered into Recognizer software of the Taxotron package (Taxolab, Institut Pasteur, Paris), as recommended (9). The "1-Jaccard" Index was calculated for each pairwise comparison of patterns (10), and the neighbor-joining algorithm was used for building trees (11).

The source of the data and its representativeness are shown in Table 1. Of 3,319 individual spoligotypes in our database, most (2,418 [73%]) were either from Europe (1,142 [34%]) or the USA (1,283 [39%]). Spoligotypes shared between the USA and Europe totaled 1,286 isolates distributed among 45 shared types (Europe, n=461; USA, n=825). A statistical

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Table 1. Source of data for 3,319 spoligotypes of *Mycobacterium tuberculosis* used to generate the database of 259 shared types

| No. of isolates | Origin[a] | Year | Reference |
|---|---|---|---|
| 136 | Denmark | 1999 | 31 |
| 147 | Italy | 1999 | 32 |
| 157 | Cuba | 1998 | 33 |
| 1[b] | Philippines | 1997 | 34 |
| 3 | Peru | 1998 | 35 |
| 18 | USA | Unpublished | R. Frothingham |
| 105 | France | 1997 | 36 |
| 167 | United Kingdom | 1997 | 37 |
| 296 | France | Unpublished | This study |
| 28 | Zimbabwe | 1998 | 38 |
| 32 | Guinea-Bissau | 1999 | 25 |
| 118 | The Netherlands | 1997 | 7 |
| 68 | Various countries | 1999 | 15 |
| 58 | France | Unpublished | J. Maïsetti & B. Carbonnelle |
| 62 | Russia | Unpublished | O. Narvskaya |
| 84 | West Africa | 1999 | 39 |
| 5 | Thailand | Unpublished | P. Palittapongarnpim |
| 14 | Romania | 1997 | 40 |
| 17 | Brazil | 1999 | 41 |
| 5[b] | Spain | Unpublished | S. Semper & C. Martin |
| 1,283 | USA | 2000 | 12 |
| 1[b] | United Kingdom | 1999 | 42 |
| 19 | The Netherlands | 1998 | 43 |
| 1[b] | The Netherlands | 1999 | 19 |
| 69 | Far East Asia | 1995 | 44 |
| 69 | Caribbean | 1999 | 6 |
| 356 | Caribbean | Unpublished | This study |

[a]Although a potential sampling bias cannot be excluded, the sampling of isolates and their representativeness (in order of description) was as follows: Denmark, of 249 isolates described with a low copy number of IS*6110* collected since 1992 (exhaustivity 93%), 24 shared types, representing 136 spoligotypes, were retained (9 other shared types, representing 49 isolates that were found exclusively in Denmark (S1,S2,S4,S19,S22,S23,S27,S30,S33), were not included in the present analysis; Italy, of 158 isolates from 156 patients in Verona collected during 1996-1997, 147 spoligotypes were retained; Cuba, of 160 isolates typed (obtained from a pool of 578 smear-positive sputa collected during 1994-1995), 157 spoligotypes described (exhaustivity 36%) were retained; Philippines, no data except for a single spoligotype available; Peru, of 29 strains isolated during 1995-1996 from the sputa of patients in Lima and Cuzco, only 3 were retained in this study since the remaining isolates shared spoligotypes with patients in Texas (12) and are included in the 1,283 Texan profiles; USA, 18 clinical isolates from the collection of R. Frothingham (representativeness unknown); France, 111 isolates from 105 hospitalized patients in Paris obtained during 1993 (patients were from three major hospitals that represented 5% of the total public hospital beds in Paris); United Kingdom, 167 isolates from all the culture-positive tuberculosis (TB) patients from three large hospitals in northwest London (without any indication of period of recruitment); France, 296 isolates sent for reference purposes during a 3-year period to the Centre National de Référence des Mycobactéries, Institut Pasteur, Paris; Zimbabwe, 28 spoligotypes obtained directly from sputum samples during a 1-month recruitment period (December 1995) of sputum-positive TB cases representing 20% of all cases; Guinea-Bissau, of 229 spoligotypes obtained from samples of 900 patients with suspected TB cases during 1989-1994, only 32 spoligotypes were fully described by the authors, and were retained for the analysis; the Netherlands, 118 isolates of unspecified representativeness from the collection of National Institute of Health (RIVM, Bilthoven); international multicenter study, 68 of 90 isolates from 38 countries representing the five continents; France, 58 isolates during a 1-year (1999) recruitment in the University Hospital of Angers; Russia, 62 isolates representing the St. Petersburg area collected during 1997-1999; West Africa, 84 isolates from Ivory Coast and around Dakar, Senegal, collected during 1994-1995; Thailand, 5 isolates from northern Thailand (unknown representativeness); Romania, 14 isolates of unknown representativeness; Brazil, 17 spoligotypes out of 91 isolates from a São Paulo hospital in 1995 (unknown representativeness); Spain, 5 multidrug-resistant isolates (unknown representativeness); USA, 1,429 clinical isolates from 1,283 patients during 1994-1999 that are part of an ongoing population-based study in Houston, Texas; United Kingdom, a single spoligotype from ancient DNA extracted from a bone sample; the Netherlands, 19 spoligotypes obtained from paraffin-wax embedded tissue samples previously collected during 1983-1993 (unknown representativeness); the Netherlands, a single spoligotype from a previous study (unknown representativeness); Far East Asia, 69 isolates from China and Mongolia obtained during 1992-1994 (unknown representativeness); Caribbean, 425 clinical isolates from a population-based ongoing study that includes all cultures isolated in Guadeloupe, Martinique, and French Guiana since 1994 and covers a 1 million population (exhaustivity 100%). Some isolates in this pool came from patients from other countries (essentially neighboring countries such as Haiti, Dominican Republic, Brazil, Commonwealth of Dominica, Barbados, and Surinam).

[b]Description of a given spoligotype without precise number of isolates within this type.

analysis was performed for the 1,286 isolates to evaluate the biogeographic specificity of the shared types and assess potential sampling bias by using a sample homogeneity test derived from the chi-square test (see below).

## Results and Discussion

### Description of Database

The 3,319 spoligotypes were grouped into 259 shared types containing 2,779 (84%) of the isolates and 540 (16%) orphan spoligotyping patterns (clinical isolates showing a unique spoligotype; results not shown; see online graphic of database, http://www.cdc.gov/ncidod/EID/vol7no3/sola_data.htm). This gives a current total of 799 distinct spoligotype patterns in our database.

The distribution of shared types, their respective sizes, and their relative distribution in different locations (distinct countries or geographic regions) are summarized in Figure 1. The 24 most frequent shared types totaled 1,804 (65%) isolates (Figure 1A); 7 types were highly frequent, representing 1,250 (45%) isolates. The Beijing type (type 1) was most frequent and represented 18% of isolates. Two types (119 and 137), which were almost exclusively found in the USA, accounted for 9% of isolates and may be specific for American populations or outbreaks (12). Types 53 and 50 accounted for 8% and 6% of isolates and were found in 17 and 15 locations, respectively. Two other types (types 42 and 47)

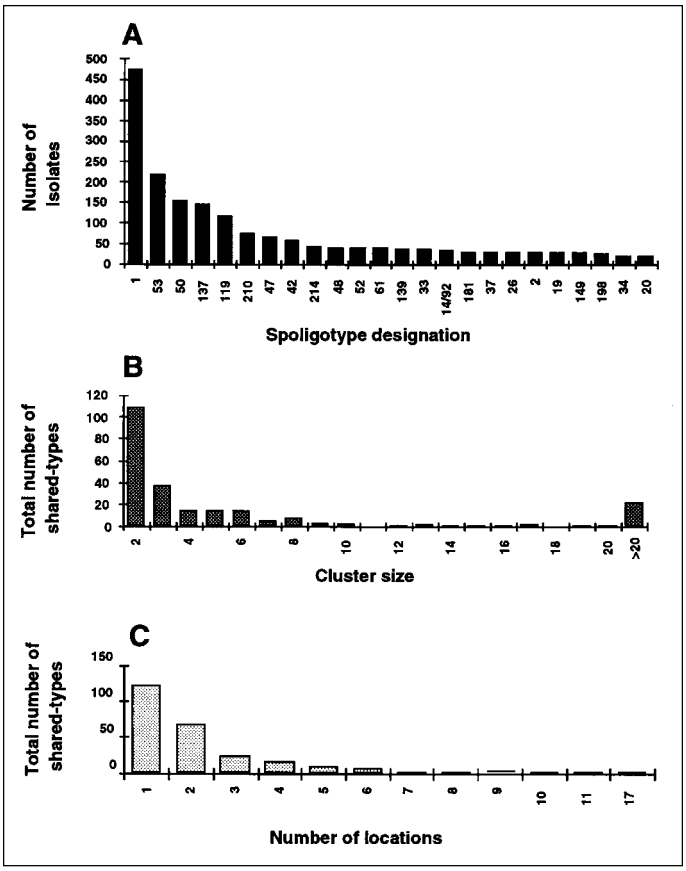


Figure 1. Histograms derived from database (graphic online at http://www.cdc.gov/ncidod/EID/vol7no3/sola_data.htm) summarizing the distribution of shared types (A), their respective sizes (B), and their relative distribution in different locations (C).

accounted for 4% of the isolates and were found in 11 countries. The remaining isolates (n=1,517) were scattered into 235 types. Figure 1B shows the relative sizes of 259 shared types; 109 shared types (42%) contained only two patients each and 38 shared types contained only three patients each. Inversely, 24 shared types containing >20 patients totaled 1,804 (65%) isolates. Finally, the distribution "unique" versus "ubiquitous" shared types (reported in one location versus found in two or more locations) is shown in Figure 1C; 122 (47%) shared types were reported from a single location, 69 (26%) were from two locations, and 25 (10%) were from three locations. Inversely, the most ubiquitous types, in increasing order of distribution, were 33 and 37, 20, 52, 42, 50, and 53. Thus, most *M. tuberculosis* shared types contained a low number of patient isolates and were confined geographically, whereas a minority contained a high number of patient isolates and were highly disseminated. The finding of identical spoligotypes in distant countries may be explained either by recent or past transmission events or by phylogenetic convergence. However, the evolution of the DR locus relies on at least three independent mechanisms, namely, homologous recombination (13), replication slippage (14, 15), and insertion sequence-mediated transposition (16-19), which does not favor a fortuitous convergence.

### Geographic Distribution of Shared Types in the Database

Analysis of geographic distribution of the shared types (see online graphic of database, http://www.cdc.gov/ncidod/EID/vol7no3/sola_data.htm) permitted us to split our collection into two broad categories: those reported in a single area (n=122, Table 2) and those reported in two or more areas (n=137). In the latter category, matching analysis for 69 spoligotypes found in four broad geographic areas, namely, Africa, the Americas (North, Central and Caribbean, and South America), Europe, and Asia (Middle East, and Far East Asia), is shown in Table 3. Contrary to ubiquitous spoligotypes such as type 1, 53, and 50, which have been found in all regions, this is an attempt to define potential inter-regional and inter-continental flow of *M. tuberculosis* isolates so far confined to limited geographic areas. The most frequent matches were found for clusters in European countries (n=17), followed by Europe and North America (n=8), Europe and Central America and the Caribbean (n=5), and Europe and South America (n=4) (Table 3). These matches may underline both recent transcontinental transmission events and the history of TB spread in the New World through European settlers.

A total of 25 shared types were reported in three countries. Among these, 8 types were exclusively found either in Europe (types 10,22,161) or the Americas (types 5,67,70,93,130); 10 types were shared between two European countries and a country of another region (types 35,49,59,86,115,118,136,138,139,150); 5 types were shared between two countries of the Americas with a country in Europe (types 92,119,168,185,190); 1 type was shared between a European country and two African countries (type 125); and 1 type was shared between Asia, Europe, and the USA (type 124). Finally, 15 types were found in four countries; 1 type (type 41) was exclusively found in Europe and may be specific for this continent. Fourteen other types were distributed as follows: Europe + Americas, 8 types (types 3,7,19,31,40,51,137,152); Europe + Africa, 1 type (type 21); Europe + Asia + Americas, 3 types (type 8,89,167); Europe + Americas + Africa, 1 type (type 64); and Europe + Africa + Asia, 1 type (type 126). Finally, 28 types were reported in five or more countries, suggesting that these types are widespread and may constitute the ubiquitous types such as the Beijing type (type 1 in our database) or the Haarlem type (type 47). The only exception in this category was type 17, which was found in six countries in the Americas and may be specific for this region. Future population studies should focus on these ubiquitous types to better define their relative prevalence in each country.

Table 2. Geographic distribution of potentially specific shared types of *Mycobacterium tuberculosis* reported in a single location (n=122)

| Region | Country | No. of types | Types |
|---|---|---|---|
| Americas | Guadeloupe | 7 | 12,13,14,15,30,103,259 |
| | French Guiana | 4 | 66,76,94,96 |
| | Cuba | 4 | 71,74,80,81 |
| | USA | 46 | 192,194,197-199,201,202, 205,206,208,210-217,219-235,237-239,241,243,246, 248,256-258 |
| Europe | The Netherlands | 4 | 9,18,28,90 |
| | United Kingdom | 6 | 16,23,27,38,43,100 |
| | France | 27 | 55,57,107-114,116,120,122, 140,141,143-148,170,171, 173,174,184,186 |
| | Italy | 9 | 155,157-160,163,165, 166,169 |
| | Spain | 2 | 104,106 |
| | Russia | 3 | 251,252,253 |
| Africa | Zimbabwe | 6 | 79,82-85,87 |
| | Guinea-Bissau | 1 | 188 |
| Asia | Philippines | 1 | 69 |
| | Mongolia | 2 | 97, 98 |

Table 3. Total number of matches found in matching analysis of the shared types (n=69) found at two geographic locations*

| | | Americas | | | | Asia* | |
|---|---|---|---|---|---|---|---|
| Matches* | Africa | North America | Central America/ Caribbean | South America | Europe | Middle East | Far East |
| Africa | 3[a] | 3[b] | 2[c] | 1[d] | 5[e] | 0 | 0 |
| North America | | NA+ | 6[f] | 4[g] | 8[h] | 0 | 1 |
| Central America | | | 2[i] | 4[j] | 5[k] | 0 | 0 |
| South America | | | | 3[l] | 4[m] | 0 | 0 |
| Europe | | | | | 17[n] | 1 | 0 |
| Asia (Middle East) | | | | | | 0 | 0 |
| Asia (Far East) | | | | | | | 0 |

*Indices a to n refer to the designation of the matching types. For full description of the matching shared type, see database (online graphic at http://www.cdc.gov/ncidod/EID/vol7no3/sola_data.htm). Spoligotyping data for isolates from Asia are scarce; hence, only two matches involving the Middle East and Far East were found (shared types 127 and 249, respectively). †NA, not applicable (matches were searched only for shared types existing between two countries or regions; as no data were available for Canada, comparison of isolates within North America was not feasible).

### Biogeographic Analysis of European Versus American Spoligotypes

Several possible scenarios could account for the introduction and spread of TB in the Americas; however, documented contact with Europeans is considered too recent to account for the widespread distribution of the disease by AD 1000 (20). One hypothesis suggests that TB may have penetrated the Americas through human migration from Asia via the Bering Strait (21). Another scenario suggests TB's initial introduction as a zoonosis that became an anthropozoonosis after cattle were domesticated (20,21). In this context, of the 259 shared types in our database, 59 were exclusively reported in the Americas, whereas 50 were found only in Europe (Table 2). This biogeographic dichotomy may signal the specific history of the disease in each continent. As enough data were present for the USA and Europe (2,418 [73%] isolates), a statistical analysis of distribution of shared types found in those two areas was performed.[1] Of 45 shared types in this category, results showed that differences in the distribution of certain shared types (1,19,20,25,26,37,44,48, 50,52,53,118,137) between the USA and Europe were highly significant, and sampling bias could not explain the differences observed (Table 4). On the other hand, the differences observed in the distribution of shared types 2,8, 33,34,47,58,62,92,138, and 139 between the USA and Europe were not statistically significant, and in this case sampling bias could not be fully excluded for the differences observed. Finally, our database described 58 isolates of the shared type 42 that were present in 11 countries (a ubiquitous type), but not a single isolate of type 42 was present among the 1,283 isolates from Texas (12).

Table 4. Analysis of distribution of shared types found in both USA and Europe[a]

| | Europe | | USA | | World | | |
|---|---|---|---|---|---|---|---|
| Type | No. (k1) | %[b] (p1) | No. (k2) | % (p2) | No. (p0) | % | $d/\sigma_{d\ quotient}$[c] |
| 1 | 21 | 1.8 | 326 | 25.5 | 476 | 14.4 | 15.3[d] |
| 2 | 6 | 0.5 | 2 | 0.2 | 28 | 0.8 | 1.6 |
| 8 | 10 | 0.9 | 7 | 0.5 | 19 | 0.6 | 0.9 |
| 19 | 1 | 0.1 | 23 | 1.8 | 27 | 0.8 | 4.2[d] |
| 20 | 8 | 0.7 | 2 | 0.2 | 20 | 0.6 | 2.1[d] |
| 25 | 13 | 1.1 | 3 | 0.2 | 17 | 0.5 | 2.7[d] |
| 26 | 22 | 1.9 | 5 | 0.4 | 28 | 0.8 | 3.6[d] |
| 33 | 13 | 1.1 | 10 | 0.8 | 38 | 1.2 | 0.9 |
| 34 | 6 | 0.5 | 9 | 0.7 | 21 | 0.6 | 0.6 |
| 37 | 17 | 1.5 | 2 | 0.2 | 28 | 0.8 | 3.7[d] |
| 44 | 12 | 1.1 | 1 | 0.1 | 15 | 0.5 | 3.3[d] |
| 47 | 25 | 2.2 | 23 | 1.8 | 65 | 2.0 | 0.7 |
| 48 | 34 | 3.0 | 7 | 0.5 | 41 | 1.2 | 4.6[d] |
| 50 | 56 | 4.9 | 32 | 2.5 | 155 | 4.7 | 3.1[d] |
| 52 | 29 | 2.5 | 7 | 0.5 | 40 | 1.2 | 4.0[d] |
| 53 | 79 | 6.9 | 46 | 3.6 | 218 | 6.6 | 3.6[d] |
| 58 | 4 | 0.4 | 7 | 0.5 | 17 | 0.5 | 0.7 |
| 62 | 7 | 0.6 | 4 | 0.3 | 15 | 0.5 | 1.1 |
| 92 | 2 | 0.2 | 8 | 0.6 | 14 | 0.4 | 1.7 |
| 118 | 8 | 0.7 | 1 | 0.1 | 9 | 0.3 | 2.5[d] |
| 119 | 2 | 0.2 | 110 | 8.6 | 115 | 3.5 | 9.6[d] |
| 137 | 10 | 0.9 | 134 | 10.5 | 146 | 4.4 | 9.7[d] |
| 138 | 5 | 1 | 1 | 0.1 | 6 | 0.2 | 1.8 |
| 139 | 19 | 1.7 | 19 | 1.5 | 38 | 1.2 | 0.3 |

[a]Results are given for 24 of 45 shared types that contained enough isolates to compare the results statistically.
[b]Percentages were calculated on the basis of 1,142 (n1), 1,276 (n2), and 3,319 individual spoligotypes reported, respectively, for Europe (p1), USA (p2), and the full database available for the world.
[c]The quotient $d/\sigma_d$ was calculated using the equation $d/\sigma_d=p_1-p_2/\sqrt{p_0q_0(1/n_1+1/n_2)}$, where d is the absolute value of the difference between p1 and p2, $\sigma_d$ is the standard deviation of the repartition law of d which follows a normal distribution and can be calculated by the equation $\sigma_d=\sqrt{p_0q_0\ (1/n_1+1/n_2)}$, and where $p_0$ is best estimated by the equation $p_0=k_1+k_2/n_1+n_2=n_1p_1+n_2p_2/n_1+n_2$ In this equation, individual sampling sizes are $n_1$ and $n_2$, the number of individuals within a given shared-type "x" are $k_1$ and $k_2$, and the representativeness for the two samples is $p_1=k_1/n_1$ and $p_2=k_2/n_2$.
[d]If the absolute value of the quotient $d/\sigma_d<2$, the variations observed in the distribution of isolates for a given shared type were not statistically significant and could be due to a sampling bias. Inversely, if $d/\sigma_d>2$, then the differences observed in the distribution of isolates for a given shared type were statistically significant and not due to a potential sample bias.

### Use of Database for Epidemiologic Studies

Essentially working in a Caribbean setting for last 6 years with systematic typing of all *M. tuberculosis* isolates from Guadeloupe, Martinique, and French Guiana, we initially focused on spoligotypes that may be specific to our region. Of 259 shared types, 85 types were present in the Caribbean. Of these, 69 were common to the Caribbean and the rest of the world, and 16 were reported only from the Caribbean (types 5,12,13,14,15,30,63,66,68,72,76,77,94, 96,103,259). Although TB has a penchant to be latent for years or decades, because of an exhaustive (nearly 100%) recruitment of isolates from the French Caribbean for last 6 years, finding a previously unreported spoligotype in our region may constitute indirect evidence for a newly imported case of TB in most instances, particularly if an epidemiologic investigation does not suggest reactivation of old disease.

As far as global epidemiologic studies are concerned, this database also emphasizes the existence of highly prevalent families of *M. tuberculosis* isolates, e.g., the Beijing type, which represents a diverse collection of clones including the notorious multidrug-resistant strain W and other W-like drug-sensitive isolates (5,22). Studies focusing on *M. tuberculosis* isolates from developing countries, where TB is highly prevalent, would improve understanding of the worldwide circulation of tubercle bacilli and provide insights into their epidemiology, phylogeny, and virulence.

### Phylogenetic Reconstruction of *M. tuberculosis*

For phylogenetic analysis (23), a neighbor-joining tree was constructed by calculating the 1-Jaccard Index (10,24). This tree (Figure 2) incorporates the data for 252 *M. tuberculosis* shared types instead of the 259 allele types

[1]For this purpose, the independent sampling sizes for Europe and the USA were taken as $n_1$ and $n_2$, the number of individuals within a given shared-type "x" was $k_1$ and $k_2$, and in this case, the representativeness of the two samples was $p_1=k_1/n_1$ and $p_2=k_2/n_2$, respectively. To assess if the divergence observed between $p_1$ and $p_2$ was due to sampling bias or the existence of two distinct populations, the percentage of individuals ($p_0$) harboring shared-type "x" in the population studied was estimated by the equation $p_0= k_1+k_2/n_1+n_2=n_1p_1+n_2p_2/n_1+n_2$. The distribution of the percentage of shared-type "x" in the sample sizes $n_1$ and $n_2$ follows a normal distribution with a mean $p_0$ and a standard deviation of $\sqrt{p_0q_0/n_1}$ and $\sqrt{p_0q_0/n_2}$, respectively, and the difference $d=p_1-p_2$ follows a normal distribution of mean $p_0-p_0=0$ and of variance $\sigma_d^2=\sigma_{p1}^2+\sigma_{p2}^2=p_0q_0/n_1+p_0q_0/n_2$ or $\sigma_d^2=p_0q_0\ (1/n_1+1/n_2)$. The two samples being independent, the two variances were additive; the standard deviation $\sigma d=\sqrt{p_0q_0\ (1/n_1+1/n_2)}$ was calculated, and the homogeneity of the samples tested was assessed using the quotient $d/\sigma_d=p_1-p_2/\sqrt{p_0q_0(1/n_1=1/n_2)}$. If the absolute value of the quotient $d/\sigma_d<2$, the two samples were considered to belong to the same population (CI 95%) and the variation observed in the distribution of isolates for given shared types could be due to a sampling bias. Inversely, if $d/\sigma_d>2$, then the differences observed in the distribution of isolates for given shared types were statistically significant and not due to potential sample bias.

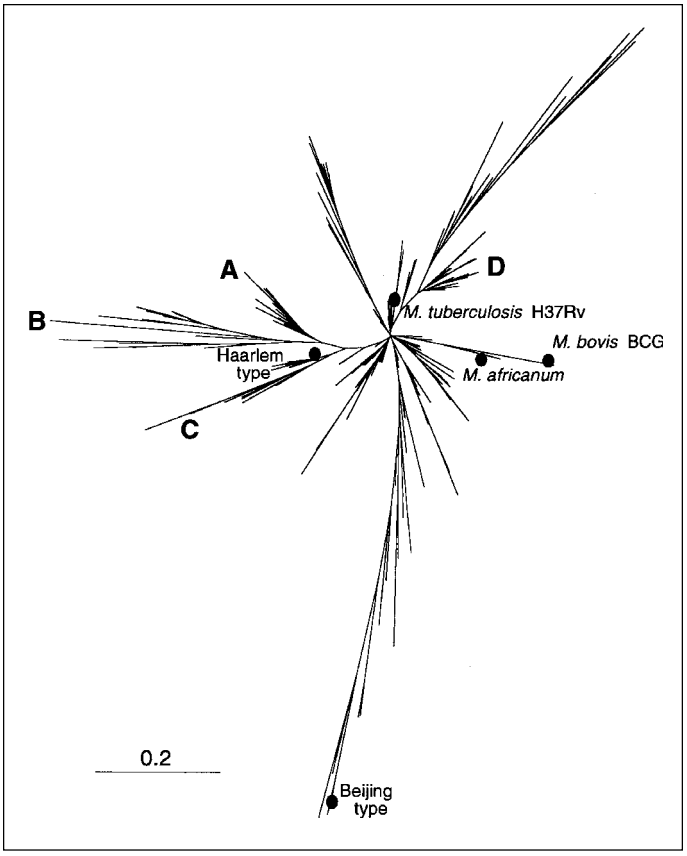


Figure 2. Phylogenetic tree of shared types of *Mycobacterium tuberculosis* constructed by pairwise comparison of patterns using the "1-Jaccard" index and the neighbor-joining algorithm. Approximately 15 branches may be visualized at an arbitrary distance of 0.2. The position of some reference strains (*M. tuberculosis* H37Rv, *M. bovis* BCG) or well-studied spoligotyping families of isolates (Beijing, Haarlem, and the *M. africanum* group) are also indicated.

described in the online database (types 253 to 259 were added recently after the completion of phylogenetic analysis). At an arbitrary distance of 0.2, one may easily distinguish nearly 15 branches that may contain significant phylogenetic information, as seen below for four selected branches (A to D) by combining results using independent genetic markers (Figure 3). As shown in Figure 2 and 3A, the homogeneous branch A (mainly present in Europe, West Africa, and South America) contains 20 types characterized by the absence of spacers 29 to 32 and 34. Such a family of isolates was recently described in Guinea-Bissau and also found to harbor a low copy number of IS*6110* (25). Information concerning *katG*283-*gyrA*95 allele combination was available for 5 of these 20 types and showed that branch A belonged to the major genotypic group 1 as defined previously (26) and may represent an ancestral clone of *M. tuberculosis* isolates originating in Africa, Asia, or both (27; this work). For this branch, VNTR information was available for 3 of 20 types and showed a high exact tandem repeat (ETR)-A copy number (between 4 to 7; Figure 3A), which is common both for *M. bovis* and *M. africanum* (8,28).

Branch B shared a common root with branch A (Figure 2) but was clearly distinct from the population in branch A, an observation corroborated both by VNTR and *katG*283-*gyrA*95 types (Figure 3B). All the isolates in branches A and B were of the major genetic group 1, as defined (26), except for a single isolate of the major genetic group 2 in branch B (type 199); the significance of this observation is not clear. Branch C was composed of two subbranches, which are likely to be of different phylogenetic significance (Figure 3C); the upper part related to the Haarlem family, as previously defined (15), and was highly homogeneous upon VNTR typing (alleles 32333), whereas the lower part was quite heterogenous (alleles 42431, 31333, 44553).

Finally, branch D comprised a subfamily of the spoligotypes that all missed spacers 33-36 (Figure 3D). This branch, which contained 30 different shared types, was easily

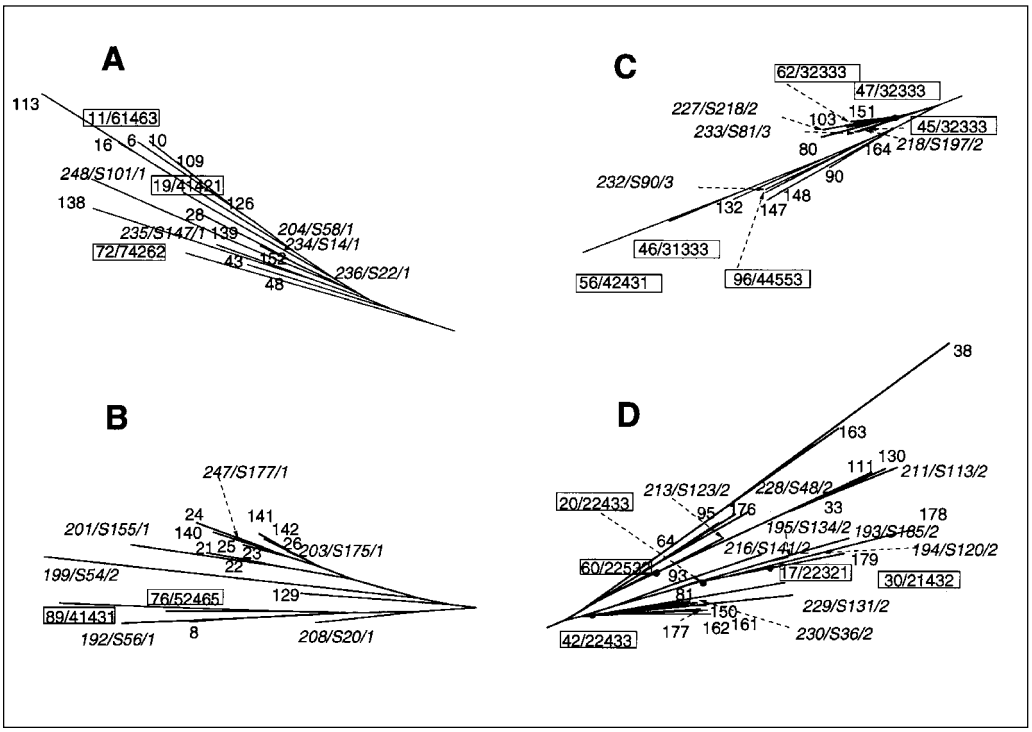


Figure 3. Enlargement of branches A to D from the *Mycobacterium tuberculosis* phylogenetic tree (Figure 2). Numbers in standard characters refer to spoligotype numbers according to our database; those in boxes describe both the spoligotype number and variable number of tandem DNA repeats (VNTR) allele designations. Italicized numbers refer to spoligotype followed by the Houston spoligotype designation (12), and the major genetic groups 1 to 3 defined on the basis of *katG*283-*gyrA*95 allele combination (24). A and B show distinct branches belonging essentially to the major genetic group 1 with a high exact tandem repeat (ETR)-A copy number; C and D show branches that include some strains of the "Haarlem family" belonging to the major genetic group 2 with a low ETR-A copy number.

characterized by simultaneous absence of spacers 21-24 and 33-36, and constitutes a highly ramified but homogeneous family on the basis of its belonging to the major genetic group 2 of Sreevatsan et al. (26), and the presence of two copies of the ETR-A allele upon VNTR typing. Frequently found in southern Europe and Central and South America, the ancestral type of this family (type 42) may have evolved by stepwise mutation to give, successively, types 20 and 17 (Figure 3D). This assumption is corroborated by the position of the respective types in the tree and their spoligotyping and VNTR patterns; type 42 (all spacers present except 21 to 24 and 33 to 36, VNTR 22433), type 20 (identical to type 42 plus a single missing spacer 3, VNTR identical to type 42), and type 17 (identical to type 20 plus a single missing spacer 13, VNTR 22321).

These results show that branches A and B are likely to be of an older evolutionary origin than branches C and D. Källenius et al. (25) hypothesized that branches A and B could find their evolutionary origin in West Africa, whereas branches C and D could be of European descent. However, since the global evolutionary rate of the DR locus may involve many independent mechanisms, this tree is likely to incorporate systematic yet unknown errors (6); therefore, a detailed analysis of the robustness of each potential phylogenetic link is under investigation.

## Conclusion

We have presented an update of a database of *M. tuberculosis* spoligotypes with a detailed description of 259 shared types. This database may help to address major aspects linked to recent mycobacterial reemergence, evolutionary history, and future epidemiologic studies. Our results demonstrate that a few major families of conserved spoligotypes are well distributed throughout the world, whereas others are specific for certain geographic regions. Thus, the current epidemiologic picture of TB appears to be based both on the persistence of ancestral clones of *M. tuberculosis* as well as those emerging more recently, e.g., the Beijing type (type 1 in our database), which also includes the MDR strain W from New York City. A future correlation between genotyping and resistance data and the respective prevalence of various clones region by region may provide more insight into the global circulation of TB and help establish priorities in TB control programs. For example, because we have typed all *M. tuberculosis* clinical isolates in our insular setting for last 6 years, introduction of a previously unreported clone in Guadeloupe may be detected and, when placed in epidemiologic context, may either be classified as a newly imported case of TB or as a reactivation. Simultaneously, an epidemiologic investigation around the case is immediately initiated by local health authorities. A comparison of the newly imported clone with those in the database sometimes suggests a probable link to a specific community or, alternatively, regional, national, or intercontinental importation of the disease.

Concerning the global phylogeny of *M. tuberculosis*, the pairwise comparison of the 252 shared types by calculation of the 1-Jaccard index and the neighbor-joining algorithm underscored phylogenetic relationships between some of the families of spoligotypes described. Four major families of spoligotypes (branches A-D) were discussed in detail, and the results were corroborated by VNTR and *katG—gyrA* polymorphism data, which support the robustness of the branchings proposed. Nevertheless, a detailed and more exhaustive analysis of evolutionary and historical spreading of the different families of tubercle bacilli is a long-term goal requiring a never-ending compilation of data. Ideally, this database could be expanded to incorporate detailed *M. bovis* and *M. africanum* results so as to infer the global phylogeny of all members of the *M. tuberculosis* complex.

It has been suggested that the evolutionary rate of *M. tuberculosis* may be strain dependent (29). In this context, our investigation also pointed out a previously unnoticed link between spoligotypes and the *katG—gyrA* polymorphism (Figure 3), i.e., the isolates in the spoligotyping-defined branch A belonged to the major genetic group 1 of Sreevatsan et al. (26), whereas those in branch D belonged to the major genetic group 2. Since the isolates in these branches came from diverse geographic areas, we suggest that the pace of the molecular clock of the DR locus might be much slower than that of other markers, such as IS*6110*. This assumption is supported by a recent study on the evolutionary origin of the DR locus of *M. tuberculosis* (19). Finally, by comparing observations with outcomes of a stepwise mutation model, the insertion sequences of the tubercle bacilli are far from equilibrium; indeed, transposition parameters appear to have a much stronger effect on IS*6110* copy number distribution than epidemic parameters and have a direct action on bacterial diversity of the *M. tuberculosis* complex (30). New studies are needed to clarify the complex relationships between epidemic parameters, selection factors, and genomic evolutionary mechanisms of the tubercle bacilli.

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### References

1. World Health Organization. Global tuberculosis control. WHO report 1999. Geneva: The Organization; 1999.
2. Slutkin G. Global AIDS 1981-1999: the response. Int J Tuberc Lung Dis 2000;4:S24-S33.
3. Snider DE, Castro KG. The global threat of drug resistant tuberculosis. N Engl J Med 1998;338:1689-90.
4. Long R, Nobert E, Chomyc S, van Embden J, McNamee C, Duran RR, et al. Transcontinental spread of multidrug-resistant *Mycobacterium bovis.* Am J Respir Crit Care Med 1999;159:2014-17.
5. Bifani PJ, Mathema B, Liu Z, Moghazeh SL, Shopsin B, Tempalski B, et al. Identification of a W variant outbreak of *Mycobacterium tuberculosis* via population-based molecular epidemiology. JAMA 1999;282:2321-7.

6. Sola C, Devallois A, Horgen L, Maïsetti J, Filliol I, Legrand E, et al. Tuberculosis in the Carribean: using spacer oligonucleotide typing to understand strain origin and transmission. Emerg Infect Dis 1999;5:404-14.
7. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 1997;35:907-14.
8. Frothingham R, Meeker-O'Connell WA. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology 1998;144:1189-96.
9. Grimont PAD. TAXOTRON instruction manual. Paris: Taxolab, Institut Pasteur; 1996.
10. Jaccard P. Nouvelles recherches sur la distribution florale. Bulletin de la Société Vaudoise de Sciences Naturelles 1908;44:223-70.
11. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406-25.
12. Soini H, Pan X, Amin A, Graviss EA, Siddiqui A, Musser JM. Characterization of *Mycobacterium tuberculosis* isolates from patients in Houston, Texas, by spoligotyping. J Clin Microbiol 2000;38:669-76.
13. Groenen PMA, Bunschoten AE, van Soolingen D, van Embden JDA. Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method. Mol Microbiol 1993;10:1057-65.
14. Hancock JM. The contribution of slippage-like processes to genome evolution. J Mol Evol 1995;41:1038-47.
15. Kremer K, van Soolingen D, Frothingham R, Haas WH, Hermans PWM, Martin C, et al. Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* strains: interlaboratory study of discriminatory power and reproducibility. J Clin Microbiol 1999;37:2607-18.
16. Fang Z, Doig C, Kenna DT, Smittipat N, Palittapongarnpim P, Watt B, et al. IS*6110*-mediated deletions of wild-type chromosomes of *Mycobacterium tuberculosis.* J Bacteriol 1999;181:1014-20.
17. Fang Z, Morrison N, Watt B, Doig C, Forbes KJ. IS*6110* transposition and evolutionary scenario of the direct repeat locus in a group of closely related *Mycobacterium tuberculosis* strains. J Bacteriol 1998;180:2102-9.
18. Filliol I, Sola C, Rastogi N. Detection of a previously unamplified spacer within the DR locus of *Mycobacterium tuberculosis*: Epidemiological implications. J Clin Microbiol 2000;38:1231-4.
19. van Embden JDA, van Gorkom T, Kremer K, Jansen R, Van der Zeijst BAM, Schouls LM. Genetic variation and evolutionary origin of the direct repeat locus of *Mycobacterium tuberculosis* complex bacteria. J Bacteriol 2000;182:2393-01.
20. Buikstra JE. Paleoepidemiology of tuberculosis in the Americas. In: Palfi G, Dutour O, Deak J, Hutas I, editors. Tuberculosis: past and present. Szeged, Hungary: Golden Book Publisher Ltd.; 1999. p. 479-94.
21. Ortner DJ. Paleopathology: implications for the history and evolution of tuberculosis. In: Palfi G, Dutour O, Deak J, Hutas I, editors. Tuberculosis: past and present. Szeged, Hungary: Golden Book Publisher Ltd; 1999. p. 255-61.
22. Kurepina NE, Sreevatsan S, Plikaytis BB, Bifani PB, Connell ND, Donneelly RJ, et al. Characterization of the phylogenetic distribution and chromosomal insertion sites of five IS*6110* elements in *Mycobacterium tuberculosis*: non random integration in the *dna*A-*dna*N region. Tuber Lung Dis 1998;79:31-42.
23. Nei M. Phylogenetic analysis in molecular evolutionary genetics. Annu Rev Genet 1996;30:371-403.
24. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406-25.
25. Källenius G, Koivula T, Ghebremichael S, Hoffner SE, Norberg R, Svensson E, et al. Evolution and clonal traits of *Mycobacterium tuberculosis* in Guinea-Bissau. J Clin Microbiol 1999;37:3872-8.
26. Sreevatsan S, Pan X, Stockbauer K, Connell N, Kreiswirth B, Whittam T, et al. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc Natl Acad Sci U S A 1997;97:9869-74.
27. Castets M, Boisvert H, Grumbach F, Brunel M, Rist N. Les bacilles tuberculeux de type africain: note préliminaire. Revue de la Tuberculose et de Pneumologie 1968;32:179-84.
28. Frothingham R, Strickland PL, Bretzel G, Ramaswamy S, Musser JM, Williams DL. Phenotypic and genotypic characterization of *Mycobacterium africanum* isolates from West Africa. J Clin Microbiol 1999;37:1921-6.
29. Warren GM, Richardson M, Sampson S, Bourn W, van der Spuy G, Hide W, et al. RFLP analysis of *M. tuberculosis* demonstrates strain-dependent evolution. Int J Tuberc Lung Dis 1999;3(Suppl. I);S38.
30. Tanaka MM, Small PM, Salamon H, Feldman MW. The dynamics of repeated elements: applications to the epidemiology of tuberculosis. Proc Natl Acad Sci U S A 2000;97:3532-7.
31. Bauer J, Andersen AB, Kremer K, Miörner H. Usefulness of spoligotyping to discriminate IS*6110* low-copy-number *Mycobacterium tuberculosis* complex strains cultures in Denmark. J Clin Microbiol 1999;37:2602-6.
32. Bonora S, Gutierrez MC, Perri GD, Brunello F, Allegranzi B, Ligozzi M, et al. Comparative evaluation of Ligation-mediated PCR and spoligotyping as screening methods for genotyping of *Mycobacterium tuberculosis* strains. J Clin Microbiol 1999;37:3118-23.
33. Diaz R, Kremer K, de Haas PEW, Gomez RI, Marrero A, Valdivia JA, et al. Molecular epidemiology of tuberculosis in Cuba outside of Havana, July 1994-June 1995: utility of spoligotyping versus IS*6110* restriction fragment length polymorphism. Int J Tuberc Lung Dis 1998;2:743-50.
34. Douglas JT, Qian L, Montoya JC, Sreevatsan S, Musser J, van Soolingen D, et al. Detection of a novel family of tuberculosis isolates in the Philippines. 97th general meeting of the American Society for Microbiology. Washington: ASM Press; 1997. p.572.
35. Escalante P, Ramaswamy S, Sanabria H, Soini H, Pan X, Valiente-Castillo O, et al. Genotypic characterization of drug-resistant *Mycobacterium tuberculosis* isolates from Peru. Tuber Lung Dis 1998;79:111-18.
36. Goguet dela Salmonière YO, Li HM, Torrea G, Bunschoten A, van Embden JDA, Gicquel B. Evaluation of spoligotyping in a study of the transmission of *Mycobacterium tuberculosis.* J Clin Microbiol 1997;35:2210-14.
37. Goyal M, Saunders NA, van Embden JDA, Young DB, Shaw RJ. Differentiation of *Mycobacterium tuberculosis* isolates by spoligotyping and IS*6110* restriction fragment length polymorphism. J Clin Microbiol 1997;35:647-51.
38. Heyderman RS, Goyal M, Roberts P, Ushewokunze S, Zishou S, Marshall BG, et al. Pulmonary tuberculosis in Harare, Zimbabwe: analysis by spoligotyping. Thorax 1998;53:346-50.
39. Niang MN, Goguet de la Salmonière YO, Samb A, Hane AA, Cisse MF, Gicquel B, et al. Characterization of *M. tuberculosis* strains from West-African patients by spoligotyping. Microbes Infect 1999;1:1189-92.
40. Popa MI, Goguet de la Salmonière YO, Teodor I, Popa L, Stefan M, Banica D, et al. Genomic profile of Romanian *M. tuberculosis* strains appreciated by spoligotyping. Rom Arch Microbiol Immunol 1997;56:63-75.
41. de C Ramos M, Soini H, Roscanni GC, Jaques M, Villares MC, Musser JM. Extensive cross-contamination of specimens with *Mycobacterium tuberculosis* in a reference laboratory. J Clin Microbiol 1999;37:916-19.
42. Taylor GM, Goyal M, Legge AJ, Shaw RJ, Young D. Genotypic analysis of *Mycobacterium tuberculosis* from medieval human remains. Microbiology 1999;145:899-904.
43. van der Zanden AG, Hoentgen AH, Heilmann FG, Weltvreden EF, Schouls LM, van Embden JD. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* complex in paraffin wax embedded tissues and in stained microscopic preparations. Mol Pathol 1998;51:209-14.
44. van Soolingen D, Qian L, de Haas PEW, Douglas JT, Traore H, Portaels F, et al. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of East Asia. J Clin Microbiol 1995;33;3234-8.

# Transmission of an Arenavirus in White-Throated Woodrats (*Neotoma albigula*), Southeastern Colorado, 1995–1999

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From 1995 to 1999, we conducted longitudinal studies of white-throated woodrats (*Neotoma albigula*) in southeastern Colorado. Forty-five (42.9%) of 105 female and 15 (26.8%) of 56 male *N. albigula* had antibodies against Whitewater Arroyo virus (WWAV). Sixteen female and three male *N. albigula* seroconverted during the study period, most of them during July-November, when population densities are highest. Analyses of longevity data, minimum numbers alive and infected, movements, and weight data suggest that the dominant mode of WWAV transmission among white-throated woodrats in Colorado is direct contact. WWAV was recently reported to cause fatal infection in humans. Our findings will lead to better assessment of the public health threat posed by infected woodrats and may be useful in predicting periods of increased risk for human infection.

Arenaviruses (order Mononegavirales, family Arenaviridae, genus *Arenavirus*) are grouped into two complexes on the basis of phylogenetic analyses of viral RNA and serology: the Old World or lymphocytic choriomeningitis-Lassa virus complex viruses and the New World or Tacaribe complex viruses (1). The principal hosts of the Old World and New World arenaviruses are murine (subfamily Murinae) and sigmodontine (subfamily Sigmodontinae) rodents, respectively, of the order Rodentia, family Muridae. Arenaviruses establish chronic infections in their specific rodent hosts.

In a recent study, antibody to arenaviruses was detected in *Neotoma* sp. rodents (2), suggesting that in the southern and western United States an unrecognized arenavirus was associated with woodrats, genus *Neotoma*. Fulhorst et al. (3) then isolated an arenavirus from a white-throated woodrat, *Neotoma albigula*, captured at Whitewater Arroyo, New Mexico. Whitewater Arroyo virus (WWAV) is antigenically and genetically most closely related to Tamiami virus, which was isolated from hispid cotton rats (*Sigmodon hispidus*) in Everglades National Park in south Florida and had been the only Tacaribe complex arenavirus known to occur in North America (4).

We conducted longitudinal mark-recapture studies of rodents at study sites in Colorado to determine the epizoology of hantaviruses at those sites. From January 1995 to November 1999, we collected blood samples from woodrats, including sequential samples from individual rats, at a short-grass prairie habitat in a canyon in southeastern Colorado. These studies showed that WWAV is highly prevalent in woodrats. We present data on the dynamics of WWAV infection and epizoologic and kinetic factors affecting the natural history of this virus.

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## Materials and Methods

### Study Sites

Rodents were trapped at the U.S. Army Pinon Canyon Maneuver Site (PCMA) located in Las Animas County, southeastern Colorado. The site (area >1,040 $km^2$) is a short-grass prairie habitat managed by the Directorate of Environmental Compliance and Management, Fort Carson, Colorado. We established two study sites, MRC (at the mouth of Red Rocks Canyon) and RRC (inside Red Rocks Canyon) (Appendix).

### Trapping and Processing

For 3 nights approximately every 6 weeks from January 1995 to December 1999, we placed 145 traps in a web pattern at the MRC site and 75 traps in an irregular grid at the RRC site. A Sherman trap, 7.6 cm x 8.9 cm x 22.9 cm, (H.B. Sherman Traps, Inc., Tallahassee, FL) was placed at each trap station, and the locations were marked. We conducted 14,790 trap-nights at the MRC site and 7,650 at the RRC site, using the mark-recapture-release methods recommended by the Centers for Disease Control and Prevention (5) as described for Colorado sites (6).

Weight classes were defined for white-throated woodrats as follows: for female rats, 50 g to 100 g = juvenile, 101 g to 150 g = subadult, and 151 g to 300 g = adult; and for male rats, 50 g to 100 g = juvenile, 101 g to 200 g = subadult, and 201 g to 300 g = adult (7).

Blood samples were stored on dry ice and transported to the Fort Collins laboratory, where they were stored at -70°C

until they were tested for antibody (8). Data were analyzed by Epi-Info, version 5.0 (9).

### Antibody Assays

All samples were tested for immunoglobulin (Ig) G antibody by indirect immunofluorescence assay (IFA) (10), using a mixture of anti-rat and anti-mouse antibodies conjugated to fluorescein isothiocyanate, and by enzyme-linked immunosorbent assay (ELISA) (11), using WWAV antigens prepared from Vero E6 cell monolayers infected with WWAV prototype strain AV 9310135 (Table 1).

We calculated relative abundance (minimum number alive [MNA]), minimum number known to have antibody to WWAV (MNI), estimated standing prevalences of antibody to WWAV (ESP [MNI/MNA]), and incidence of infection (number of new infections/100 rats/month) for *N. albigula*. If a sample had discordant results by the two methods, we did not consider it positive. Seroconversions were defined as fourfold or greater increases or decreases in IgG antibody titer as determined by ELISA.

## Results

The first woodrats were captured in May 1995 at each site. Of 306 samples tested, 207 (67.6%) were negative in both assays, and 37 (12.1%) were positive at 4+ for IFA and >51,200 for ELISA (Table 1). Antibody, as determined by both IFA and ELISA, was detected in all three woodrat species (*N. albigula*; *N. mexicana*, Mexican woodrat; and *N. micropus*, southern plains woodrat) captured at PCMS (Table 2). Forty-five (41.7%) of 108 female and 15 (26.8%) of 56 male *N. albigula* (60 [36.6%] of 164 seropositive rats) captured at least once had antibody to WWAV. Of these 164 rodents, 108 (65.9%) were female, representing 45 (75%) of all seropositives. These proportions did not differ significantly (Yates corrected chi square 1.19, p = 0.28). Two of 25 Mexican woodrats and 2 of 11 southern plains woodrats had antibody to WWAV (Table 3). Seroconversion was detected in 16 *N. albigula* (13 female and 3 male; Figure 1); most seroconversions (in 9 female and 3 male rats) occurred from July to November.

The range of MNA for *N. albigula* during this study was 0 to 23 (August 1995), with 11, 17, and 10 later in that year (Figure 2). Peak MNAs were in August 1995, August to September 1996, July 1997, June and September 1998, and August to September 1999, indicating months of peak population density. Highest MNIs were also during August to November 1995 (Figure 2). ESPs peaked during November 1995 to May 1996 and generally declined thereafter until late summer 1999 (Figure 2).

Weights varied considerably from capture to capture, with increases being the greatest in younger woodrats, a few increasing 20 g to 50 g in a 3- to 4-month period. A few adult woodrats lost weight, as much as 20 g within a 6-week period. Most large weight losses occurred during late summer to the end of autumn or in mid-winter (data not shown). However, for most of the observation time, the woodrats' weight either increased 5 g to 15 g or remained stable.

Ten (18.9%) of 53 juvenile female woodrats and none of 17 juvenile male woodrats had antibody to WWAV. Thirty-two (45.1%) of 71 subadult female rats and 4 (28.6%) of 14 subadult male rats were seropositive. Of adults, 34 (55.7%) of

Table 2. Antibody to Whitewater Arroyo virus in woodrats (*Neotoma* spp.) by sex, Piñon Canyon Maneuver Site, Colorado, 1995-1999

| *Neotoma* spp. | | Single samples Female | Single samples Male | Multiple samples Female | Multiple samples Male |
|---|---|---|---|---|---|
| *albigula* | Pos. | 21 (33.3%) | 10 (22.7%) | 24 (53.3%) | 5 (41.7%) |
| | Neg. | 42 (66.7%) | 34 (77.3%) | 21 (46.7%) | 7 (58.3%) |
| *mexicana* | Pos. | 0 (0) | 2 (20%) | 0 | 0 |
| | Neg. | 9 (100%) | 8 (80%) | 4 | 2 |
| *micropus* | Pos. | 1 (16.7%) | 1 | 0 | 0 |
| | Neg. | 5 (83.3%) | 1 | 2 | 1 |

Table 3. Prevalence[a] of antibody to Whitewater Arroyo virus in rats collected in southeastern Colorado, 1995-1999

| Site | *Neotoma albigula* | *N. mexicana* | *N. micropus* | *Sigmodon hispidus* |
|---|---|---|---|---|
| Mouth of Red Rocks Canyon | 26/64 (40.6%) | 0 | 1/2 (50%) | 0/86 (0) |
| Inside Red Rocks Canyon | 12/36 (33.3%) | 2/25 (8.0%) | 1/9 (11.1%) | 0/46 (0) |
| Total | 38/100 (38%) | 2/25 (8.0%) | 2/11 (13.2%) | 0/132 (0) |

[a]Number of rats seropositive/number of rats sampled (%).

Table 1. Indirect fluorescent antibody[a] (IFA) and enzyme-linked immunosorbent assays[b] (ELISA) for antibody to Whitewater Arroyo virus (WWAV) in 306 blood samples from white-throated woodrats (*Neotoma albigula*) captured in southeastern Colorado, 1995-1999

| | ELISA | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| IFA | <100 | 100 | 200 | 400 | 800 | 1,600 | 3,200 | 6,400 | 12,800 | 25,600 | >51,200 |
| 0 | 206 | 4 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1+ | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2+ | 3 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3+ | 3 | 0 | 1 | 1 | 4 | 1 | 0 | 0 | 1 | 0 | 0 |
| 4+ | 1 | 1 | 2 | 1 | 0 | 2 | 12 | 5 | 7 | 9 | 37 |

[a]Twelve-well IFA spot slides were coated with test antigens consisting of a mixture of Vero E6 cells infected with WWAV prototype strain AV 9310135 and Vero E6 cells infected with Amapari virus prototype strain BeAn 70563; uninfected Vero E6 cells were controls. Blood samples were diluted 1:100 and tested for positivity at that dilution, providing somewhat less sensitivity but greater specificity than using a lower starting dilution. Each sample was scored as a negative (0 or 1+) or positive (2+, 3+, or 4+) serologic reaction. Antibody-positive samples were titrated in twofold dilutions to determine endpoints.

[b]All samples were tested for immunoglobulin (Ig) G antibody by ELISA. The infected cells were sonicated and detergent (Triton X-100; Sigma Chemical Co., St. Louis, MO) treated; control antigens were prepared from uninfected Vero E6 cells. The working dilution of the test antigen was determined by box-titration against a homologous hyperimmune mouse ascitic fluid prepared against WWAV. Test and control antigens were diluted in 0.01 M phosphate-buffered saline, pH 7.4, and coated onto flat-bottom wells in 96-well polyvinyl chloride assay plates (Becton Dickinson Labware, Oxnard, CA). For screening purposes, 1:100 dilutions of bloods were tested; positives were titrated in serial twofold dilutions. Bound antibody was detected by using a mixture of goat anti-*Rattus rattus* IgG peroxidase conjugate and goat anti-*Peromyscus leucopus* IgG peroxidase conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and ABTS (Microwell Peroxidase Substrate System, Kirkegaard & Perry) was used as the enzyme indicator. Optical densities (OD) at 410 nm (reference = 490 nm) were measured with a Dynatech MR 5000 microplate reader (Dynatech Industries, Inc., McLean, VA). A blood sample was considered positive if the OD with the test antigen was at least 0.300 and at least twice that of the same sample with the control antigen, providing the OD with the control antigen was <0.150.

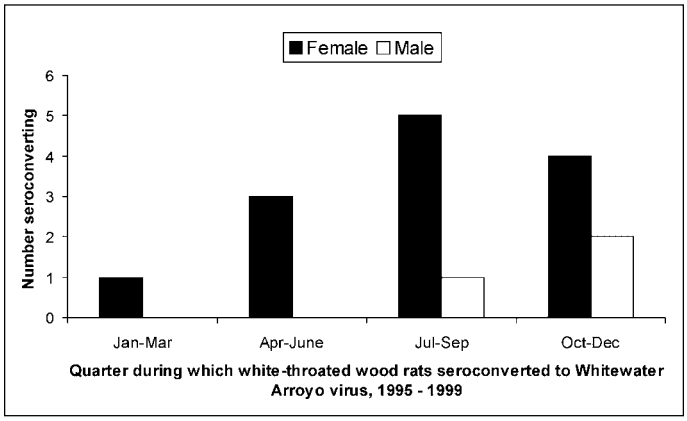


Figure 1. Quarter in which white-throated woodrats were first noted to have acquired antibody to Whitewater Arroyo virus in MRC and RRC sites, southeastern Colorado, 1995–1999.

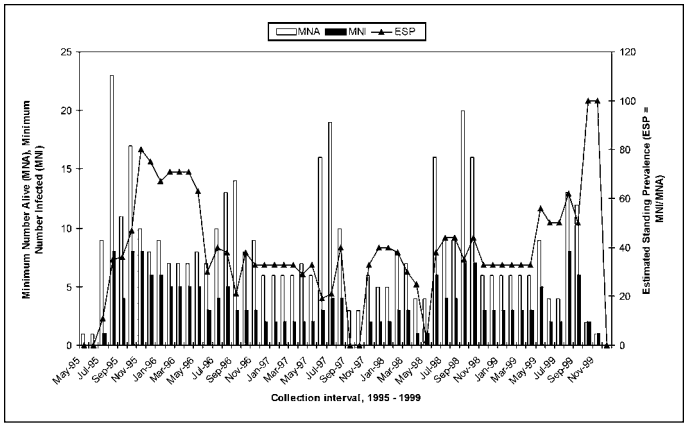


Figure 2. Minimum number alive (MNA), minimum number infected (MNI) with Whitewater Arroyo virus, and estimated standing prevalence (ESP=MNI/MNA), white-throated woodrats, southeastern Colorado, 1995–1999.

61 female rats and 18 (45%) of 40 male rats were seropositive. In all, 14.3% of juveniles, 42.3% of subadults, and 51.5% of adult *N. albigula* had antibody to WWAV. None of eight gravid (as determined by increased weight and palpation) adult female rats and one that delivered a litter in the trap had antibody to WWAV.

Among 54 female and 33 male *N. albigula* captured only once, mean apparent longevity (number of months from first time captured and seropositive to last time captured) of 44 seropositive female rats was 7.8 months (2 to 19 months) and 10 seropositive male rats was 8.1 months (2 to 22 months). Mean apparent longevity (calculated from first to last time captured) of all seronegative female *N. albigula* was 8.1 months (2 to 12 months) and of all seronegative male *N. albigula* was 7.9 months (2 to 20 months). Mean longevities of uninfected and infected *N. albigula* did not differ significantly (Yates corrected chi-square 0.43, p = 0.51).

From 1995 to 1999 at the MRC site, *N. albigula* were captured in 52 of 145 traps. At the RRC site, rats were captured in 20 of 25 traps on the north line and 14 of 25 traps on the south line. Incidence was calculated by using the month in which antibody was first detected in recaptured *N. albigula*; data for both sites were pooled. In summary, 5-year incidence rates from January to December were 0, 0, 3.8, 0, 3.6, 0, 2.0, 5.9, 3.3, 4.4, 9.7, and 0. Overall incidences were 1.2 for December to July and 5.8 for August to November.

## Conclusion

The characteristics and geographic distribution of WWAV have been described (2,3). Kosoy et al. (2) detected anti-arenaviral antibody in 1 of 7 white-throated woodrats trapped near Hesperus, La Plata County, southwestern Colorado. Arenaviruses are associated with persistent infections in the natural vertebrate host, even when the host is producing antibody (12). WWAV may also be maintained transseasonally in long-lived, persistently infected woodrats.

The prototype isolate of WWAV was from an *N. albigula* captured in McKinley County, New Mexico, approximately 500 km southwest of our study site. Kosoy et al. (2) did not detect anti-arenaviral antibody in three other *N. albigula*, five *N. cinerea* (bushy-tailed woodrats), two Mexican woodrats, or nine southern plains woodrats in Colorado, although they did detect antibody in Mexican woodrats collected in Arizona, New Mexico, and Utah. More than one arenavirus may be occurring in rats of the genus *Neotoma* and in deer mice and other rodents in southeastern Colorado. We have provided the first evidence that Mexican and southern plains woodrats in Colorado are infected with a WWAV-like arenavirus. Kosoy et al. did not detect anti-arenaviral antibody in eight hispid cotton rats from Colorado, and we did not detect antibody to WWAV in 132 of these rodents from PCMS. We detected antibody to WWAV in 1 of 2 white-throated woodrats collected in Oklahoma (R. Nisbett et al., unpub. data). Thus, WWAV or a related virus is distributed widely in various *Neotoma* species in the southwestern United States, and serologic evidence strongly suggests that *N. albigula* is the primary rodent host of WWAV virus in southeastern Colorado.

Fulhorst (unpub. data) has shown that the arenavirus at PCMS differs genetically from the prototype strain, but it is clearly more similar to WWAV than to either Tamiami or Bear Canyon virus, the latter having been isolated from *Peromyscus* sp. rodents in California. Furthermore, the arenavirus associated with deer mice that had antibody to WWAV was also confirmed to be WWAV and may represent spillover transmission from infected rats.

White-throated woodrats have been described as solitary animals, with little social interaction among adults except for mating, which occurs mostly during late winter and spring until mid-June (7). The rate of new infections at PCMS is highest when the rat populations are largest (in late summer and fall), suggesting that mating may not be central to the transmission of this virus. Olsen (13) found that shelter-site selection by these rodents is based on quantity of ground-level vegetation and debris for cover. As neither the large area at the MRC site (traps 109 to 143) nor the central area of the RRC site include such flora or much debris, it is not surprising that we did not capture white-throated woodrats in these areas. Nevertheless, if white-throated woodrats gather in plots of preferred habitat, rat-to-rat contact is likely to increase, particularly when vegetative growth is reduced during the late fall-spring period. At high densities of populations of competing rat species, the probability of agonistic encounters increases. The Mexican woodrat, *N. mexicana,* may compete with *N. albigula* for den sites in

some areas (14), although the latter species appears dominant at the MRC and RRC sites. Such agonistic encounters could be a possible mechanism for interspecific WWAV transmission. Alternatively, another virus antigenically distinct from those used for the IFA (WWAV and Amapari virus) may be occurring in these species, and lack of sufficient cross-reactivity of antibody to this virus may explain the few positives detected.

The duration of this study allowed us to obtain data regarding the epizoology of WWAV. The high overall prevalence (60/164 = 36.6%) of antibody to WWAV at PCMS and the fact that adults (51.5% seropositive) were at higher risk for having antibody to WWAV indicate that infection with this virus is common and that older rats are more likely to be infected. Susceptibility of juvenile rats may be delayed as a result of protection by maternal antibody or because the behavior patterns of juvenile rats do not put them at risk for exposure to a contagious adult. The entrance of a cohort of young nonsusceptible rats may reduce the prevalence of antibody in the overall population.

Analyses of crude night-to-night movements showed no obvious difference between movement by white-throated woodrats with antibody to WWAV and those without, further suggesting that infection does not affect behavior. Increased rates of transmission are more likely during periods of higher population density because of increased overlapping home ranges and, therefore, increased rodent-to-rodent contact and potential for transmission.

Horizontal modes of transmission by rodent-to-rodent contact, particularly between mature rats, may be central to the maintenance of this virus in its rodent reservoir, but contact associated with mating is unlikely to be an important mechanism for transmission. The reason for this is unknown but could be related to sampling bias. The presence of long-lived, virus-infected rodents appears important for transseasonal persistence of hantaviruses (15) and may be equally important for arenaviruses. Our observations may support such a hypothesis, given that white-throated woodrats at PCMS lived for as long as 22 months, some of them with antibody living longer than 1 year. Nonetheless, serologic data alone cannot determine if the woodrats sampled in this study were chronically infected and, if so, what type of infection resulted.

Arenaviruses characteristically produce chronic infections and can cause measurable physiologic effects in their reservoir hosts (12). Persistent infection after intranasal infection with Junin virus can increase deaths, reduce growth, and markedly reduce reproductive efficiency in its reservoir host, *Calomys musculinus* (16). A similar cyclical relationship has been observed between *C. callosus* and Machupo virus (12). In our study, none of 8 gravid adult *N. albigula* at PCMS (four each at MRC and RRC sites) had antibody to WWAV, whereas 34 (60%) of 57 other adult female *N. albigula* that were not apparently gravid were seropositive. This may indicate that WWAV infection influences breeding in female *N. albigula* at these sites. Given that only 10 (14.3%) of 70 juvenile *N. albigula* were seropositive and that WWAV-infected female rats may have reduced reproductive capacity, vertical transmission of WWAV is unlikely to cause infection rates as high as we observed. Although persistent infection of reservoir hosts is a hallmark of arenaviruses, not all viruses in this family establish chronic infections in all vertebrate hosts and such infections may be difficult to detect. Tamiami virus can be detected in kidneys and other organ systems of experimentally infected hispid cotton rats and virus can be recovered readily from urine (17,18), but a chronic viremic carrier state does not develop. Determining whether the long-lived white-throated woodrats with antibody at PCMS continue to shed WWAV will be key to our understanding of the persistence of this virus.

If the reproductive capacity of white-throated woodrats is compromised by infection with WWAV and the virus is more readily spread among rats when the population is large, we would expect subsequent generations to be smaller. This could be the result of decreased reproductive capacity in a significant portion of the mating female population ([65.9% of the adult population was female] x [41.7% of adult females infected] = 27.5% of the total adult female population potentially, if temporarily, infertile), as adults are culled from the population by predators, disease, environmental hazards, and aging. The female preponderance in the *N. albigula* populations at MRC and RRC sites might indicate a sex-biased reduction in male rats resulting from intermittent food deprivation, as occurs with eastern woodrats (19).

Two types of observation bias are likely to have occurred in this study design. Most rats included in this longitudinal study were captured only once, and approximately 40% of first captures were in the first weight class. Rats captured only once, then, tend to be young. As rats observed for >13 weeks appear to have a greater relative risk for infection, and if age and season influence behavior, long-term survivors are likely to differ from those captured only once with respect to both exposures and presence of antibody. Consequently, the associations observed between the exposures and presence of antibody are likely to be biased towards a stronger association. Second, recording data subjectively, as in detecting pregnancy in female white-throated woodrats, may lead to misclassification bias. Some female rats thought not to be gravid may have been in the early stages of pregnancy or for other reasons may not have been correctly identified as gravid. Since palpation of fetuses was the main criteria for defining pregnancy, it is less likely that rats were incorrectly identified as gravid when they were not.

This study provided further information about the geographic distribution of arenaviruses associated with *Neotoma* sp. rodents. The data contribute to our understanding of the intraspecific transmission of this virus and the mechanisms underlying viral maintenance in natural populations of the white-throated woodrat, the probable rodent reservoir for WWAV in southeastern Colorado. WWAV was recently associated with fatal hemorrhagic infections in humans (20). Thus, knowledge of factors influencing WWAV transmission and changes in prevalence of infection of this virus in its natural host, the white-throated woodrat, may be useful in predicting periods of increased risk for human infections.

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## References

1. Peters CJ, Buchmeier M, Rollin PE, Ksiazek TG. Arenaviruses. In: Fields BN, Knipe DM, Howley PM, editors. Fields virology. 3rd ed. Philadelphia: Lippincott-Raven Publishers; 1996. p. 1521-44.
2. Kosoy MY, Elliott LH, Ksiazek TG, Fulhorst CF, Rollin PE, Childs JE, et al. Prevalence of antibodies to arenaviruses in rodents from the southern and western United States: evidence for an arenavirus associated with the genus *Neotoma*. Am J Trop Med Hyg 1996;54:570-6.
3. Fulhorst CF, Bowen MD, Ksiazek TG, Rollin PE, Nichol ST, Kosoy MY, et al. Isolation and characterization of Whitewater Arroyo virus, a novel North American arenavirus. Virology 1996;224:114-20.
4. Calisher CH, Tzianabos T, Lord RD, Coleman PH. Tamiami virus, a new member of the Tacaribe group. Am J Trop Med Hyg 1970;19:520-6.
5. Mills JN, Yates TL, Ksiazek TG, Peters CJ, Childs JE. Long-term studies of hantavirus reservoir populations in the southwestern United States: rationale, potential, and methods. Emerg Infect Dis 1999;5:95-101.
6. Calisher CH, Sweeney W, Mills JN, Beaty BJ. Natural history of hantaviruses in western Colorado, 1994-97. Emerg Infect Dis 1999;5:126-34.
7. Fitzgerald JP, Meaney CA, Armstrong DM. Mammals of Colorado. Niwot (CO): University Press of Colorado; 1994. 467 pages.
8. Feldmann HA, Sanchez A, Morzunov S, Spiropoulou CF, Rollin PE, Ksiazek TG, et al. Utilization of autopsy RNA for the synthesis of the nucleocapsid antigens of a newly recognized virus associated with hantaviral pulmonary syndrome. Virus Res 1993;30:351-67.
9. Dean AG, Dean JA, Burton AH, Dicker RC. Epi Info, Version 5: a word processing, database, and statistics program for epidemiology on microcomputers. Atlanta: Centers for Disease Control; 1990. p. 1-384.
10. Wulff H, Lange JV, Webb PA. Interrelationships among arenaviruses measured by indirect immunofluorescence. Intervirology 1978;9:344-50.
11. Childs JE, Ksiazek TG, Spiropoulou CF, Krebs JW, Morzunov S, Maupin GO, et al. Serologic and genetic identification of *Peromyscus maniculatus* as the primary rodent reservoir for a new hantavirus in the southwestern United States. J Infect Dis 1994;169:1271-80.
12. Childs JE, Peters CJ. Ecology and epidemiology of arenaviruses and their hosts. In: Salvato MS, editor. The arenaviridae. New York: Plenum Press; 1993; p. 331-84.
13. Olsen RW. Shelter-site selection in the white-throated wood rat, *Neotoma albigula*. J Mammal 1973;54:594-610.
14. Howe R. Agonistic behavior of three sympatric species of wood rats (*Neotoma mexicana, N. albigula,* and *N. stephensi*). J Mammal 1978;59:780-6.
15. Mills JN, Ksiazek TG, Peters CJ, Childs JE. Long-term studies of hantavirus reservoir populations in the southwestern United States: a synthesis. Emerg Infect Dis 1999;5:135-42.
16. Vitullo AD, Hodara VL, Merani MS. Effect of persistent infection with Junin virus on growth and reproduction of its natural reservoir, *Calomys musculinus*. Am J Trop Med Hyg 1987;37:663-9.
17. Murphy FA, Winn WC Jr, Walker DH, Flemister MR, Whitfield SG. Early lymphoreticular viral tropism and antigen persistence: Tamiami virus infection in the cotton rat. Lab Invest 1976;34:125-40.
18. Jennings WL, Lewis AL, Sather GE, Pierce LV, Bond JO. Tamiami virus in the Tampa Bay area. Am J Trop Med Hyg 1970;19:527-36.
19. McClure PA. Sex-biased litter reduction in food-restricted wood rats (*Neotoma floridana*). Science 1981;211:1058-60.
20. Centers for Disease Control and Prevention. Fatal illnesses associated with a New World arenavirus—California, 1999-2000. MMWR Morb Mortal Wkly Rep 2000;49:709-11.

## Appendix

Complete descriptions of this tract (1,2) have characterized the area as follows: "Climate is classified as dry continental and elevation ranges from 1,300 to 1,700 m. Topography consists of broad, moderately sloping uplands bordered by the Purgatoire River Canyon on the east, limestone hills on the west, and a basaltic hogback on the south. Vegetation is dominated by short grass prairie and piñon pine (*Pinus edulis*)-one-seeded juniper (*Juniperus monosperma*) woodland" (3). The piñon-juniper plant association is concentrated along the Purgatoire River Canyon and its side canyons, in the limestone hills, and on parts of the basaltic hogback.

Two trapping sites were established in January 1995, one at the mouth of Red Rocks Canyon (MRC) and one within Red Rocks Canyon (RRC). The MRC site was a 3.14-ha web, containing twelve 100-m transects radiating from a central point, resembling the spokes of a wheel; additional details have been published (4). The MRC site, located at N 37° 32' 48.8", W 103° 49' 34.3", 1,675 m elevation, is in an area characterized as recovering grassland, disturbed by previous human habitation. The center of the web is essentially flat, but the eastern and western sides are rocky, the eastern side comprising mostly rock outcroppings and the western side sloping steeply to a mesa, which is a piñon-juniper habitat. A natural spring forms a pond at the northwest side of the site; the water flows from this pond in a creek that runs southeast, bisecting the site, until it dissipates in the usually dry soil. From January 1995 until December 1999, there was water in the pond and for approximately 100 m downstream. Only once, after an exceedingly heavy rain, did we observe water further down the creek bed. The soils at this site range from sandstone-derived slopes to sandy loam flat areas, with some marshy areas near the pond and its outlet. The slopes are 10%-70% bare ground; the western slope, being closer to the piñon-juniper mesa, is populated by sparse piñon pines and one-seeded junipers, and the eastern slope is mostly rocky outcroppings with sparse grasses and a few trees. Approximately 70% of the flora of the flatter areas is grasses, mostly Japanese brome (*Bromus japonicus*), blue grama (*Bouteloua gracilis*), and sideoats grama (*B. curtipendula*); 5% shrubs, mostly Louisiana sagebrush (*Artemesia ludoviciana*), milkvetch (*Astragalus crassicarpus*), and other sages (*Artemesia* sp.); 20% forbs, mostly skunkbrush sumac (*Rhus trilobata*), broom snakeweed, (*Gutierrezia sarothrae*), and rabbitbrush (*Chrysothamnus nauseosus*); and 5% cacti, mostly soapweed (*Yucca glauca*) and plains prickly pear (*Opuntia polycantha* var. *polycantha*).

Because the canyon at the RRC site is too narrow to install a web, we placed three parallel east-west lines of traps approximately 50 m apart, on the north side, in the middle, and on the south side of the canyon. Twenty-five traps were spaced 10 m apart on each line. The center of the middle line was located at N 37° 32' 19.3", W 103° 49' 12.5", 1,500 m elevation.

The RRC site is a vacant former homestead that still contains residences and outbuildings, with an east-west gravel road traversing it. An arroyo, dry most of the time, originates west of the site and bisects the site roughly west to east. The north line of traps

was placed on a south-facing slope, which has sandstone-derived soil. This area mostly comprises rocky outcroppings, some piñon pine and one-seeded junipers, very sparse grasses (mostly Japanese brome and blue grama), forbs (mostly skunkbrush sumac and rabbitbrush), shrubs (mostly sages and skunkbrush sumac), and cacti (essentially all tree cholla, *O. imbricata*); 20% of the ground was bare during most seasons and most years. The south line (at the base of a north-facing slope), also on sandstone-derived soil, was similar to the north line, with somewhat more rocks and shrubs (mostly skunkbrush sumac). The middle line was placed in an area that had been severely disturbed from decades of vegetation and water utilization and hoof compaction but is now recovering. It is primarily deep sandy loam bottomland, with alluvial soils. Grasses comprised 10% of the flora, mostly Japanese brome); forbs 55%, mostly pinnate tansymustard (*Descurainia pinnata*); shrubs 15%, mostly rabbitbrush; and cacti 5%, mostly plains prickly pear); 15% of the middle line area was bare ground.

**References**


1. Shaw RB, Anderson SL, Schultz KA, Diersing VE. Plant communities, ecological checklist, and species list for the U.S. Army Piñon Canyon Maneuver Site, Colorado. Fort Collins (CO): Colorado State University, Science Series No. 37; 1989. p. 71.
2. U.S. Department of the Army. Draft environmental impact statement for training land acquisition. Fort Carson, Colorado, 1980.
3. Costello DF. Vegetation zones in Colorado. In: Harrington HD, editor. Manual of the plants of Colorado. Chicago: Swallow Press, Inc.; 1954. p. iii-x.
4. Mills JN, Yates TL, Ksiazek TG, Peters CJ, Childs JE. Long-term studies of hantavirus reservoir populations in the southwestern United States: rationale, potential, and methods. Emerg Infect Dis 1999;5:95-101.

# Geographic Distribution and Genetic Diversity of Whitewater Arroyo Virus in the Southwestern United States

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The purpose of this study was to extend our knowledge of the geographic distribution and genetic diversity of the arenavirus(es) associated with *Neotoma* species (woodrats) in the southwestern United States. Infectious arenavirus was recovered from 14 (3.3%) of 425 woodrats. The virus-positive species included *N. albigula* in New Mexico and Oklahoma, *N. cinerea* in Utah, *N. mexicana* in New Mexico and Utah, and *N. micropus* in Texas. Analyses of viral nucleocapsid protein gene sequence data indicated that all the isolates were strains of the Whitewater Arroyo virus, an arenavirus previously known only from northwestern New Mexico. Analyses of the sequence data also indicated that there can be substantial genetic diversity among strains of Whitewater Arroyo virus from conspecific woodrats collected from different localities and substantial genetic diversity among strains from different woodrat species collected from the same locality.

The virus family *Arenaviridae* comprises two serocomplexes. The lymphocytic choriomeningitis-Lassa (Old World) complex includes lymphocytic choriomeningitis (LCM), Lassa, Mopeia, Mobala, and Ippy viruses. The Tacaribe (New World) complex includes Tamiami (TAM), Whitewater Arroyo (WWA), Pichindé (PIC), Amapari, Flexal, Guanarito, Junin, Latino, Machupo, Oliveros, Parana, Pirital, Sabiá, and Tacaribe viruses.

The arenaviruses have bipartite, single-stranded RNA genomes (1). The large (L) genomic segment encodes the viral RNA-dependent RNA polymerase and a zinc-binding protein. The small (S) genomic segment encodes the nucleocapsid (N) protein and glycoprotein precursor. The most comprehensive knowledge of the phylogeny of the family *Arenaviridae* is based on a fragment of the N protein gene (2-4).

Six arenaviruses are known to cause severe disease in humans. LCM virus is an agent of acute central nervous system disease (5) and congenital malformations (6). Lassa, Junin, Machupo, Guanarito, and Sabiá viruses are etiologic agents of hemorrhagic fever in western Africa, Argentina, Bolivia, Venezuela, and Brazil, respectively (7).

The arenaviruses known to occur in North America are LCM, TAM, and WWA. LCM virus was introduced into the Americas along with its principal rodent host, *Mus musculus* (house mouse) (8). TAM virus is known only from *Sigmodon hispidus* (hispid cotton rat) in southern Florida (9-11). WWA virus was originally recovered from *Neotoma albigula* (white-throated woodrat) collected from northwestern New Mexico (12).

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In a recent study (13), antibody to an arenavirus was found in five *Neotoma* species in the southwestern United States: *N. albigula* in Arizona, Colorado, and New Mexico; *N. stephensi* (Stephen's woodrat) in Arizona and New Mexico; *N. mexicana* (Mexican woodrat) in Arizona and Utah; and *N. fuscipes* (dusky-footed woodrat) and *N. lepida* (desert woodrat) in California. The purpose of the present study was to extend our knowledge of the geographic distribution and genetic diversity of the arenavirus(es) associated with *Neotoma* rodents in the southwestern United States.

## Materials and Methods

All work with rodent tissues and infectious arenavirus was performed in a biosafety level 3 laboratory at the Centers for Disease Control and Prevention (Atlanta, GA) or University of Texas Medical Branch, Galveston.

### Rodent Tissues

Five hundred sixty-six tissue specimens (74 spleen, 225 liver, and 267 kidney) from 425 woodrats were tested for infectious arenavirus. The specimens were from the Museum of Texas Tech University (Lubbock, TX) or Museum of Southwestern Biology (University of New Mexico, Albuquerque, NM). The specimens from the Museum of Southwestern Biology were chosen to represent localities in which antibody to an arenavirus was found in one or more *Neotoma* species in a previous study (13).

### Virus Assay

Tissue specimens were tested for infectious arenavirus as described previously (12). Briefly, 0.2 mL of a 10% w/v crude tissue homogenate was inoculated onto a monolayer of Vero E6 cells in a 25-cm$^2$ plastic culture flask (Corning, Inc.,

Corning, NY). The inoculum was incubated on the cell monolayer at 37°C for 60 minutes; then the monolayer was overlaid with 7.0 mL of a minimum essential medium containing Earle's salts, 1.5 mg/mL sodium bicarbonate, 2% v/v heat-inactivated (56ºC for 30 minutes) fetal bovine serum, 0.29 mg/mL L-glutamine, 100 U/mL penicillin G, 100 µg/mL streptomycin sulfate, and 100 U/mL nystatin. The cell culture was maintained at 37°C in a humidified atmosphere of 5% $CO_2$ in air for 13 days. Half the culture medium was replaced with fresh maintenance medium on day 6 or 7 after inoculation. Cells were scraped from the monolayer on day 13 after inoculation and coated onto 12-well glass microscope slides (Cel-Line Associates, Inc., Newfield, NJ). The cell spots were air-dried, fixed in cold acetone, and then tested for arenaviral antigen by using an indirect fluorescent antibody test (12). In that test, cell spots were stained with a hyperimmune mouse ascitic fluid prepared against the WWA virus prototype strain AV 9310135, and mouse immunoglobulin G (IgG) bound to cell-associated arenaviral antigen was detected by using a goat anti-mouse IgG fluorescein isothiocyanate conjugate (Cappel Laboratories, West Chester, PA).

### Genetic Characterization of Viral Isolates

The nucleotide sequence of a fragment of the N protein gene of each of 12 isolates was determined. Four of the 12 isolates were from the spleens and kidneys of two animals, rodents 62425 and 62439 (Table 1). Total RNA was extracted from monolayers of infected Vero E6 cells by using TRIzol Reagent (Life Technologies, Inc., Grand Island, NY). Reverse transcription of RNA from isolates AV 96010149, AV 96010151, AV 96010025, and AV 96010024 was carried out by using Superscript II RTase (Life Technologies) in conjunction with oligonucleotide ARE-3'END (14). This oligonucleotide apparently is complementary to the 19-nt fragment at the 3' terminus of the S genomic segment of all arenaviruses. Polymerase chain reaction (PCR) amplification of the first-strand cDNA was carried out by using *Taq* DNA polymerase (Promega Corp., Madison, WI) in conjunction with oligonucleotides 1010C and NW1696R (2-3), which flank a 616-nt region of the N protein gene of WWA virus prototype strain AV 9310135 (12). Reverse transcription and PCR (RT-PCR) amplification of a fragment of the N protein gene of each of the eight other isolates was carried out by using the Access RT-PCR Kit (Promega Corp.) in conjunction with oligonucleotides AVNP1 (5'-CCCTTCTTYTTNYTCTTRATGACTA-3') and AVNP2 (5'-GGKAGRGCNTGGGAYAACAC-3'). AVNP1 and AVNP2 flank a 518-nt region in the fragment of the WWA virus N protein gene that is amplified by using oligonucleotides 1010C and NW1696R. They were designed based on N protein gene sequence data for the WWA virus prototype strain AV 9310135 (GenBank Accession No. U52180), WWA virus strains AV 96010149, AV 96010151, AV 96010025, and AV 96010024, TAM virus strain W-10777 (U43690), and PIC virus strain An 3739 (K02734). Size separation of PCR products was done by agarose gel electrophoresis; the products of the expected size were purified from gel slices by using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA). One strand of each 1010C-NW1696R PCR product was sequenced directly by using the dye termination cycle sequencing technique (Applied Biosystems, Inc., Foster City, CA) in conjunction with oligonucleotide 1010C. The sequence of the other (i.e., complementary) strand of each of these products was determined by cloning the PCR product in the TA cloning vector PCRII (Invitrogen Corp., Carlsbad, CA) and then using a plasmid-specific oligonucleotide (M13) to initiate the cycle sequencing reaction. Both strands of the AVNP1-AVNP2 PCR products were sequenced directly by using the same oligonucleotides that were used to prime the RT-PCR, i.e., AVNP1 and AVNP2. The 12 nucleotide sequences generated in this study were deposited with the GenBank nucleotide sequence database under Accession Nos. AY012710-AY012721.

### Data Analysis

The analyses of nucleotide sequence data were restricted to the 518-nt fragment of the WWA virus N protein gene that is flanked by oligonucleotides AVNP1 and AVNP2. The GenBank database sequences included in the analyses were Accession Nos. U52180 (WWA virus, strain AV 9310135), U43690 (TAM, W-10777), K02734 (PIC, An 3739), U43689 (Parana, 12056), U43687 (Flexal, BeAn 293022), U62561 (Pirital, VAV-488), U43688 (Latino, 10924), U34248 (Oliveros, 3229-1), U70802 (Junin, XJ), X62616 (Machupo, AA288-77), U43686 (Guanarito, INH-95551), U41071 (Sabiá, SPH 114202), U43685 (Amapari, BeAn 70563), M20304 (Tacaribe, TRVL 11573), M20869 (LCM, Armstrong), and U80004 (Lassa, LP). The computer software package

Table 1. Recovery of infectious arenavirus from tissues of virus-positive woodrats (*Neotoma* species)

| Rodent | Species | Date collected | Collected from: County | Collected from: State | Virus (strain) recovered from[a]: Spleen | Kidney | Liver |
|---|---|---|---|---|---|---|---|
| 1627 | *N. albigula* | 07/15/93 | McKinley | NM | AV 9310041 | **AV 9310135** | nt |
| 1626 | *N. albigula* | 07/15/93 | McKinley | NM | nt | AV 9310040 | nt |
| 62415 | *N. mexicana* | 09/24/94 | Socorro | NM | nt | AV 96010149 | nt |
| 62425 | *N. mexicana* | 09/24/94 | Socorro | NM | nt | AV 96010151 | AV 98360019 |
| 62439 | *N. mexicana* | 09/24/94 | Socorro | NM | nt | AV 96010154 | AV 98360020 |
| 28731 | *N. albigula* | 10/12/85 | Cimarron | OK | nt | AV 98490013 | Negative |
| 28742 | *N. albigula* | 10/12/85 | Cimarron | OK | nt | AV 97130039 | TVP-6038 |
| 84648 | *N. micropus* | 07/18/99 | Dimmit | TX | Negative | AV A0400098 | nt |
| 84703 | *N. micropus* | 07/19/99 | Dimmit | TX | AV A0400337 | AV A0400135 | nt |
| 84708 | *N. micropus* | 07/19/99 | Dimmit | TX | nt | AV A0400140 | nt |
| 84761 | *N. micropus* | 07/18/99 | Dimmit | TX | AV A0400373 | AV A0400174 | nt |
| 84816 | *N. micropus* | 07/20/99 | La Salle | TX | AV A0400412 | AV A0400212 | nt |
| 36287 | *N. cinerea* | 07/06/94 | San Juan | UT | nt | AV 96010025 | AV 96010206 |
| 36282 | *N. mexicana* | 07/05/94 | San Juan | UT | nt | AV 96010024 | AV 96010205 |

[a]nt = not tested. The WWA virus prototype strain is bolded. Isolates (strains) included in the (phylo-) genetic analyses are underlined.

CLUSTAL W1.7 (15) was used to construct an alignment of the predicted amino acid sequences, and the computer program TransAlign (16) was used to generate a multiple nucleotide sequence alignment from the amino acid sequence alignment. Pairwise genetic distances were computed by using the p distance model as implemented in the computer program MEGA, version 1.02 (17). Percent sequence identities were calculated by subtracting the genetic distances from 1.0 and multiplying by 100. Phylogenetic analysis was carried out on the multiple amino acid sequence alignment by using the neighbor-joining method (gamma model, alpha = 2) as implemented in MEGA, version 1.02. Bootstrap support (18) for the results of the phylogenetic analysis was based on 500 pseudoreplicate datasets generated from the original multiple amino acid sequence alignment.

## Results

### Viral Isolates

Twenty-three arenaviral isolates were recovered from tissues of 14 (3.3%) of 425 *Neotoma* rodents (Table 1). The 23 isolates included three WWA virus strains (AV 9310135, AV 9310041, and AV 9310040) that were reported previously (12).

The virus-positive animals included two *N. albigula* from McKinley County, northwestern New Mexico; two *N. albigula* from Cimarron County, western Oklahoma; three *N. mexicana* from Socorro County, central New Mexico; five *N. micropus* from the Chaparral Wildlife Management Area (Dimmit and La Salle counties), southern Texas; and one *N. mexicana* and one *N. cinerea* from San Juan County, southeastern Utah (Table 2, Figure 1). The virus-positive animals from McKinley County were two (50%) of four woodrats (all *N. albigula*) collected on July 15, 1993, from Whitewater Arroyo. The positive *N. albigula* from Cimarron County were two (22.2%) of nine woodrats (seven *N. albigula* and two *N. mexicana*) collected on October 12, 1985, from a site near Kenton. The positive *N. mexicana* from Socorro County were three (42.9%) of seven woodrats (all *N. mexicana*) collected on September 24, 1994, from a site in the Magdalena Mountains. The positive *N. micropus* from Dimmit County were 4 (13.8%) of 29 woodrats (all *N. micropus*) collected in a 3-day period (July 17 through July 19, 1999) from the western region of the Chaparral Wildlife Management Area. The positive *N. micropus* from La Salle County was one (25.0%) of four woodrats (all *N. micropus*) collected on July 20, 1999, from the eastern region of the Chaparral Wildlife Management Area. The positive *N. mexicana* and *N. cinerea* from San Juan County were 2 (12.5%) of 16 woodrats (11 *N. mexicana*, 2 *N. cinerea*, and 3 *N. albigula*) collected in an 8-day period (June 29 through July 6, 1994) from Natural Bridges National Monument. Information from the Museum of Southwestern Biology indicated that the positive *N. mexicana* and *N. cinerea* were collected from different sites in Natural Bridges National Monument.


Figure 1. Locations of 14 arenavirus-positive *Neotoma* rodent collections. San Juan County, southeastern Utah = *N. cinerea* and *N. mexicana* (one virus-positive animal each species); Cimarron County, western Oklahoma = *N. albigula* (2); McKinley County, northwestern New Mexico = *N. albigula* (2); Socorro County, central New Mexico = *N. mexicana* (3); Dimmit and La Salle counties (Chaparral Wildlife Management Area), southern Texas = *N. micropus* (5). The map inset shows the location of study area.

Table 2. Results of virus isolation attempts on tissues from 425 woodrats

| | | *Neotoma* species[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| County[b] | State | Nalb | Ncin | Nflo | Nme | Nmic | Nste | Total |
| Apache (1) | AZ | - | - | - | 0/6 | - | 0/5 | 0/11 |
| Cochise (2) | AZ | 0/31 | - | - | - | - | - | 0/31 |
| Coconino (2) | AZ | 0/6 | - | - | - | - | 0/3 | 0/9 |
| Maricopa (1) | AZ | 0/23 | - | - | - | - | - | 0/23 |
| Navajo (1) | AZ | 0/29 | 0/1 | - | 0/5 | - | 0/7 | 0/42 |
| Yavapai (2) | AZ | 0/5 | - | - | 0/2 | - | - | 0/7 |
| McKinley (3) | NM | 2/16 | - | - | - | - | - | 2/16 |
| Otero (9) | NM | 0/33 | - | - | 0/9 | 0/35 | - | 0/77 |
| Socorro (3) | NM | 0/31 | - | - | 3/10 | 0/1 | - | 3/42 |
| Cimarron (4) | OK | 2/11 | - | - | 0/5 | - | - | 2/16 |
| Major (4) | OK | - | - | 0/45 | - | 0/38 | - | 0/83 |
| McIntosh (2) | OK | - | - | 0/12 | - | - | - | 0/12 |
| Pottawatomie (2) | OK | - | - | 0/7 | - | - | - | 0/7 |
| Dimmit (1) | TX | - | - | - | - | 4/29 | - | 4/29 |
| La Salle (1) | TX | - | - | - | - | 1/4 | - | 1/4 |
| San Juan (8) | UT | 0/3 | 1/2 | - | 1/11 | - | - | 2/16 |
| Total | | 4/188 | 1/3 | 0/64 | 4/48 | 5/107 | 0/15 | 14/425 |

[a]Nalb = *Neotoma albigula,* Ncin = *N. cinerea*, Nflo = *N. floridana*, Nmex = *N. mexicana*, Nmic = *N. micropus*, Nste = *N. stephensi.* Values are the number positive/number tested; "-" = none tested.
[b]Number in parentheses indicates the number of sites sampled in the county.

The nucleotide sequences of the isolates from rodent 62425 (one isolate each from kidney and liver; strains AV 96010151 and AV 98360019, respectively) were identical. In contrast, the nucleotide sequences of the isolates from rodent 62439 (again, one isolate each from kidney and liver; strains AV 96010154 and AV 98360020, respectively) were 99.6% identical. Further study is needed to determine whether the differences between the isolates from rodent 62439 represent the coexistence of multiple virus genotypes (alleles) in the same rodent. An alternative explanation is that the sequence differences are the result of adaptation of the isolates to growth in cultured (Vero E6) cells or manipulation of viral nucleic acid extracted from cultured cells.

Nucleotide and amino acid sequence identities among WWA virus prototype strain AV 9310135 and 12 other

isolates from *Neotoma* rodents ranged from 74.7% to 100.0% and 84.9% to 100.0%, respectively (Table 3). When compared with other arenaviruses, the isolates from the *Neotoma* rodents exhibited 69.9% to 73.7% nucleotide sequence identity with TAM virus, 61.0% to 63.3% identity with PIC virus, and less than 62.0% sequence identity with all other arenaviruses.

Phylogenetic analysis of N protein amino acid sequence data indicated that isolates from *Neotoma* rodents represent a phylogenetic lineage (viral species) that is in a sister relationship to the lineage represented by TAM virus (Figure 2). We concluded that all isolates recovered from the *Neotoma* rodents were strains of WWA virus.

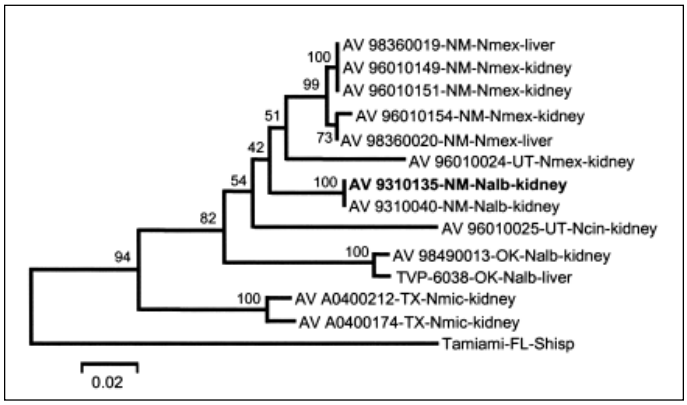


Figure 2. Phylogeny of the North American arenaviruses based on a neighbor-joining analysis of nucleocapsid protein amino acid sequence data. Distances and groupings were determined by using the gamma distance algorithm (alpha = 2). Branch lengths are proportional to the gamma distance between amino acid sequences. Numbers indicate the percentage of 500 bootstrap replicates that supported each labeled interior branch. The WWA virus prototype strain AV 9310135 is in bold type. Nmex = *Neotoma mexicana*, Nalb = *N. albigula*, Ncin = *N. cinerea*, Nmic = *N. micropus*, and Shisp = *Sigmodon hispidus*.

## Discussion

Before the present study, WWA virus was known only from *N. albigula* in northwestern New Mexico (12). The present work provides unequivocal evidence that the virus also is naturally associated with *N. cinerea*, *N. mexicana*, and *N. micropus*, and that it occurs in Utah, central New Mexico, Oklahoma, and Texas. The recovery of WWA virus strains AV 98490013 and TVP-6083 from *N. albigula* is the first evidence that a Tacaribe complex virus occurs in Oklahoma. Likewise, the recovery of strains AV A0400174 and AV A0400212 from woodrats collected from southern Texas (Chaparral Wildlife Management Area) is the first evidence that *N. micropus* is naturally associated with a Tacaribe complex virus and that WWA virus occurs in Texas.

In a previous study (13), antibody to an arenavirus was found in *N. fuscipes* and *N. lepida* in southern California; *N. albigula*, *N. mexicana*, and *N. stephensi* in Arizona; and *N. albigula* in southwestern Colorado. Although the results of the present study indicate that WWA virus is geographically widely distributed in association with *Neotoma* rodents, further work is needed to determine whether the arenavirus associated with *Neotoma* rodents in California, Arizona, and Colorado is in fact WWA virus.

The results of the present study indicate that there can be substantial genetic heterogeneity among strains of WWA virus from different woodrat species from the same locality and among strains from conspecific woodrats collected from different localities. For example, nucleotide sequence identity between the strains recovered from *N. mexicana* and *N. cinerea* from Natural Bridges National Monument (San Juan County, Utah; strains AV 96010024 and AV 96010025, respectively) was 82.8%, and nucleotide sequence identity between strain AV 96010024 and the three strains recovered from *N. mexicana* collected from the Magdalena Mountains (Socorro County, New Mexico; strains AV 96010149, AV 96010151, and AV 96010154) was from 85.1% to 85.5%. In contrast, nucleotide sequence identity in strains recovered from conspecific rodents collected from the same locality (e.g., strains AV 9310135 and AV 9310040 from *N. albigula* from Whitewater Arroyo, and strains AV A0400174 and AV

Table 3. Nucleotide and amino acid sequence identities among 13 arenavirus isolates recovered from 11 woodrats and Tamiami virus[a]

| | | Virus or strain | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Virus[b] | Strain | AV 93 10135 | AV 93 10040 | AV 96 010149 | AV 96 010151 | AV 98 360019 | AV 96 010154 | AV 98 360020 | AV 98 490013 | TVP-6038 | AV A0 400212 | AV A0 400174 | AV 96 010025 | AV 96 010024 | TAM |
| WWA | AV 9310135 | -- | 100.0 | 86.5 | 86.5 | 86.5 | 85.3 | 85.3 | 82.3 | 82.6 | 79.1 | 79.1 | 83.4 | 85.1 | 71.6 |
| WWA | AV 9310040 | 100.0 | -- | 86.5 | 86.5 | 86.5 | 85.3 | 85.3 | 82.3 | 82.6 | 79.1 | 79.1 | 83.4 | 85.1 | 71.6 |
| WWA | AV 96010149 | 95.3 | 95.3 | -- | 100.0 | 100.0 | 98.1 | 98.5 | 80.1 | 80.3 | 79.5 | 78.8 | 84.4 | 85.5 | 73.7 |
| WWA | AV 96010151 | 95.3 | 95.3 | 100.0 | -- | 100.0 | 98.1 | 98.5 | 80.1 | 80.3 | 79.5 | 78.8 | 84.4 | 85.5 | 73.7 |
| WWA | AV 98360019 | 95.3 | 95.3 | 100.0 | 100.0 | -- | 98.1 | 98.5 | 80.1 | 80.3 | 79.5 | 78.8 | 84.4 | 85.5 | 73.7 |
| WWA | AV 96010154 | 94.8 | 94.8 | 98.8 | 98.8 | 98.8 | -- | 99.6 | 81.3 | 81.5 | 79.3 | 78.6 | 84.4 | 85.1 | 73.2 |
| WWA | AV 98360020 | 95.3 | 95.3 | 99.4 | 99.4 | 99.4 | 99.4 | -- | 81.3 | 81.5 | 79.5 | 78.8 | 84.4 | 85.1 | 73.4 |
| WWA | AV 98490013 | 91.9 | 91.9 | 90.7 | 90.7 | 90.7 | 90.7 | 91.3 | -- | 99.4 | 79.0 | 77.8 | 80.9 | 80.7 | 72.2 |
| WWA | TVP-6038 | 91.9 | 91.9 | 90.7 | 90.7 | 90.7 | 90.7 | 91.3 | 98.8 | -- | 79.1 | 78.0 | 81.1 | 80.1 | 72.4 |
| WWA | AV A0400212 | 88.4 | 88.4 | 88.9 | 88.9 | 88.9 | 88.9 | 89.5 | 86.6 | 86.6 | -- | 95.7 | 77.2 | 77.0 | 69.9 |
| WWA | AV A0400174 | 88.9 | 88.9 | 89.5 | 89.5 | 89.5 | 89.5 | 88.9 | 85.5 | 85.5 | 98.3 | -- | 74.7 | 75.5 | 69.9 |
| WWA | AV 96010025 | 90.7 | 90.7 | 91.3 | 91.3 | 91.3 | 91.3 | 91.3 | 87.8 | 87.8 | 84.9 | 84.9 | -- | 82.8 | 71.6 |
| WWA | AV 96010024 | 93.0 | 93.0 | 94.2 | 94.2 | 94.2 | 94.2 | 94.2 | 88.4 | 88.4 | 86.0 | 86.0 | 89.5 | -- | 72.2 |
| TAM | W-10777 | 77.9 | 77.9 | 78.5 | 78.5 | 78.5 | 78.5 | 78.5 | 78.5 | 78.5 | 80.2 | 80.2 | 77.3 | 78.5 | -- |

[a]Nucleotide and amino acid sequence identities are listed above and below the dashes, respectively.
[b]WWA = Whitewater Arroyo, TAM = Tamiami.

A0400212 from *N. micropus* from the Chaparral Wildlife Management Area) was >95.0%.

The results of previous studies (3,19,20) suggested that the present-day diversity of the arenaviruses is a product of long-term coevolution of the various viruses with their respective principal rodent hosts. In the present study, WWA viral strains AV 9310135 and AV 9310040 (both from *N. albigula*, northwestern New Mexico) appeared to be phylogenetically more closely related to strain AV 96010024 (*N. mexicana*, southeastern Utah) than to strains AV 98490013 and TVP-6038 (both from *N. albigula*, western Oklahoma). This situation suggests that the present-day association of WWA virus with *N. albigula* and *N. mexicana* does not represent a long-term shared evolutionary relationship between virus and rodent species. However, this conclusion assumes that recovery of WWA virus from a rodent represents a principal virus-host relationship. Perhaps some of the virus-positive rodents in the present study were infected by contact with other *Neotoma* species or even non-*Neotoma* rodent species.

The geographic range of the genus *Neotoma* extends from western Canada south to Guatemala, Honduras, and Nicaragua, and includes 33 states in the contiguous United States and 26 of the 32 states in Mexico (21). Thus, if the present-day association of WWA virus with the genus *Neotoma* represents a long-term shared evolutionary relationship between virus and rodent host, the geographic range of the virus may extend far beyond the southwestern United States. WWA virus recently was associated with several human deaths in California (22). Further study is needed to assess the human health significance of this virus in the southwestern United States and other regions in North America in which woodrats are indigenous.

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### References

1. Southern PJ. *Arenaviridae*: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, et al., editors. Fields virology. 3rd ed. Philadelphia: Lippincott-Raven Publishers; 1996. p. 1505-19.
2. Bowen MD, Peters CJ, Nichol ST. The phylogeny of New World (Tacaribe complex) arenaviruses. Virology 1996;219:285-90.
3. Bowen MD, Peters CJ, Nichol ST. Phylogenetic analysis of the *Arenaviridae*: patterns of virus evolution and evidence for cospeciation between arenaviruses and their rodent hosts. Mol Phylogenet Evol 1997;8:301-16.
4. Weaver SC, Salas RA, de Manzione N, Fulhorst CF, Duno G, Utrera A, et al. Guanarito virus (*Arenaviridae*) isolates from endemic and outlying localities in Venezuela: sequence comparisons among and within strains isolated from Venezuelan hemorrhagic fever patients and rodents. Virology 2000;266:189-95.
5. Jahrling PB, Peters CJ. Lymphocytic choriomeningitis virus. A neglected pathogen of man [editorial]. Arch Pathol Lab Med 1992;116:486-8.
6. Barton LL, Budd SC, Morfitt WS, Peters CJ, Ksiazek TG, Schindler RF, et al. Congenital lymphocytic choriomeningitis virus infection in twins. Pediatr Infect Dis J 1993;12:942-6.
7. Peters CJ, Buchmeier M, Rollin PE, Ksiazek TG. Arenaviruses. In: Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, et al., editors. Fields virology. 3rd ed. Philadelphia: Lippincott-Raven Publishers; 1996. p. 1521-51.
8. Childs JE, Peters CJ. Ecology and epidemiology of arenaviruses and their hosts. In: Salvato MS, editor. The Arenaviridae. New York: Plenum Press; 1993. p. 331-84.
9. Berge T, editor. International catalogue of arboviruses and certain other viruses of the world (supplement). Tamiami (TAM), strain W-10777. Am J Trop Med Hyg 1970;19:1157-8.
10. Calisher CH, Tzianabos T, Lord RD, Coleman PH. Tamiami virus, a new member of the Tacaribe group. Am J Trop Med Hyg 1970;19:520-6.
11. Jennings WL, Lewis AL, Sather GE, Pierce LV, Bond JO. Tamiami virus in the Tampa Bay area. Am J Trop Med Hyg 1970;19:527-36.
12. Fulhorst CF, Bowen MD, Ksiazek TG, Rollin PE, Nichol ST, Kosoy MY, et al. Isolation and characterization of Whitewater Arroyo virus, a novel North American arenavirus. Virology 1996;224:114-20.
13. Kosoy MY, Elliot LH, Ksiazek TG, Fulhorst CF, Rollin PE, Childs JE, et al. Prevalence of antibodies to arenaviruses in rodents from the southern and western United States: evidence for an arenavirus associated with the genus *Neotoma*. Am J Trop Med Hyg 1996;54:570-5.
14. Gonzalez JP, Sanchez A, Ricco-Hesse R. Molecular phylogeny of Guanarito virus, an emerging arenavirus affecting humans. Am J Trop Med Hyg 1995;53:1-6.
15. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choices. Nucleic Acids Res 1994;22:4673-80.
16. Weiller GF. TransAlign, version 1.0. Canberra, Australia: Research School of Biological Sciences; 1999.
17. Kumar S, Tamura K, Nei M. MEGA: Molecular Evolutionary Genetics Analysis package, version 1.02. University Park (PA): Pennsylvania State University; 1993.
18. Felsentein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 1985;39:783-91.
19. Gonzalez JP, Georges AJ, Kiley MP, Meunier DM, Peters CJ, McCormick JB. Evolutionary biology of a Lassa virus complex. Med Microbiol Immunol 1986;175:157-9.
20. Johnson KM, Webb PA, Justines G. Biology of Tacaribe-complex viruses. In: Lehman-Grube F, editor. Lymphocytic choriomeningitis virus and other arenaviruses. Berlin: Springer-Verlag; 1973. p. 241-58.
21. Musser GG, Carleton MD. Family Muridae. In: Wilson DE, Reeder DM, editors. Mammal species of the world. A taxonomic and geographic reference. Washington: Smithsonian Institution Press; 1993. p. 501-755.
22. Centers for Disease Control and Prevention. Fatal illnesses associated with a New World arenavirus-California, 1999-2000. MMWR Morb Mortal Wkly Rep 2000;49:709-11.

# Is High Prevalence of *Echinococcus multilocularis* in Wild and Domestic Animals Associated with Disease Incidence in Humans?

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We investigated a focus of highly endemic *Echinococcus multilocularis* infection to assess persistence of high endemicity in rural rodents, explore potential for parasite transmission to domestic carnivores, and assess (serologically) putative exposure versus infection frequency in inhabitants of the region. From spring 1993 to spring 1998, the prevalence of *E. multilocularis* in rodents was 9% to 39% for *Arvicola terrestris* and 10% to 21% for *Microtus arvalis*. From June 1996 to October 1997, 6 (7%) of 86 feral dogs and 1 of 33 cats living close to the region tested positive for intestinal *E. multilocularis* infection. Testing included egg detection by coproscopy, antigen detection by enzyme-linked immunosorbent assay (ELISA), and specific parasite DNA amplification by polymerase chain reaction. Thus, the presence of infected domestic carnivores can increase *E. multilocularis* exposure risk in humans. A seroepidemiologic survey of 2,943 blood donors in the area used specific Em2-ELISA. Comparative statistical analyses of seroprevalence and clinical incidence showed an increase in Em2-seroprevalence from 1986 and 1996-97 but no increase in clinical incidence of alveolar hydatid disease.

Alveolar hydatid disease (AHD) in humans is caused by infection with the proliferative larval stage of the small fox tapeworm *Echinococcus multilocularis*. Once the infection becomes successfully established, AHD is one of the most lethal helminthic diseases in humans (1). Infection sources, risk, and rates for humans may be related to prevalence in wild and domestic animals. In a recent study, we described highly endemic *E. multilocularis* in a small area of the canton of Fribourg, Switzerland (2). An *E. multilocularis* prevalence of 47% to 56% per year was found in the fox population. Prevalence in the local *Arvicola terrestris* population fluctuated annually between 11% and 39%. The wide distribution of *E. multilocularis* eggs in the study area, reflected by the high prevalence in rodents, may have represented a considerable risk for humans in the densely populated periurban regions of Switzerland. Recently, high *E. multilocularis* prevalence was also found in urban fox and rodent populations, which may represent an even higher infection risk for humans (3). However, in spite of high prevalence in the definitive host in most parts of Switzerland (north of the Alps), disease in humans is relatively rare. In recent decades, the annual death rate for AHD in Switzerland has been 0.18 cases per 100,000 inhabitants (4). Another study in the United States found no association between high prevalence of *E. multilocularis* in wild canids and deaths in trappers from South Dakota (5). A link between prevalence in natural definitive and intermediate wildlife hosts and domestic definitive hosts with infection risk and prevalence of disease in humans has not been examined in Switzerland.

On the basis of our previous findings (2,6,7), we designed the present study to determine the effect of a naturally occurring persistent high prevalence in wildlife hosts on domestic animals (dogs and cats) and assess (by serologic and clinical tests) the exposure rate of humans living in the area.

## Methods

The study was carried out from 1993 to 1998 in a periurban area north of the city of Fribourg. The primary (or rodent) study site has been described (2,7). A secondary area for studying the *E. multilocularis* infection rate in cats and dogs was delineated by an approximate 5-km radius around the primary site. A tertiary area for studying seroprevalence in human blood donors included approximately 20% of the surface area of the canton of Fribourg (representing approximately 400 $km^2$ surrounding the primary site).

### Survey in Intermediate Hosts (Rodents)

The rodent survey has been ongoing since spring 1993. The study period assessed in this article is spring 1993 to

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spring 1998. *A. terrestris* and *Microtus arvalis* were caught as described (7) by using standardized 100-m trap lines to obtain density estimates (8). Each trapped animal was assessed for biologic variables and *E. multilocularis* infection status as described (2,7). Liver parts were preserved in 70% ethanol and 4% buffered paraformaldehyde after microscopy examination to detect metacestode lesions. All preserved lesions were subsequently assessed by immunohistochemistry or polymerase chain reaction (PCR) (2).

### Survey in Definitive Hosts (Dogs and Cats)

Three local veterinarians participated in the survey by distributing information and diagnostic fecal containers to dog and cat owners. This part of the study took place from June 1996 to October 1997. Fecal samples were examined by the following methods: Taeniid egg detection by microscopy following a flotation enrichment (9); identification of isolated *E. multilocularis* eggs by PCR (10); and detection of *E. multilocularis* antigens by sandwich-enzyme-linked immunosorbent assay (ELISA) (9). Owners of *E. multilocularis*-positive dogs or cats obtained detailed information and support by their veterinarians for treating *E. multilocularis*-infected animals and taking the necessary safety precautions, which included a recommendation to inform their physicians about previous exposure risk and to have all household members undergo serologic testing (procedures described in the next section).

### Survey in Humans

The seroepidemiologic survey was done in collaboration with the Blood Transfusion Centre, Cantonal Hospital, Fribourg. Serum samples were obtained from 2,943 blood donors in October 1996 to August 1997. The blood donation collection area included 38 villages located in the periphery of the initial rodent study area. In Switzerland, blood donation is voluntary and not compensated financially. Blood donation statistics have shown that a constant rate of 15% of the population (independent of geographic area) participates in blood donation (men four times and women three times a year). The sampling strategy was designed by the blood donation center to obtain all registered donors of the study area and avoid double donations by the same person. An identical sampling procedure and strategy had been used earlier, which also had covered approximately 15% of the study area (6). Informed consent was obtained from blood donors. All sera were stored at -30°C until tested. Em2-ELISA was performed as described, including the respective data management processing (2,6). For complementary serologic investigations, the serum samples were also tested in parallel for reactivity with an *E. granulosus* hydatid fluid antigen (EgHF) ELISA (11). If Em2-serologic test results were positive, a second blood sample was obtained through the responsible physician to rule out errors in handling the first sample and confirm the first serologic test result. If the result was confirmed, hepatic ultrasonography was performed in a regional imaging center. In cases where sonography showed hepatic lesions, the patient was referred to the University Hospital in Bern for further assessment by computed tomography (CT).

### Statistical Analyses

Comparative statistical analyses of the present seroepidemiologic data and those of a previous study (6) were done with SAS v.6.12 and Fisher's exact test (2-tail); p values were <0.005, unless otherwise stated.

## Results

*E. multilocularis* were repeatedly found in intermediate hosts (rodents). *A. terrestris* and *M. arvalis* were captured in the same period from 1993 to 1998. The presence of *E. multilocularis* metacestodes in the liver was determined by microscopy and, if required, was confirmed by immunohistochemistry and PCR. Prevalence data (Table 1) indicate that during the study period, the area maintained a relatively constant high infection rate (prevalence 9% to 39% for *A. terrestris* and 10% to 23% for *M. arvalis*). The yearly fluctuation of prevalence of *A. terrestris* was different from that of *M. arvalis*. For *A. terrestris,* a significant interannual temporal effect (p <0.005) was observed. No significant interannual effect was observed for *M. arvalis.*

### *E. multilocularis* in Definitive Hosts (Dogs and Cats)

The prevalence of intestinal *E. multilocularis* infection in dogs and cats living near the area under investigation is provided in Table 2. The definitive prevalence of 7% for dogs and 3% for cats may be underestimated as the veterinarians involved in the collection of samples informed regional cat and dog owners about the possibility of preventing infection by monthly administration of praziquantel (5 mg per kg body weight). Bias was suggested by the fact that most *E. multilocularis*-positive dogs were detected at the beginning of this study in 1996.

Table 1. Prevalence of *Echinococcus multilocularis* in *Arvicola terrestris* and *Microtus arvalis* captured in spring 1993 and 1998

| Year | No. of *A. terrestris* trapped | No. positive by microscopy/immunochemistry[a] PCR[b] | (%) | [95% CI] | No. of *M. arvalis* trapped | No. positive by microscopy/immunohistochemistry PCR | (%) | [95% CI] |
|---|---|---|---|---|---|---|---|---|
| 1993 | 28 | 11 | (39) | [21-57] | nt | -- | – | -- |
| 1994 | 44 | 5 | (11) | [ 2-20] | 20 | 2 | (10) | [ 3-23] |
| 1995 | 67 | 6 | ( 9) | [ 2-16] | 61 | 13 | (21) | [11-32] |
| 1996 | 49 | 10 | (20) | [ 9-21] | 55 | 9 | (16) | [ 7-26] |
| 1997 | 59 | 4 | ( 7) | [ 1-13] | 52 | 12 | (23) | [12-35] |
| 1998 | 46 | 4 | ( 9) | [ 1-17] | 32 | 5 | (16) | [3-28] |
| Totals | 293 | 40 | (14) | [ 1-18] | 220 | 41 | (19) | [13-24] |

[a]Positivity is based on a primary microscopy lesion detection and subsequent confirmation of *E. multilocularis* by immunohistochemistry and PCR.

[b]PCR, polymerase chain reaction; nt, not trapped.

Table 2. Prevalence of *Echinococcus multilocularis* in dogs and cats, 1996[a]

| Animal | No. of animals tested | No. positive for taeniid eggs | No. positive by PCR | No. positive by ELISA | Final *E. multilocularis* positive diagnosis (%) |
|---|---|---|---|---|---|
| Feral dogs | 86 | 7[b] | 6[bc] | 6[bc] | 6/86 (7) |
| Cats | 33 | 1 | 1 | 1 | 1/33 (3) |

[a]Polymerase chain reaction (PCR) was performed directly on eggs following a taeniid egg isolation (9).
[b]One dog had a borderline coproantigen reactivity. Subsequent investigations provided a negative *Echinococcus*-PCR by the presence of taeniid eggs. Thus, final test interpretation did not indicate *E. multilocularis* infection.
[c]PCR- and copro-Ag-positivity refers to all the same animals. The one dog exhibiting a borderline copro-Ag-reactivity is not in these group of animals.

### *E. multilocularis* in Humans

Sera from 2,943 healthy blood donors were tested by Em2-ELISA (Table 3). All six Em2-positive blood donors had a relatively high anti-Em2 antibody concentration (>15 antibody units, AU) (11). The donors were referred to their physicians for hepatic imaging analyses by ultrasonography. Four of the six donors agreed to this procedure; in three of the four, no lesions could be detected by the initial ultrasonography. The procedure was repeated approximately 6 months and 24 months after immunodiagnosis and then annually. No lesions have been detected in these three donors; one, however, had a small echodense lesion of a few centimeters in diameter in the right liver lobe (Figure, A). The donor was referred to the outpatient clinic of the University Hospital in Bern for an abdominal CT, which confirmed the presence of a small hypodense lesion assumed to be fully calcified (Figure, B). No other pathologic changes of the liver were observed. As the morphologic features of this lesion matched the criteria for nonviable, so-called "died-out" or "abortive" lesions (1,12,13), the case was classified in this category. Further CT investigations using contrast enhancement detected a very small hypodense area in the periphery of the calcified herd, which could have harbored a putatively still viable parasitic zone (Figure, C). The patient was reexamined by CT at the same time intervals as described above for the other seropositive donors. No changes in size and morphologic features of the calcified lesion and the peripheral hypodense zone have been demonstrated. The patient received no treatment at any time. The inert lesion was definitively rated as abortive in 1999.

Table 3. Specific seroprevalences for *Echinococcus multilocularis* in blood donors[a]

| | No. | (%) | [95%-CI] |
|---|---|---|---|
| Blood donors tested (total) | 2,943 | | |
| Em2-positive blood donors[b] | 6[b] | (0.2) | [0.04-0.36] |
| EgHF-positive blood donors but negative by Em2 | 33[c] | (1.1) | [0.5-2.1] |

[a]Assessed primarily by Em2-enzyme-linked immunosorbent assay (ELISA), complementary serologic data were obtained with the *E. granulosus* hydatid fluid antigen-ELISA. Blood samples were collected from October 1996 to July 1997.
[b]Four of six Em2-positive blood donors received imaging investigations, one of which was computed tomography-positive for an alveolar hydatid disease (AHD) lesion. Two Em2-positive donors refused subsequent imaging investigations but have had no signs of AHD.
[c]Ten donors with high *E. granulosus* hydatid fluid antigen-titers but negative in Em2-ELISA were selected for pilot imaging investigations. One had a cystic hepatic lesion of 2.5 cm in diameter. Morphologic features were compatible with those of *E. granulosus* cysts.

In parallel, all sera were serologically tested by EgHF-ELISA to compare data with those from a former study performed in Switzerland in 1985-86 (2). Of 2,943 sera, 33 were seropositive in this EgHF ELISA (including the six Em2-positive sera described above). Ten EgHF ELISA-positive (but Em2-negative) persons were arbitrarily selected for a subsequent hepatic ultrasonography investigation. Nine persons had negative ultrasonography results. One person, however, had a typical hepatic *E. granulosus* hydatid cyst of 5 cm in diameter and was referred to his physician for further clinical testing and treatment.

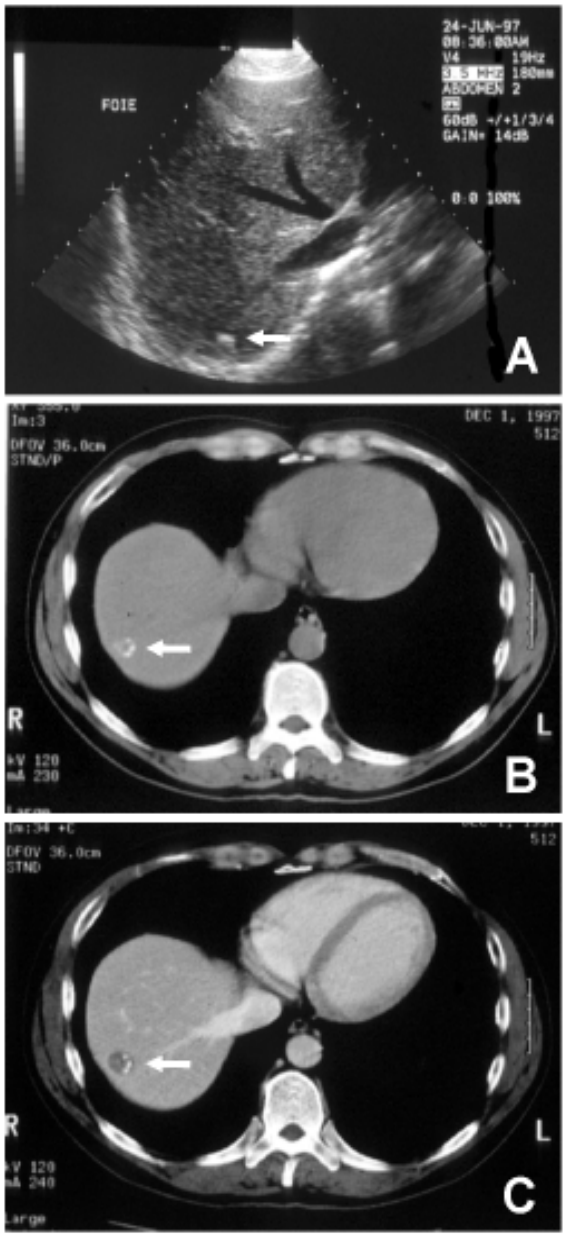


Figure. Abortive alveolar echinococcosis detected in a 44-year-old blood donor in Switzerland. A, ultrasonography of the liver demonstrates the presence of a small echodense lesion (arrow). B, a liver computed tomography scan shows a small, hypodense, apparently fully calcified lesion (arrow). C, after contrast enhancement, a very small hypodense area in the periphery of the calcified herd was detected (arrow).

### Retrospective Statistical Comparative Analyses

Seroprevalence and clinical findings obtained in the present study (Table 3) were directly compared with those obtained in a previous study, which had used similar immunodiagnostic tools and blood donor population (6). The key data used from this previous study originated from 17,166 blood donors, six Em2-ELISA-positive (two of these with hepatic lesions by CT). Subsequent surgery confirmed the presence of active *E. multilocularis* metacestodes. Of 5,166 blood donors additionally tested with EgHF-ELISA, 16 Em2-ELISA-negative donors had EgHF seropositivity. Echinococcosis tests and standard laboratory tools were the same in both studies (11): Diagnostic sensitivity of the Em2-ELISA was 95%, and specificity was 100%. Diagnostic sensitivity of the EgHF-ELISA was 96%, and specificity was 97%. By these key data and the 2-tailed Fisher's exact test, a significant increase of seroprevalence from 1986 to 1996-97 became apparent for both the Em2-ELISA ($p < 0.005$) and the EgHF-ELISA ($p < 0.01$). Conversely, clinical findings (CT hepatic lesions compatible with *E. multilocularis* infection) were not significantly different between the two studies ($p = 0.378$).

## Conclusion

Recent findings have clearly documented a persistently high prevalence of *E. multilocularis* in rural and urban fox populations of Switzerland (3,9,11,14). These data contrasted to a persistently low annual incidence of AHD (8 to 10 new cases per year) in accidentally infected humans (6,15,16). Until 1996, little information was available about the prevalence of *E. multilocularis* in the intermediate rodent host. In 1996 (2), we reported a focus of high *E. multilocularis* prevalence not only in foxes (annual mean 51%) but also in rodents (annual mean 25%). The latter represented the highest prevalence ever reported for rodents in central Europe. The discovery and documentation of an area highly endemic for *E. multilocularis* in definitive and intermediate hosts raised the following question: Does high endemicity have implications for the rate of infection in domestic animals living near *E. multilocularis*-endemic areas and for human health? To address this question, we first had to demonstrate persistence of high endemicity in the rodent population. A 6-year follow-up showed that—independent of interannual fluctuations significant for *A. terrestris* but not for *M. arvalis*—both species remained infected at an exceptionally high level. Consequently, the local dog and cat populations hunting rodents were at a persistently high infection risk. This risk may result in a relatively high exposure risk for human populations in the vicinity. The number of dogs and cats in Switzerland is relatively high (approximately 500,000 dogs and 1.2 million cats). Local veterinarians assessed the dog and cat populations of our study area as within the Swiss average (1 dog per 10 inhabitants and 1 cat per 5 inhabitants). Therefore, any person in our study area may have had direct contact with pet dogs or cats or may have been to locations contaminated by their feces. The veterinarians confirmed that dog and cat owners in the study area did not exhibit any peculiarities in comparison to owners in other areas of Switzerland, including the area and period covered by an earlier study (6). As a precaution, all pet owners visiting veterinary practices in the study area were given information on *E. multilocularis*. The information indicated that carnivores eating rodents in this specific area could be prophylactically treated every 28 days with a therapeutic dose (5 mg per kg body weight) of praziquantel. Assessment of the effectiveness of this therapeutic regimen will be the topic of a separate study.

When we compared our study to other European studies (17,18), we confirmed (as a consequence of the persistently high prevalence in intermediate rodent hosts) an exceptionally high prevalence of intestinal *E. multilocularis* infections, especially in dogs. High prevalence among foxes, dogs, cats, and rodents reflects high environmental contamination with *E. multilocularis* eggs, putatively brought into the households by dogs and cats. Thus, we examined the extended exposure risk of the local human population for increases in seroprevalence, by comparing current data with those collected in an earlier study (6). As expected, Em2-ELISA exhibited a significantly higher seroprevalence. (Higher seroprevalence obtained with the EgHF-ELISA will not be further discussed because the lower specificity of the test may be due to other nonspecific parameters.) However, the number of reported clinical cases did not increase. This lack of increase in cases was underlined by the unique detection of one abortive (died-out) case of AHD (first such case documented in Switzerland). Earlier, we had postulated that the time between infection and clinical manifestation was 5 to 15 years (4,6). However, we know that, in experimental infections of rodents, seroconversion to the Em2-antigen occurs as early as 4 to 6 weeks after peroral inoculation of *E. multilocularis* eggs (unpub. data). As our study covered a 4- to 5-year period of high endemicity (1993 to 1996-97) until the human population was assessed, a significant increase in clinical cases, including asymptomatic (early) cases, which are detectable by ultrasonography, should have been observed in our study. However, high prevalence of *E. multilocularis* in wild (and domestic animals) seemed not to be associated with a higher prevalence of AHD in humans living in the same region.

Epidemiologic data similar to ours have recently been reported with regard to a rural community in southwestern Germany where a high prevalence of *E. multilocularis* (75%) had been observed in foxes (13). Screening of the human population (2,560 participants) found one case of active AHD and nine cases of seropositivity to specific antibodies without detectable liver lesions.

The human population in our study area exhibited low susceptibility to AHD: The relatively high seroprevalence observed in the population was associated with the documented high exposure rate, but the disease rate did not increase. (Disease cases included early cases putatively detectable by ultrasonography.) In addition to low susceptibility, the persistently low incidence of AHD in our study area may also be accounted for by increased immunity, which may protect a large proportion of infected persons. For more detailed and definitive conclusions on disease incidence in humans, we will continue to monitor the affected population. A long-term assessment of the same human population is already planned in the Fribourg area over the next 4 to 10 years. By using the same tools as described in this project, it will be possible to document more subtle changes in disease prevalence in humans.

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## References

1. Gottstein B, Felleisen R. Protective immune mechanisms against the metacestode of *Echinococcus multilocularis.* Parasitol Today 1995;11:320-6.
2. Gottstein B, Saucy F, Wyss C, Siegenthaler M, Jacquier P, Schmitt M, et al. Investigations on a Swiss area highly endemic for *Echinococcus multilocularis.* Appl Parasitol 1996;37:129-36.
3. Hofer S, Gloor S, Müller U, Mathis A, Hegglin D, Deplazes P. High prevalence of *Echinococcus multilocularis* in urban red foxes (*Vulpes vulpes*) and voles (*Arvicola terrestris*) in the city of Zürich, Switzerland. Parasitology 2000;120:135-42.
4. Ammann RW, Hoffmann AF, Eckert J. Swiss study of chemotherapy of alveolar echinococcosis—review of a 20-year clinical research project. Schweiz Med Wochenschr 1999;129:323-32.
5. Hildreth MB, Sriram S, Gottstein B, Wilson M, Schantz PM. Failure to identify alveolar echinococcosis in trappers from South Dakota in spite of high prevalence of *Echinococcus multilocularis* in wild canids. J Parasitol 2000;86:75-7.
6. Gottstein B, Lengeler C, Bachmann P, Hagemann P, Kocher P, Brossard M, et al. Sero-epidemiological survey for alveolar echinococcosis (by Em2-ELISA) of blood donors in an endemic area of Switzerland. Trans R Soc Trop Med Hyg 1987;81:960-4.
7. Schmitt M, Saucy F, Wyborn S, Gottstein B. Befall von Schermäusen (*Arvicola terrestris*) mit Metazestoden von *Echinococcus multilocularis* im Kanton Freiburg (Schweiz). Schweiz Arch Tierheilkd 1997;139:84-93.
8. Pascal M, Meylan A. L'échanillonage linéaire des populations de la forme fouisseuse du Campagnol terrestre (*Arvicola terrestris scherman* (Shaw)). Défense des Végétaux 1986;225:36-44.
9. Deplazes P, Alther P, Tanner I, Thompson RC, Eckert J. *Echinococcus multilocularis* coproantigen detection by enzyme-linked immunosorbent assay in fox, dog, and cat populations. J Parasitol 1999;85:115-21.
10. Mathis A, Deplazes P, Eckert J. An improved test system for PCR-based specific detection of *Echinococcus multilocularis* eggs. J Helminthol 1996;7:219-22.
11. Gottstein B, Jacquier P, Bresson-Hadni S, Eckert J. Improved primary immunodiagnosis of alveolar echinococcosis in humans by an enzyme-linked immunosorbent assay using the Em2plus-antigen. J Clin Microbiol 1993;31:373-6.
12. Rausch RL, Wilson JF, Schantz PM, McMahon BJ. Spontaneous death of *Echinococcus multilocularis*: cases diagnosed serologically by Em2-ELISA and clinical significance. Am J Trop Med Hyg 1987;36:576-85.
13. Romig T, Kratzer W, Kimmig P, Frosch M, Gaus W, Flegel WA, et al. An epidemiological survey of human alveolar echinococcosis in southwestern Germany. Am J Trop Med Hyg 1999;6:566-73.
14. Ewald D, Eckert J, Gottstein B, Straub M, Nigg H. Parasitological and serological studies on the prevalence of *Echinococcus multilocularis* Leuckart, 1863 in red foxes (*Vulpes vulpes* Linnaeus, 1758) in Switzerland. Rev Sci Techn 1992;11:1057-61.
15. Ammann RW, Ilitsch N, Marincek B, Freiburghaus AU. Effect of chemotherapy on the larval mass and the long-term course of alveolar echinococcosis. Swiss Echinococcosis Study Group. Hepatol 1994;19:735-42.
16. Eckert J, Deplazes P. Alveolar echinococcosis in humans: the current situation in Central Europe and the need for countermeasures. Parasitol Today 1999;15:315-9.
17. Viel JF, Giraudoux P, Abrial V, Bresson-Hadni S. Water vole (*Arvicola terrestris* scherman) density as risk factor for human alveolar echinococcosis. Am J Trop Med Hyg 1999;61:559-65.
18. Petavy AF, Deblock S, Walbaum S. Life cycles of *Echinococcus multilocularis* in relation to human infection. J Parasitol 1991;77:133-7.

# Goat-Associated Q Fever: A New Disease in Newfoundland

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In the spring of 1999 in rural Newfoundland, abortions in goats were associated with illness in goat workers. An epidemiologic investigation and a serologic survey were conducted in April 1999 to determine the number of infections, nature of illness, and risk factors for infection. Thirty-seven percent of the outbreak cohort had antibody titers to phase II *Coxiella burnetii* antigen >1:64, suggesting recent infection. The predominant clinical manifestation of Q fever was an acute febrile illness. Independent risk factors for infection included contact with goat placenta, smoking tobacco, and eating cheese made from pasteurized goat milk. This outbreak raises questions about management of such outbreaks, interprovincial sale and movement of domestic ungulates, and the need for discussion between public health practitioners and the dairy industry on control of this highly infectious organism.

*Coxiella burnetii* is an obligate intracellular pathogen known to be the causative agent of Q fever, a zoonosis with a worldwide occurrence (1). The organism has been found in many wild and domestic animals (1-3). The most common reservoirs of infection in humans are domestic farm animals such as cattle, goats, and sheep (4-6). *C. burnetii* is shed in urine, feces, and milk from infected animals and has a particularly high concentration in products of conception (7). The organism is highly infectious: Only one organism is required to produce infection under experimental conditions (8,9). Inhalation of aerosolized microorganisms is thought to be the most important route of infection in humans. However, ingestion of raw milk products has also been implicated (6).

Although *C. burnetii* can cause abortion and stillbirth, most animals have a persistent, relatively asymptomatic subclinical infection (10). Infection in humans usually manifests as a self-limiting febrile illness, pneumonia, or hepatitis (11). Most patients have an uneventful recovery; however, chronic infections such as Q-fever endocarditis and chronic hepatitis are uncommon but well-documented sequelae (12).

The diagnosis of Q fever is usually established by demonstrating seroconversion to *Coxiella* antigens in conjunction with an appropriate clinical history (13). *C. burnetii* can have two distinct antigenic presentations or phases; animals and humans develop antibody responses to both phases. In humans, phase II gives rise to the predominant antibody response in acute infection, while response to phase I antigen is dominant during chronic infections (14).

In the spring of 1999, abortions were noted in goats on one farm belonging to a newly formed cooperative in rural Newfoundland. Aborted placenta had histologic evidence of *C. burnetii* infection. At the same time a number of farmers and their workers had a nonspecific febrile illness associated with severe headaches. Serologic testing revealed that these persons had recent infection with *C. burnetii*. No documented case of Q fever had previously been reported in Newfoundland. An epidemiologic investigation and serologic survey were started in April 1999 to determine the extent of the outbreak in animals and humans, the nature of the clinical illness, and risk factors for Q fever associated with this outbreak.

## Methods

### Identification of Cases

The cooperative consisted of eight goat farms within a 170-km$^2$ area of rural Newfoundland, with a population of approximately 8,000 people (Figure 1). In April 1999, farmers, workers, and contacts (family members of the farmers or workers and other persons who may have had contact with the farms) were interviewed by using a detailed questionnaire. Workers included persons who were involved directly with animal care as well as carpenters and other farm laborers. Serum samples were drawn to determine the presence of antibodies to *C. burnetii*. Family physicians in the area submitted serum samples from all patients in their practices who had been seen with symptoms compatible with Q fever.

The diagnosis of acute *C. burnetii* infection in participants was based solely on serologic findings as

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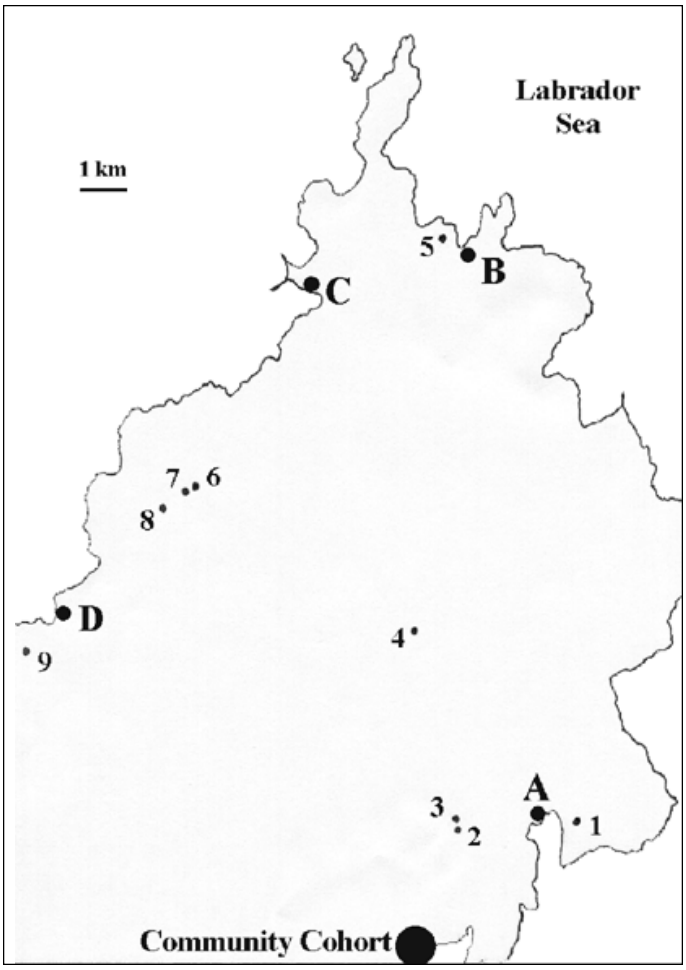


Figure 1. Map of the Newfoundland goat cooperative showing the farms (2-9) in relationship to the surrounding communities (A-D, the area from which the community cohort was derived). (Note: farm 1 is not directly involved with the cooperative.)

described below. In July 1999, follow-up serum samples were obtained to determine further evidence of seroconversion. In addition, 2 weeks earlier, serum was collected from 154 volunteers from adjacent communities (community cohort) and a questionnaire was completed for comparison with the outbreak cohort.

Serum samples were collected in May 1999 from 387 random blood donors, primarily from urban areas. These samples were used to determine the background seroprevalence of *C. burnetii* infection in Newfoundland.

### Source of Animals and Identification of *C. burnetii* Infection in Animals

Although a few locally raised goats were present in the community before the cooperative was established, the eight farms received shipments of goats from Ontario, Nova Scotia, Prince Edward Island, and Maine in the summer and fall of 1998. At the time of the outbreak, 174 goats were within the cooperative, with 10 to 38 animals per herd. Serum samples were obtained from 147 goats to determine the extent of *C. burnetii* infections in the animals.

Serum samples were collected from livestock from other farms throughout Newfoundland to determine the background seroprevalence of Q fever in farm animals in Newfoundland.

### Laboratory Studies

Antibody titers (immunoglobulin G [IgG]) to *C. burnetii* phase I and phase II antigens were determined (15). Antibodies were detected by using indirect immunofluorescence with whole cells of the Nine-Mile strain of *C. burnetii*. An IgG antibody titer of $\geq$1:8 was considered seropositive, indicating prior exposure to *C. burnetii*. Acute *C. burnetii* infection was characterized by a phase II IgG titer of 1:64 or a fourfold rise in titer between two separate serum samples.

Placenta samples from goats were sent to Dr. D. Raoult in France, where they were processed for polymerase chain reaction (PCR) using established protocols (16).

### Epidemiologic Studies

A standardized questionnaire was administered to participants who submitted a serum sample. Demographic data, a detailed history of exposure to goats, clinical history, and symptoms were collected by direct interview. Where available, charts of patients were reviewed to collect additional clinical and laboratory data.

To construct epidemic curves, date of onset of symptoms was considered the date of infection. When this date was unavailable (in asymptomatic cases and participants lacking clinical data), date of infection was based on date of the first serum sample (if it had a diagnostic titer) or the halfway point in those who demonstrated a fourfold rise in antibody titer between acute- and convalescent-phase serum samples.

### Statistical Analysis

Differences between infected and uninfected participants were tested for statistical significance by using the chi-square test for proportions and Student *t* test for means. Independent risk factors for infection were determined by using a backward logistical regression analysis. Variables with a p value of $<0.05$ on univariate analysis were entered into the regression analysis. All data were analyzed by using SPSS for Windows version 8.0 (SPSS Inc. 1989-1999); results were considered significant when p was $<0.05$.

## Results

### Clinical Illness in Goats and Humans

Kidding began January 6 and ended April 24, 1999. Although occasionally it was restricted to dedicated pens, most birthing took place in a communal pen on each farm. *Coxiella* was identified in placental samples examined by using electron microscopy and light microscopy (Gimenez stain), and *C. burnetii* DNA was demonstrated in all three placental samples with PCR. A total of 30 abortions were recorded at six of the eight farms. (Some farms had incomplete records.) The first abortion occurred in December before the kidding season began; the others took place between January 14 and April 24, with abortion rates of 16%-22% per farm. There was no relationship between seropositivity in goats and frequency of abortion.

The epidemic curves differed from farm to farm. Evidence of a continuous common source of infection was seen at one farm (Figure 2), while other evidence suggested a point source (Figure 3). The overall epidemic curve suggested a continuous

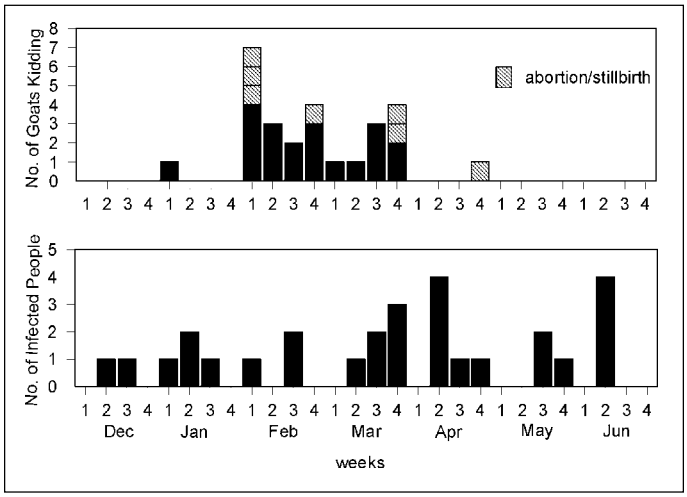


Figure 2. Epidemic curve for farm no. 4, showing the timing of human infection with kidding and abortions.

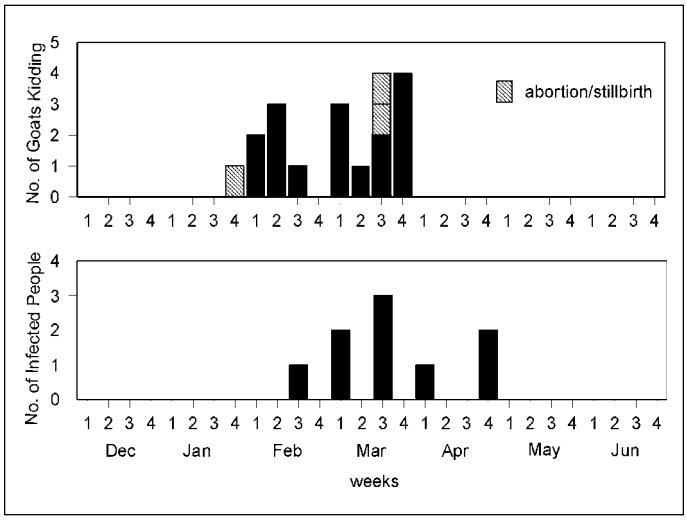


Figure 3. Epidemic curve from farm no. 6, showing the timing of human infection with kidding and abortions.

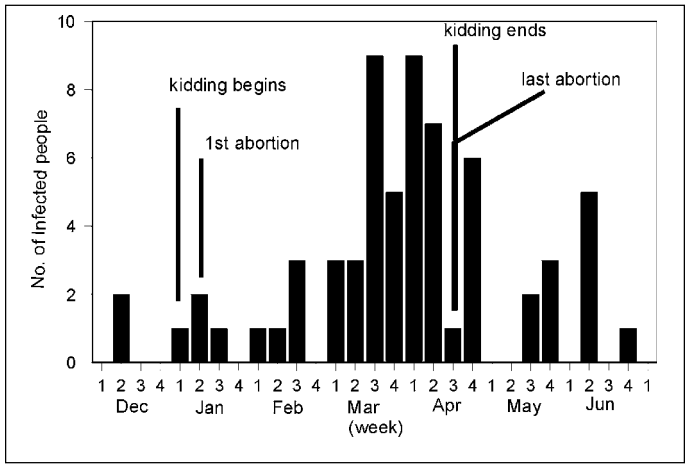


Figure 4. Overall epidemic curve for Q fever outbreak associated with Newfoundland goat cooperative.

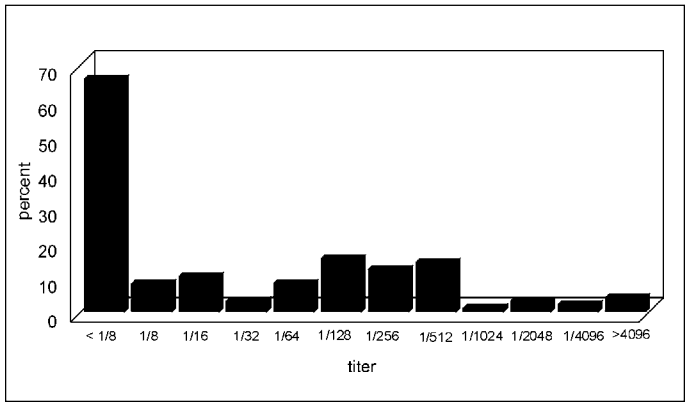


Figure 5. Antibody titers to *Coxiella burnetii* phase II antigen in the outbreak cohort. Of the infected cohort, 24/66 had a fourfold rise in antibodies to phase II (24/66 of the infected cohort had a fourfold rise in antibodies to phase II antigen)

source or reservoir for infection that had a peak during the kidding season (Figure 4).

Illness in goat farmers or their workers was noted in March 1999. Serologic data were available for 179 farmers, workers, and contacts (outbreak cohort). Eighty (44.7%) outbreak cohort participants had antibodies against the phase II antigen. Sixty-six (36.9%) had phase II titers of ≥1:64 or had a fourfold rise in titer, suggesting recent infection (Figure 5). The seroprevalence of infected workers (including farmers) on each farm ranged from 0 (farm 5) to 87.5% (farm 4). In comparison, 35 (22.7%) of 154 community cohort participants were seropositive ($p<0.001$), and 2 (1.3%) had titers of antibodies to phase II antigen of >1:64 (Figure 6). Seroprevalence in blood donors (8.3%) (Table 1) was significantly lower than that of the control ($p<0.001$) and outbreak ($p<0.001$) cohorts. Five blood donors (1.3%) had titers to phase II antigen ≥1:64.

Questionnaires were completed by 146 (81.6%) farm workers or contacts who provided blood samples. The remaining 33 could not be reached for questioning. Of the 146 participants, 9 (6.2%) were farmers, 58 (39.7%) were workers, and 79 (54.1%) were contacts. Demographic data were collected (Table 2). The infected and noninfected groups had equal numbers of men and women. Infected persons tended to be slightly older, were more likely to have been ill in the past 2 months (odds ratio [OR] 3.53), and to have visited their doctor during that time (OR 3.13). Symptoms associated with infection included sweats, chills, headache, weight loss, malaise, fever, fatigue, myalgias, dyspnea, nausea, and diarrhea (Table 2).

The incubation period for Q fever was difficult to determine as most people had many contacts with goats. However, three persons could recall the date of a specific high-risk activity such as assisting with the delivery of a stillborn kid. Incubation periods for these three persons were 21, 31, and 36 days.

A family physician performed clinical laboratory tests on 25 of the infected persons. Four (16%) of these had transaminase levels >1.5x, the upper limit of normal. Eight had X rays; one had pneumonia.

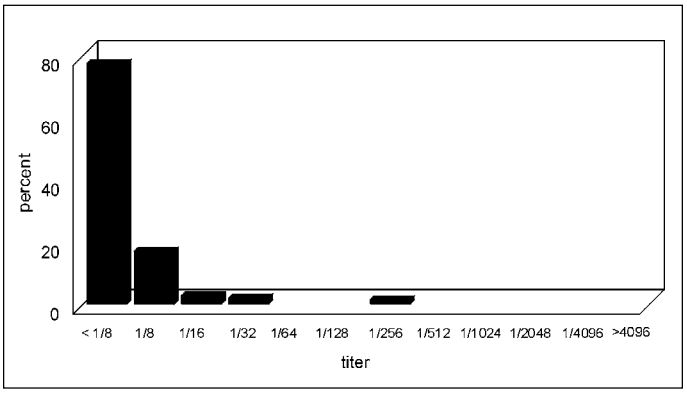


Figure 6. Antibody titers to *Coxiella burnetii* phase II antigen in the community cohort.

Table 1. Seroprevalance of antibodies to *Coxiella burnetii* phase II antigen in random blood donors from Newfoundland

| Region | Seropositivity no. (%) |
|---|---|
| St. John's Center | 12/155 (7.7) |
| Cornerbrook | 1/31 (3.2) |
| St. John's | 7/57 (12.3) |
| Norman's Cove | 4/40 (10.0) |
| Foxtrap | 6/71 (8.5) |
| Conception Bay Central | 2/33 (6.1) |
| Total | 32/387 (8.3) |

Table 2. Demographic features and symptoms associated with human *Coxiella burnetii* infection in Newfoundland outbreak

| Features and symptoms | Infected no. (%) | Noninfected no. (%) | Odds ratio (95% CI) |
|---|---|---|---|
| Male | 31/58 (53.4) | 46/88 (52.3) | 1.05 (0.154-2.04) |
| Female | 27/58 (46.6) | 42/88 (47.7) | |
| Mean age | 38.48±11.76 | 33.47±19.34 | p=0.054 |
| Sick in the past 2 months | 49/60 (81.7) | 48/86 (55.8) | 3.53 (1.62–7.70) |
| Symptoms | | | |
| Sweats | 11/12 (91.7) | 3/9 (33.3) | 22.0 (1.86–260.5) |
| Chills | 12/14 (85.7) | 4/10 (40.0) | 9.00 (1.27–63.90) |
| Headache | 43/60 (71.7) | 20/78 (25.6) | 7.34 (3.44–15.64) |
| Malaise | 44/59 (74.6) | 28/77 (36.4) | 5.13 (2.43–10.84) |
| Weight loss | 8/31 (25.8) | 3/43 (7.0) | 4.64 (1.12–19.24) |
| Fever | 40/60 (66.7) | 25/79 (31.6) | 4.32 (2.11–8.84) |
| Fatigue | 39/58 (67.2) | 27/79 (34.2) | 3.95 (1.93–8.11) |
| Myalgias | 26/58 (44.8) | 15/76 (19.7) | 3.30 (1.54–7.11) |
| Dyspnea | 19/60 (31.7) | 12/78 (15.4) | 2.55 (1.12–5.79) |
| Nausea | 30/60 (50.0) | 22/80 (27.5) | 2.24 (1.30–5.34) |
| Diarrhea | 23/60 (38.3) | 18/81 (22.2) | 2.18 (1.04–4.55) |
| Cognitive[a] | 14/30 (46.7) | 4/15 (26.7) | 2.41 (0.62–9.29) |
| Sputum | 13/46 (22.0) | 11/78 (14.1) | 1.72 (0.71–4.18) |
| EOM pain | 8/15 (53.3) | 4/9 (44.4) | 1.43 (0.27–7.52) |
| Neck stiffness | 16/22 (72.7) | 9/14 (64.3) | 1.43 (0.27–7.52) |
| Vomiting | 12/60 (20.0) | 15/81 (18.5) | 1.10 (0.47–2.56) |
| Pleuritic pain | 8/57 (14.0) | 10/76 (13.2) | 1.08 (0.40–2.93) |
| Cough | 22/60 (36.7) | 28/81 (34.6) | 1.10 (0.55–2.20) |
| Loss of libido | 13/32 (40.6) | 5/12 (41.7) | 0.96 (0.25–3.38) |

[a]Cognitive problems, including changes in concentration, memory, or temper. EOM, extra-ocular eye movement; CI, confidence intervals.

### Risk Factors for Q Fever

Risk factors associated with human infection on univariate analysis included being a farmer, milking goats, assisting with kidding, handling placentas, shoveling manure, having direct contact with goats, eating cheese made from goat milk, petting goats, feeding goats, being a worker, smoking tobacco, and drinking alcohol (Table 3). When only a multivariate analysis was used, the following were significant risk factors for infection with *C. burnetii*: contact with the placenta (p<0.001), smoking history (p=0.001), and eating cheese made from goat milk (p= 0.022). Both infected persons in the community cohort also had direct contact with goats.

Overall, 82 (55.8%) of the 147 goats were seropositive (range from 10% to 100%, depending on the farm); antibody titers ranged from 1:8 to >1:4,096. Although 8 (50%) of 16 goats from other areas in eastern Newfoundland had antibodies to *C. burnetii*, the highest titer was 1:16. In contrast, titers in goats in the outbreak ranged from 1:8 to >1:4,096. In the goats in the cooperative, 63 (43%) and 30 (20%), respectively, had an antibody titer of ≥1:64 to phase I and phase II antigen. Correlation between *C. burnetii* infection in goats and the geographic origin of the animals or determination of a relationship between seropositivity of goats and the number of persons infected on each farm was not feasible because of insufficient data.

Table 3. Exposure risks associated with *Coxiella burnetii* infection in the Newfoundland outbreak

| Risk factors | Infected no.(%) | Noninfected no.(%) | Odds ratio (95% CI) |
|---|---|---|---|
| Visited a barn | 57/60 (95.0) | 63/86 (73.3) | 6.94 (1.98–24.34) |
| Direct contact with goats | 54/60 (90.0) | 54/86 (62.8) | 5.33 (2.06–13.79) |
| Milking | 19/60 (31.7) | 3/85 (3.5) | 12.67 (3.54–45.29) |
| Assisting with kidding | 29/60 (48.3) | 6/85 (7.1) | 12.32 (4.66–32.57) |
| Handling placenta[a] | 31/60 (51.6) | 7/86 (8.1) | 12.06 (4.79–30.39) |
| Shoveling manure | 37/60 (61.7) | 19/84 (22.6) | 5.50 (2.65–11.41) |
| Feeding | 39/57 (68.4) | 29/85 (34.2) | 4.17 (2.06–8.43) |
| Petting goats[a] | 52/60 (86.7) | 51/85 (60.0) | 4.33 (1.83–10.26) |
| Farmer | 8/60 (13.3) | 1/85 (1.2) | 12.92 (1.57–106.32) |
| Farm worker | 34/60 (56.7) | 24/86 (27.9) | 3.38 (1.67-6.77) |
| Household contact, visited farm | 13/60 (21.7) | 35/85 (41.2) | 0.40 (0.19-0.84) |
| Household contact, no farm visit | 2/60 (3.3) | 23/85 (27.1) | 0.09 (0.02–0.41) |
| Ate goat cheese[a] | 17/60 (28.3) | 6/86 (7.0) | 5.27 (1.94–14.35) |
| Smoked[a] | 36/58 (62.1) | 28/84 (33.3) | 3.27 (1.63–6.58) |
| Drank alcohol | 37/57 (64.9) | 38/84 (45.2) | 2.08 (1.04–4.13) |
| Have liver problems | 5/53 (9.4) | 2/72 (2.8) | 3.65 (0.68-19.57) |
| Have cats | 25/59 (42.4) | 30/86 (34.9) | 1.37 (0.70–2.71) |
| Drink goat milk | 19/60 (31.7) | 27/86 (31.4) | 1.01 (0.50–2.06) |

[a]By logistic regression model, the following were statistically significant: Contact with placenta (p <0.001); smoking history (p=0.001); eating goat cheese (p=0.022); and petting goats (p=0.055).

## Conclusion

Goats have been implicated in outbreaks of Q fever in the United States, Ontario, Bulgaria, Slovakia, Greece, and Australia, and have replaced sheep and cattle as the most common source of human infection with *C. burnetii* in Bulgaria (17-19). An estimated 20% of Ontario's dairy goat population have antibodies to *C. burnetii* (20).

The incubation period and clinical illness seen in the Newfoundland outbreak were consistent with those reported for other outbreaks (5,15,21,22). The most common manifestation of *C. burnetii* infection was an acute febrile illness. Although dyspnea was an associated feature of our outbreak, only one of eight patients with X rays had pneumonia. This is in contrast to what is typically seen in Nova Scotia, where *C. burnetii* pneumonia is common after exposure to infected parturient cats (15,23).

Although the patients reported here are the first documented cases of Q fever in Newfoundland, serologic results from blood donors suggest that infection with this organism is present elsewhere in this province but goes unrecognized. The seroprevalence of *C. burnetii* in Newfoundland blood donors (8.3%) is consistent with results from blood donors in other Atlantic Canadian provinces (24,25). The higher seroprevalence in the population from communities surrounding the outbreak area (22.7%) could be due to their close proximity to the outbreak area or may reflect a difference in prevalence, which is often higher in rural areas (11).

The eight farms in the cooperative house their goats in small, uninsulated, naturally ventilated barns, many of which have concrete floors. The winter and spring months in Newfoundland can be quite cold, so to provide better insulation the hay spread on floors of the pens is packed down instead of being disposed of regularly. The resulting "manure pack" would be heavily contaminated by *C. burnetii* in feces, urine, and products of conception. Removing the bedding would generate aerosols containing *C. burnetii*. *Coxiella* is very hardy and resists desiccation, remaining viable in soil for several years (26).

Contaminated hay and manure were also spread on the rocky ground to fertilize small pastures next to the barns. This method of disposal represents potential sources of exposure for surrounding communities. Inhalation of *C. burnetii* from contaminated environments is well documented, and contaminated fields and roads often serve as reservoirs for airborne spread of *C. burnetii* (5,18,22,27,28). Studies from Europe demonstrate that wind can spread *C. burnetii* >18 km from its source (29). These newly developed pastures in the Newfoundland cooperative may explain the higher seroprevalence rate in the community cohort compared with that in blood donors from across the province.

Kidding took place in isolated pens but also occurred in communal areas of the barn. Placental tissue and aborted kids were disposed of by incineration or burial. Although the workers usually did not handle the placenta, they would often help clean and dry newborn goats covered in amniotic fluid without the protection of masks or gloves. Exposure to the birth products of infected animals has been consistently shown to be a risk factor in other Q fever outbreaks (28). Given that *Coxiella* is shed in high numbers in birth products (7) and aerosolization of the microorganism can persist for days after parturition, despite immediate removal of the highly infectious placenta (30), it is not surprising that exposure to the placenta was an independent risk factor for infection ($p<0.001$).

In our study, smoking tobacco was an independent risk factor for infection ($p=0.001$). This could be due to contaminated hands touching cigarettes, resulting in ingestion of *Coxiella*. Smoking does impair pulmonary host defenses and thus may have contributed to this finding (31). In addition, some barns did not have running water and washrooms until late in the spring, contributing to poor hygienic practices in some instances.

The role of drinking unpasteurized milk in *C. burnetii* infection is controversial. *C. burnetii* has been recovered from milk from infected cows and goats and from butter (17,32). Epidemiologic studies suggest that ingestion of unpasteurized milk has been a source of *Coxiella* infection for humans (6,17,33). Experimental evidence to support a causal relationship is sparse. Asymptomatic seroconversion and infection were noted in inmates fed raw milk from a Q fever infected herd (33). In another study, volunteers who drank naturally infected unpasteurized milk did not develop symptoms or an immunologic response to suggest infection (34). These authors suggest that the lack of seroconversion in their study may have been related to exposure to a different *Coxiella* strain than the one that caused infection in the inmate population (33,34). Pasteurization will effectively kill *Coxiella* in raw milk (35). However, in our study, ingestion of cheese made from pasteurized goat milk was identified as an independent risk factor for infection ($p=0.022$) even though consumption of goat milk itself was not associated with an increased risk of infection (OR 1.07). This is the first time a pasteurized dairy product has been implicated in an outbreak of Q fever. However, 21 (14%) of 154 members of the community cohort ate the product but were not infected. The reason for the association between ingesting goat cheese and developing Q fever is not clear and suggests further study is needed. At present, this is an epidemiologic association only, as *C. burnetii* has not been recovered from the goat cheese.

In Canada, *C. burnetii* infection is not a reportable disease in animals (36). Serbezov (19) suggests that "goats may pose a threat to human health as a source of *C. burnetii* infection in every country in which they are raised extensively and are in close contact with humans." Goats in the Newfoundland cooperative originated from four different sources—Maine, Nova Scotia, Prince Edward Island, and Ontario. Although the sale and movement of infected animals have been implicated in spreading the disease (4), there was no relationship between the seroprevalence rate of goats originating from one area compared to another, making it difficult to determine if one group of imported animals was responsible for initiating the outbreak. However, goats tend to remain chronically infected, and once infection is established it can spread rapidly through the remaining herds (37). Once *C. burnetii* infection was identified in the herd, only four goats on one farm in the cooperative were treated with antibiotics.

These are the first cases of Q fever in Newfoundland. The small barns and poor ventilation created confined conditions

and an environment that facilitated infection. Although exposure to goats and eating unpasteurized milk have been implicated in causing *C. burnetii* infection in the past, this is the first time that a product made from pasteurized milk has been associated epidemiologically as a risk factor. Outbreaks of Q fever in research institutions as a result of exposure to infected parturient sheep and goats has led to number of recommendations (38-41). These recommendations include using only *C. burnetii*-seronegative animals in research; vaccinating seronegative animals; using protective clothing and masks while working with animals (especially pregnant ones); restricting access to animals; properly decontaminating surfaces with formalin or bleach solutions; properly disposing of waste by incineration; and using caution, culling, confinement, or chemotherapy in herds with a rate of >20% seropositivity containing animals with titers ≥1:32.

Some of these measures are difficult to carry out on a dairy farm; however, since data suggest that human infection can be prevented by vaccination with formalin-inactivated phase I *C. burnetti*, persons at risk from occupational exposure should be offered the vaccine (41).

Our experience raises many questions about management of *C. burnetii* outbreaks in the dairy industry, the interprovincial sale and movement of domestic ungulates, and the need for discussion between public health practitioners and the dairy industry on control of this highly infectious organism.

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**References**

1. Kaplan MM, Bertagna P. The geographical distribution of Q fever. Bull World Health Org 1955;13:829-60.
2. Marrie TJ, Embil J, Yates L. Seroepidemiology of *Coxiella burnetii* among wildlife in Nova Scotia. Am J Trop Med Hyg 1993;49:613-15.
3. Marrie TJ, Van Buren J, Fraser J, Haldane EV, Faulkner RS, Williams JC, et al. Seroepidemiology of Q fever among domestic animals in Nova Scotia. Am J Public Health 1985;75:763-6.
4. Luoto L, Pickens EG. A resume of recent research seeking to define the Q-fever problem. Am J Hyg 1961;74:43-9.
5. Dupuis G, Petite J, Peter O, Vouilloz M. An important outbreak of human Q fever in a Swiss alpine valley. Int J Epidemiol 1987;16:282-7.
6. Fishbein DB, Raoult D. A cluster of *Coxiella burnetii* infections associated with exposure to vaccinated goats and their unpasteurized dairy products. Am J Trop Med Hyg 1992;47:35-40.
7. Abinanti FR, Lennette EH, Winn JF, Welsh HH. Q fever studies XVVII. Presence of *Coxiella burnetii* in the birth fluids of naturally infected sheep. Am J Hyg 1953;58:358-88.
8. Tigertt WD, Benenson AS, Gochenour WS. Airborne Q fever. Bacteriol Rev 1961;25:285-93.
9. Ormsbee R, Peacock M, Gerloff R, Tallent G, Wike D. Limits of rickettsial infectivity. Infect Immun 1978;19:239-45.
10. Moore JD, Barr BC, Daft BM, O'Connor MT. Pathology and diagnosis of *Coxiella burnetii* infection in a goat herd. Vet Pathol 1991;28:81-4.
11. Tissot Dupont H, Raoult D, Brouqui P, Janbon F, Peyramond D, Weiller PF, et al. Epidemiologic features and clinical presentation of acute Q fever in hospitalised patients: 323 French cases. Am J Med 1992;93:427-34.
12. Raoult D, Tissont-Dupont H, Foucault C, Gouvernet J, Fournier PE, Bernit E, et al. Q fever 1985-1998. Clinical and epidemiologic features of 1383 infections. Medicine 2000;79:109-23.
13. Fournier PE, Marrie TJ, Raoult D. Diagnosis of Q fever. J Clin Microbiol 1998;36:1823-34.
14. Peacock MG, Philip RN, Williams JC, Faulkner RS. Serologic evaluation of Q fever in humans: Enhanced phase I titres of immunoglobulins G and A are diagnostic for Q fever endocarditis. Infect Immun 1983;41:1089-98.
15. Marrie TJ, MacDonald A, Durant H, Yates L, McCormick L. An outbreak of Q fever probably due to contact with a parturient cat. Chest 1988;93:98-103.
16. Willems HD, Thiele D, Frolich-Ritter R, Krauss H, et al. Detection of *Coxiella* in cow's milk using the polymerase chain reaction. J Vet Med 1994;41:580-7.
17. Lennette EH, Clark WH. Observations on the epidemiology of Q fever in northern California. JAMA 1951;145:306-9.
18. Spicer AJ, Crowthier RW, Vella EE, Bengtsson E, Miles R, Pitzolis G. Q fever and animal abortion in Cyprus. Trans R Soc Trop Med Hyg 1977;71:16-20.
19. Serbezov V, Kazar J, Novkirishki V, Gatcheva N, Kovacova E, Voynova V. Q fever in Bulgaria and Slovakia. Emerg Infect Dis 1999;5:388-94.
20. Lang GH. Serosurvey of *Coxiella burnetii* infection in dairy goat herds in Ontario. A comparison of two methods of enzyme-linked immunosorbent assay. Can J Vet Res 1988;52: 37-41.
21. Clark WH, Lennette EH, Railsback OC, Romer MS. Q-fever in California vii. Clinical features in one hundred eighty cases. Arch Intern Med 1951;88:155-67.
22. Derrick EH. The course of infection with *Coxiella burnetii*. Med J Aust 1973;1:1051-7.
23. Marrie TJ, Durant H, Williams JC, Mintz E, Waag DM, et al. Exposure to parturient cats: A risk factor for acquisition of Q fever in Maritime Canada. J Infect Dis 1988;158:101-8.
24. Marrie TJ, VanBuren J, Faulkner RS, Haldane EV, Williams JC, Kwan C. Seroepidemiology of Q fever in Nova Scotia and Prince Edward Island. Can J Microbiol 1984;30:129-34.
25. Marrie TJ. Seroepidemiology of Q fever in New Brunswick and Manitoba. Can J Microbiol 1988;34:1043-5.
26. Reimer LG. Q fever. Clin Microbiol Rev 1993;6:193-8.
27. Tissot-Dupont H, Torres S, Nezri M, Raoult D. Hyperendemic focus of Q fever related to sheep and wind. Am J Epidemiol 1999;150:67-74.
28. Hart RJC. The epidemiology of Q fever. Postgrad Med J 1973;49:535-8.
29. Hawker JI, Ayres JG, Blair I, Evans MR, Smith DL, Smith EG, et al. A large outbreak of Q fever in the West Midlands: windborne spread into a metropolitan area? Commun Dis Public Health 1998;1:180-7.
30. Welsh HH, Lennette EH, Abinanti FR, Winn JF. Airborne transmission of Q fever: The role of parturition in the generation of infective aerosols. Ann NY Acad Sci 1958;70:528-40.
31. Daniele RP, Dauber JH, Altose MD, Rowlands DT, Gorenberg DJ. Lymphocyte studies in asymptomatic cigarette smokers: A comparison between lung and peripheral blood. Am Rev Respir Dis 1977;116:997-1005.
32. Biberstein EL, Behymer DE, Bushnell R, Crenshaw G, Riemann HP, Franti CE. A survey of Q fever (*Coxiella burnetii*) in California dairy cows. Am J Vet Res 1974;35:1577-82.
33. Benson WW, Brock D, Mather J. Serologic analysis of a penitentiary group using raw milk from a Q fever infected herd. Public Health Rep 1963;78:707-10.
34. Krumbiegel ER, Wisniewski HJ. Consumption of infected raw milk by human volunteers. Arch Environ Health 1970;21:63-5.

35. Enright HB, Sadler WW, Thomas RC. Pasteurisation of milk containing the organism of Q fever. Am J Pub Health 1957;47:695-700.
36. Lang GH. Serosurvey of the occurrence of *Coxiella burnetii* in Ontario cattle. Can J Public Health 1988;79:56-9.
37. Wisniewski HJ, Krumbiegel ER. Q fever in the Milwaukee area—I. Q fever in Milwaukee. Arch Environ Health 1970;21:58-65.
38. Simor AE, Brunton JL, Salit IE, Vellend H, Ford-Jones L, Spence LP. Q fever: hazard from sheep used in research. Can Med Assoc J 1984;130:1013-6.
39. Graham CJ, Yamauchi T, Rountree P. Q fever in animal laboratory workers: An outbreak and its investigation. Am J Infect Control 1989;17:345-8.
40. Perry S, Dennie CJ, Coblentz CL, Cleland S. Minimizing the risk of Q fever in the hospital setting. Can J Infect Control 1994;9:5-8.
41. Maurin R, Raoult D. Q fever. Clin Microbiol Rev 1999;12:518-53.

# Molecular Epidemiology of Serogroup A Meningitis in Moscow, 1969–1997

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Molecular analysis of 103 serogroup A *Neisseria meningitidis* strains isolated in Moscow from 1969 to 1997 showed that four independent clonal groupings were responsible for successive waves of meningococcal disease. An epidemic from 1969 to the mid-1970s was caused by genocloud 2 of subgroup III, possibly imported from China. Subsequent endemic disease through the early 1990s was caused by subgroup X and then by subgroup VI, which has also caused endemic disease elsewhere in eastern Europe. A 1996 epidemic was part of the pandemic spread from Asia of genocloud 8 of subgroup III. Recent genocloud 8 epidemic disease in Moscow may represent an early warning for spread of these bacteria to other countries in Europe.

Until the mid-1970s, *Neisseria meningitidis* expressing the serogroup A capsular polysaccharide caused numerous epidemics of meningitis and septicemia, with annual incidence rates >100/100,000 (1). During serogroup A epidemics, these bacteria can also be isolated from the nasopharynx of a considerable proportion of the healthy population (2). After the mid-1970s, large serogroup A epidemics became rare, except in the so-called Meningitis Belt in the Sahel region of Africa (3,4). In 1997, *N. meningitidis* caused approximately 500,000 cases of disease and 50,000 deaths worldwide, half of them in Africa (5). Serogroup A meningococci are currently isolated only rarely in western Europe and the United States, and a rise in either the healthy carriage rate or the proportion of serogroup A cases would be cause for concern.

Epidemic isolates of *N. meningitidis* have been assigned to a limited number of clonal groupings, historically by multilocus enzyme electrophoresis (MLEE) (4) and currently by multilocus sequence typing (MLST) (6). For MLST, sequences of housekeeping gene fragments are assigned different numeric allele designations, even if they differ by only one nucleotide. The combination of alleles from seven housekeeping gene fragments is called the sequence type (ST). Serogroup A bacteria have also been assigned to clonal groupings by random amplification of polymorphic DNA (RAPD) (7). These three methods yield largely concordant results, except that MLST is the most conservative and RAPD differentiates the largest number of isolates. The clonal groupings among serogroup A meningococci have been designated subgroups I through IX according to MLEE (7,8). Some of these subgroups have not been differentiated by RAPD analysis (I vs. II, III vs. VIII) (7) or MLST.

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Recent epidemic serogroup A disease in Europe has largely reflected the pandemic patterns of spread of subgroup III meningococci. After a large subgroup III epidemic in China in the mid-1960s (8), these bacteria caused an outbreak of meningococcal disease in 1969 in western Norway (9), followed in the mid-1970s by a major epidemic in Finland (10). During the 1970s, subgroup III meningococci were isolated throughout western and northern Europe (11). In 1987, serogroup A, subgroup III meningococci caused a meningitis epidemic during the Hajj pilgrimage to Mecca, Saudi Arabia (12), and healthy pilgrims carried these bacteria throughout the world. Localized serogroup A disease was associated with former pilgrims for several years in the United Kingdom (13) and France (14), but these meningococci have since largely disappeared from western Europe. Rare endemic serogroup A disease was associated with subgroup VI in East Germany in the 1980s (8) and with subgroup IX in the Netherlands in the early 1990s (7).

Meningococci are naturally transformable and can import novel alleles from neisseriae that colonize the nasopharynx (15,16). However, these recombinant strains are usually lost during the bottlenecks caused by limited numbers of bacteria being transmitted during epidemic spread (17). Occasionally, variants pass through these bottlenecks first, and all subsequent progeny have a common genetic background different from that of the parental bacteria (17). Most subgroup III bacteria isolated from the mid-1960s to the mid-1980s ("pre-Mecca") had particular alleles of the hypervariable *opaB* and *opaD* loci encoding Opa (opacity) proteins and the *iga* locus encoding IgA1 protease. In contrast, subgroup III bacteria isolated after the 1987 Mecca epidemic ("post-Mecca") had other alleles at these three loci (17). Exceptional isolates with still other alleles at one or more of these loci were also found in several countries, but these variants all disappeared during subsequent epidemic spread (17).

Data in the English literature about meningococci from Russia and other areas of eastern Europe are scarce. In recent decades, most patients with meningitis in Moscow (population 8.6 million during 1990 to 1999) have been treated at the Second Moscow Hospital for Infectious Diseases. Our review of their records shows that from 1980 to 1999, the annual incidence rates of meningitis caused by *Haemophilus influenzae* and *Streptococcus pneumoniae* were approximately 0.2/100,000 and 0.4/100,000, respectively. In contrast, several epidemics of meningococcal disease with much higher incidence rates occurred in Moscow from 1924 to 1998 (Figure 1). Large serogroup A epidemics occurred in 1931 and 1940, followed by a decline in disease incidence until

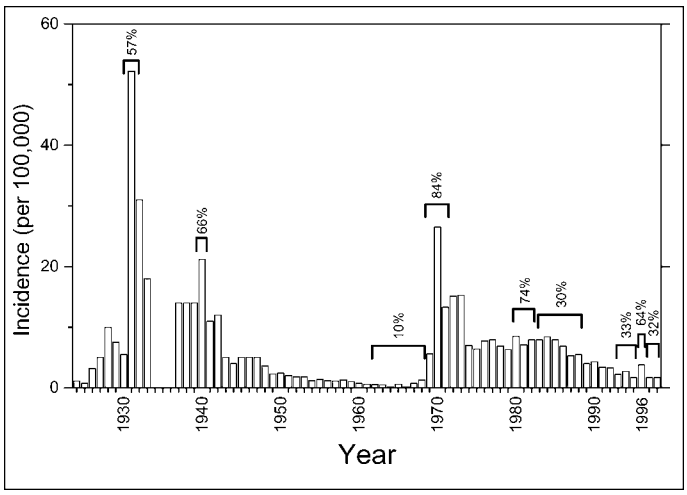


Figure 1. Annual incidence rates of meningococcal meningitis in Moscow from 1924 to 1998. Incidence rates were extracted from the compilation by Bolshakov (18) and data published in annual reports of the Russian Ministry of Public Health or obtained from 1969 to 1998 by the Central Research Institute of Epidemiology from epidemiologic investigations in Moscow. The percentages of serogroup A meningococci among all meningococci isolated from disease were compiled from various sources [number of strains]: 1924-1969, Bolshakov (18); 1962-1971, Kostyukova (19) [300]; 1980-1988, Demina (20) [1,000]; and 1993-1998, Koroleva, laboratory records [500].

the late 1960s. In 1968, a serogroup A epidemic began in Lipezk, Russia, where factory workers had recently arrived from Vietnam. These immigrants had traveled by rail through China, where subgroup III disease was prevalent (8). The incidence rate of meningococcal disease in Moscow and the proportion of serogroup A isolates remained high until the 1980s. In 1996, a new outbreak of serogroup A disease began, again initially associated with the Vietnamese community. During the outbreak peak in early 1996, 50 (34%) of 147 patients with meningococcal disease were Vietnamese, and all their disease isolates were serogroup A. During the rest of 1996, only 8 (6%) of 145 patients with meningococcal disease belonged to the Vietnamese community. During 1997 to 1999, hundreds of thousands of Muscovites were immunized with A polysaccharide vaccine. The incidence rate and the proportion of serogroup A isolates decreased and have remained stable through 1999.

We present data on the molecular epidemiology and genetic relationships of serogroup A meningococci isolated in Moscow from 1969 to 1997. The data show that four clonal groupings have been responsible for successive waves of disease and that epidemic disease in Moscow since 1994 is associated with a new subgroup III pandemic.

## Materials and Methods

### Serogroup A Isolates from Moscow

One hundred three strains of *N. meningitidis* were isolated in Moscow from the cerebrospinal fluid or blood of patients with systemic meningococcal disease or from throat swabs of their close contacts (seven subgroup VI strains, 1988-89). Strains were serogrouped by immune precipitation with specific sera and lyophilized. The bacteria we describe represent almost all the viable serogroup A bacteria still available in the laboratory collections at the Central Research Institute of Epidemiology and the Gamaleya Institute of Epidemiology and Microbiology, with the following exceptions: Only one third of the bacteria available from 1969 to 1972 and half the bacteria from 1996 were tested to avoid bias toward these two periods. The lyophilized bacteria were purified again by single colony isolation. These bacterial cultures were then serogrouped, serotyped, and subtyped by using whole-cell enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (8). DNA was prepared as described (21) and used for molecular fine typing and MLST.

### MLST

Both strands of fragments of *abcZ*, *adk*, *aroE*, *gdh*, *pdhC*, *pgm*, and *fumC* were sequenced as described (http://www.mlst.net), except that primers O1101 (5'TCCGGCTTGCCGTTTGTCAG) and O1102 (5'TTGTAGGCGGTTTTGGCGAC) were used for *fumC*. At the beginning of this project, the MLST database inadequately represented the diversity of serogroup A meningococci because it contained data for only 36 strains, predominantly from MLEE subgroups I, III, and IV-1 (6). To enlarge the database, MLST was performed with 66 serogroup A isolates from diverse sources that had previously been assigned to subgroups II through IX by MLEE (7,8) and in some cases by RAPD analysis (7). MLST was also performed with 42 serogroup A isolates from Moscow chosen from the most diverse branches of the RAPD tree. Other laboratories had deposited data for eight serogroup A strains in six novel STs, resulting in 152 serogroup A strains in 31 STs (Table 1). The results, including strain description, source, and MLEE assignments, are publicly available (http://www.mlst.net).

### Molecular Fine Typing of Subgroup III

Alleles of the four chromosomal *opa* genes were determined by restriction analysis of polymerase chain reaction (PCR) products generated by using one primer in the upstream conserved flanking DNA and a second HV2-specific primer, as described (17). The complete *opa* genes were also sequenced from selected representative strains.

A fragment of *iga* was amplified from chromosomal DNA by using primers O103 and O115 and tested for a *Bfa*I site that is present in *iga1* but not in *iga2* or *iga3* (17). A different fragment of *iga* was amplified with primers O113 and O151 and tested for a *Dde*I site that is present in *iga3* but not *iga2* or *iga1* (17). In addition, the entire *iga* gene was amplified with primers O103 and O152 and sequenced from one ST5 and one ST7 strain.

Table 1. Multilocus sequence typing analysis of serogroup A meningococci

| ST[a] | MLEE/ RAPD | Sources | No. | *fumC* | *abcZ* | *adk* | *aroE* | *gdh* | *pdhC* | *pgm* |
|---|---|---|---|---|---|---|---|---|---|---|
| 21 | other | UK (1941) | 1 | 1 | 1 | 5 | 1 | 2 | 16 | 17 |
| 1 | I, II | Pandemic (1963-80) | 15 | 1 | 1 | 3 | 1 | 1 | 1 | 3 |
| 75 | X | Moscow (1983-94) | 3 | 1 | 2 | 3 | 25 | 1 | 1 | 3 |
| 78 | X | Moscow (1997) | 1 | 1 | 2 | 3 | 25 | 1 | 1 | 17 |
| 76 | X | Moscow (1983-85) | 4 | 17 | 2 | 3 | 1 | 1 | 1 | 3 |
| 77 | X | Moscow (1984-85) | 3 | 1 | 2 | 3 | 1 | 1 | 1 | 3 |
| 69 | VI | Moscow (1970) | 1 | 28 | 1 | 3 | 4 | 1 | 1 | 3 |
| 2 | VI | Moscow, GDR (1980-95) | 17 | 7 | 1 | 3 | 4 | 1 | 1 | 3 |
| 68 | VI | Moscow (1994-96) | 2 | 7 | 1 | 3 | 4 | 21 | 1 | 17 |
| 70 | VI | Moscow (1984-86) | 2 | 7 | 1 | 3 | 4 | 22 | 21 | 3 |
| 73 | VI | Moscow (1995) | 1 | 7 | 1 | 3 | 4 | 1 | 21 | 3 |
| 58 | VI | GDR (1986) | 1 | 7 | 1 | 3 | 21 | 1 | 1 | 3 |
| 71 | VI | Moscow (1985) | 1 | 7 | 1 | 3 | 10 | 1 | 1 | 3 |
| 72 | VI | Moscow (1987) | 1 | 7 | 1 | 3 | 7 | 1 | 1 | 3 |
| 57 | II | USA (1930-43) | 2 | 1 | 1 | 3 | 1 | 1 | 2 | 3 |
| 4 | IV-1,IV-2 | Pandemic (1917-90) | 37 | 1 | 1 | 3 | 3 | 4 | 2 | 3 |
| 80 | | Gambia (1983) | 1 | 31 | 1 | 3 | 15 | 4 | 2 | 3 |
| 203 | | Gambia (1996) | 1 | 1 | 1 | 3 | 2 | 3 | 2 | 3 |
| 5 | III,VIII | Pandemic (1963-98) | 24 | 1 | 1 | 1 | 2 | 3 | 2 | 3 |
| 6 | III | China (1966) | 1 | 1 | 1 | 1 | 2 | 3 | 2 | 11 |
| 7 | III | Pandemic (1992-96) | 9 | 1 | 1 | 1 | 2 | 3 | 2 | 19 |
| 3 | V,VII | China (1963-87) | 11 | 1 | 1 | 3 | 1 | 1 | 23 | 13 |
| 59 | V | China (1980) | 1 | 22 | 1 | 14 | 1 | 1 | 23 | 13 |
| 103 | | Greece (1996) | 1 | 17 | 8 | 4 | 6 | 5 | 18 | 2 |
| 60 | IX | Holland (1989-92) | 4 | 17 | 17 | 5 | 19 | 3 | 26 | 2 |
| 61 | IX | Holland (1992) | 1 | 17 | 17 | 10 | 19 | 3 | 26 | 2 |
| 79 | other | Moscow (1995) | 1 | 26 | 12 | 5 | 6 | 9 | 22 | 8 |
| 299 | | Czechoslovakia (1972) | 1 | 26 | 2 | 29 | 36 | 26 | 18 | 20 |
| 300 | | Czechoslovakia (1972) | 1 | 26 | 2 | 29 | 9 | 26 | 18 | 20 |
| 388 | | Czechoslovakia (1972) | 2 | 35 | 57 | 2 | 2 | 6 | 68 | 12 |
| 400 | | Czechoslovakia (1972) | 1 | 43 | 12 | 16 | 2 | 3 | 37 | 7 |

[a]ST = sequence type; GDR = German Democratic Republic (East Germany); MLEE = multilocus enzyme electrophoresis; RAPD = random amplification of polymorphic DNA.

The IS*1106*A locus near *opcA* was amplified by using primers in conserved DNA flanking this locus (22). Two sizes of PCR products (4.8 and 3.5 kb) were obtained among the subgroup III strains. (One strain yielded a 12-kb product that was not investigated further.) An internal, polymorphic 307-bp stretch was sequenced from one product of each size and found to differ by 19 bp, including four restriction sites for *Hae*III, *Cla*I, and *Bcl*I. The allele in the 4.8-kb fragment was named IS*1106*A10, and the allele in the 3.5-kb fragment was named IS*1106*A7. PCR products from all the subgroup III strains were tested for the four distinctive restriction sites, but no additional diversity was identified. Details on alleles at this locus in subgroup III bacteria from sources other than Moscow have been described (23).

The 2.1-kb *tbpB* gene was amplified by using an internal primer located near the ATG start codon (3'-Met2) and a primer located in the intergenic region between *tbpB* and *tbpA* (5'-Inter1), as described (16). Different alleles of *tbpB* were recognized by digesting the 2.1-kb fragment with *Hae*III, *Msp*I, and *Ssp*I and by sequencing the entire fragment from two ST5 strains and one ST7 strain. The *tbpB* allele designations are congruent with those described for a 600-bp 5'-terminal region that has been examined from numerous other strains (16).

To distinguish between *pgm3* and *pgm19* (6), the *pgm* locus was PCR-amplified by using primers O781 (5'-CGGCGATGCCGACCGCTTGG) and O782 (5'GGTGAT-GATTTCGGTTGCGCC). The PCR products were tested for an *Hinf*I site that is absent in *pgm3* and present in *pgm19*.

## Results

### Genetic Relationships of Serogroup A Strains from Moscow

One hundred three serogroup A *N. meningitidis* strains isolated in Moscow during 1969 to 1998 were investigated by RAPD analysis to elucidate their genetic relationships. The data (Figure 2) revealed three primary clusters of isolates, two of which (subgroups III and VI) were assigned to known clonal groupings by comparison with previous data (7). The third cluster (24 isolates) differs from previously defined subgroups and was therefore tentatively designated subgroup X. Two singleton strains were labeled IV (a strain formerly assigned to subgroup IV-2 by MLEE) and ST79.

Forty-two strains that represent the diversity revealed by RAPD were analyzed by MLST. These data were combined with data from other strains as described in Materials and Methods, resulting in a database containing 152 serogroup A isolates (Table 1, Figure 3). MLST did not distinguish several subgroups defined by MLEE (I and II, IV-1 and IV-2, III and VIII, V and VII). Otherwise, the subgroup structure in a neighbor-joining (NJ) tree of the fraction of alleles differing between STs (Figure 3) was largely concordant with prior subgroup assignments from MLEE (8) or RAPD (7). Only STs 1 and 57 clustered inappropriately in the NJ tree (Figure 3).

The MLST results confirmed the assignments of isolates from Moscow to subgroups III and VI. They also demonstrated that subgroup X is distinct from previously defined subgroups and is closely related to subgroups I and II. Individual STs of

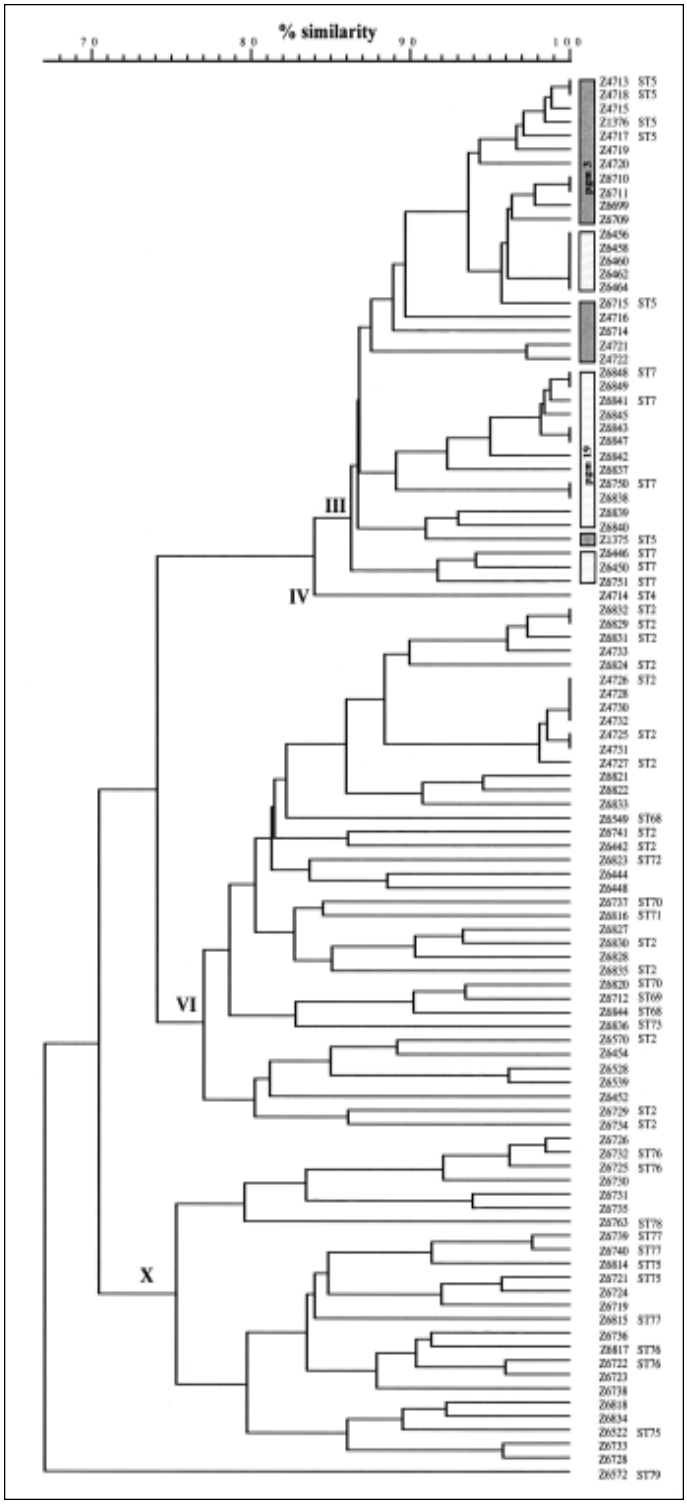


Figure 2. UPGMA tree of band differences between random amplified polymorphic DNA (RAPD) patterns from 103 serogroup A strains from Moscow. RAPD tests were performed with primers 1254, 1281, NM03, and NM04 as described (7), generating four patterns for each strain. The combination of the differences between these patterns was used to generate a UPGMA tree with the program GelCompar (Applied Maths, Kortrijk, Belgium), as described (7). Subgroup designations are indicated within the tree. The strain designations and the sequence type (ST) assignments from MLST analysis are shown at the right. For subgroup III bacteria, dark gray rectangles indicate *pgm3* and light gray rectangles indicate *pgm19*. Strain Z4714 is indicated as belonging to subgroup IV because RAPD does not distinguish between subgroups IV-1 and IV-2.

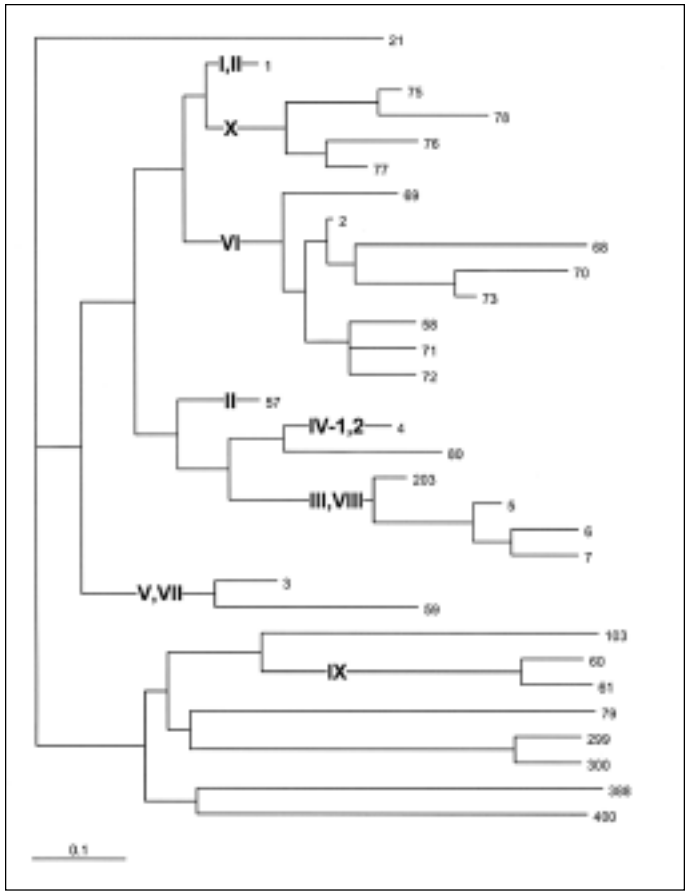


Figure 3. Midpoint rooted neighbor-joining (NJ) tree of the proportion of seven housekeeping gene fragments that differed between individual sequence types (STs) among 152 serogroup A isolates. The ST designations are indicated at the right of each twig, and the subgroup designations are shown in bold print in the tree. A scale bar showing the distance of 0.1 is at the lower left. STs 1 and 57, containing six subgroup II strains, are widely separated in this tree although they differ only at the *pdhC* locus (Table 1). These two STs were on adjacent twigs in a UPGMA tree (data not shown), but that tree separated STs 3 and 59 (subgroup V), that differ only at two of the seven loci (Table 1) and are on neighboring twigs in the NJ tree. Given the low number of allelic differences between STs 1 and 57 and STs 3 and 59, their aberrant relative positions in one or the other tree were ignored and their former subgroup assignments have been retained.

these three subgroups share identical alleles for up to six of the seven gene fragments sequenced (Table 1).

### Inconsistent Fine Typing by RAPD and MLST

Subgroup III isolates from Moscow were isolated during more than one epidemic wave, raising the question whether these isolates shared a common source. However, the concordance between RAPD and MLST did not extend below the subgroup level. The two major RAPD branches in subgroup III initially seemed to correspond to MLST ST5 and ST7. These STs are identical except for their *pgm* alleles (ST5, *pgm3*; ST7, *pgm19*,) which differ by 19 (4%) of 450 bp, including a distinctive restriction site. Genotyping of *pgm* from all subgroup III isolates by PCR-restriction fragment length polymorphism (PCR-RFLP) showed that both major RAPD branches contained both *pgm* alleles (Figure 2). Therefore, additional powerful fine typing methods, such as

multilocus PCR-RFLP, were needed to resolve whether all bacteria within a subgroup originated from the same sources.

### Serologic Properties

In some cases, serologic serotyping and serosubtyping can be used to enable rapid recognition of clonal groupings. The serologic properties of all the serogroup A bacteria from Moscow were determined by ELISA with monoclonal typing antibodies. The results confirmed previous results for subgroups III and VI (8) and showed that subgroup X is 4,21:P1.10 or 21:P1.10 (Table 2). Subgroup X is therefore not distinguishable by serologic testing from serogroup A subgroups I, II, IV-2 and VI, which are also 4,21:P1.10. The subgroup III strains uniformly expressed the class I and IIa pilin epitopes (8); however, the pilin epitopes of subgroups VI and X were not uniform: half of each group of strains expressed class I and IIa epitopes and the other half the class IIb epitope. Thus, except for subgroup III, serologic typing would not be useful for distinguishing these various subgroups.

Table 2. Serologic properties of serogroup A meningococci from Moscow

| Subgroup | Proportion (%) | Serotype | Serosubtype |
|---|---|---|---|
| III | 34/38 (89) | 21 | P1.20,9 |
| VI | 6/39 (15) | 21 | P1.10 |
| VI | 12/39 (31) | 4,21 | P1.10 |
| VI | 17/39 (44) | 4,21 | P1.5,10 |
| X | 17/24 (71) | 4,21 | P1.10 |
| X | 6/24 (25) | 21 | P1.10 |

### Successive Waves Caused by Different Clonal Groupings

The three subgroups were isolated in Moscow during four successive waves of disease caused, respectively, by ST5 of subgroup III (1969 to 1977, 17 of 19 isolates), subgroup X (1983 to 1985; 21 of 27), subgroup VI (1986 to 1995; 32 of 36) and ST7 of subgroup III (1996-97; 19 of 21) (Table 3). The isolation of subgroup III bacteria correlated with epidemic peaks of disease in 1970 and 1996 (Figure 1); in both cases, subgroup III bacteria were also isolated in Moscow 1 to 2 years before the peak epidemic year (Table 3). In contrast, the isolation of subgroup X or subgroup VI did not correlate with an increase in the incidence rate for serogroup A disease. The slight increase in disease incidence in the mid-1980s was associated with serogroup B meningococci.

### Molecular Fine Typing of Subgroup III Isolates

Both pre-Mecca and post-Mecca isolates are ST5, while the subgroup III meningococci isolated in Moscow since the mid-1990s are ST7. PCR-RFLP of the *opaB, opaD* and *iga* loci distinguished pre-Mecca from post-Mecca bacteria (17) but did not allow unambiguous assignment of the isolates from Moscow to either of these two waves. To resolve the fine structure of subgroup III, we identified three additional distinctive molecular markers, *tbpB*, IS*1106*A, and *pgm*, which were tested by PCR-RFLP plus sequencing for numerous subgroup III isolates from other countries (23). PCR-RFLP of these six variable loci subdivides subgroup III bacteria into nine so-called genoclouds, each containing a spectrum of related genotypes, and shows that a third wave of pandemic disease caused by genocloud 8 began in eastern

Table 3. Years of isolation of individual subgroups of serogroup A *Neisseria meningitidis* in Moscow

| | Number of strains | | | | | |
|---|---|---|---|---|---|---|
| Year | III (ST5[a]) | X | VI | III (ST7) | other | Total |
| 1969 | 1 | | | | | 1 |
| 1970 | 9 | | 1 | | | 10 |
| 1971 | 4 | | | | 1 | 5 |
| 1973 | 1 | | | | | 1 |
| 1977 | 2 | | | | | 2 |
| 1983 | | 4 | | | | 4 |
| 1984 | | 14 | 3 | | | 17 |
| 1985 | | 3 | 3 | | | 6 |
| 1986 | | | 3 | | | 3 |
| 1987 | | | 3 | | | 3 |
| 1988 | | | 8 | | | 8 |
| 1991 | | | 8 | | | 8 |
| 1993 | | | 2 | | | 2 |
| 1994 | | 1 | 4 | 1 | | 6 |
| 1995 | | | 4 | 1 | 1 | 6 |
| 1996 | | | 1 | 17 | | 18 |
| 1997 | | 1 | | 2 | | 3 |
| Total | 17 | 23 | 40 | 21 | 2 | 103 |

[a]ST = sequence type

Subgroup assignments are according to data from random amplification of polymorphic DNA (Figure 2). For strains of subgroup III where a complete multilocus sequence typing analysis was not performed, bacteria were assigned to ST5 and ST7 on the basis of results with *pgm* polymerase chain reaction/restriction analysis. Other: 1971, subgroup IV-2; 1995, ST79.

Asia in the early 1990s. Genocloud 8 bacteria differ from other subgroup III meningococci in having the *tbpB55, pgm19*, and IS*1106*A7 alleles (Table 4). All 21 subgroup III bacteria isolated in Moscow from 1994 to 1997 also have these alleles (Table 4) and thus represent an extension of the third pandemic wave.

The subgroup III ST5 meningococci isolated in Moscow from 1969 to 1977 are distinct from all other subgroup III meningococci and define genocloud 2 (Table 4). The most closely related genocloud is genocloud 1, isolated in China in the mid-1960s, which contains the same IS*1106*A10 element but differs at *tbpB* and *opaB*. The genocloud 3 bacteria that caused outbreaks in northern Europe concurrently with the epidemic in Moscow differed in IS*1106*A (IS*1106*A4) as well as *tbpB* and *opaB*. Other subgroup III genoclouds, including genocloud 5 (post-Mecca), differed at even more loci (Table 4).

### Phylogenetic Ancestry of Genocloud 2

The unique *tbpB* allele in ST5 subgroup III bacteria from Moscow is not informative for phylogenetic descent: It was not found in any other genocloud and was probably imported by DNA transformation (16) shortly before or after these bacteria arrived in Moscow. However, the phylogenetic history of the unique *opaB* alleles in the subgroup III bacteria from Moscow can be reconstructed from their sequences. Genocloud 1 bacteria from China have the *opa132, opa92, opa131*, and *opa101* alleles at the *opaA*, *B*, *D*, and *J* loci, respectively. The novel alleles at the *opaB* and *opaD* loci in genocloud 2 bacteria from Moscow probably represent gene conversion with the *opaJ101* and *opaA132* alleles (Figure 4). The *opaB140* allele, which was present in all but one strain from Moscow (Table 4), differs from *opaJ101* by only one nucleotide (Figure 4). The *opaB113* allele (one strain) can be explained by gene conversion of *opaB140* with DNA from the *opaA132* allele. *opaD131* is present in genocloud 1 and was

Table 4. Hypervariable alleles among subgroup III meningococci isolated in Moscow

| Strains | No. | Locus | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *tbpB* | *iga* | *pgm* | IS*1106* | *opaA* | *opaB* | *opaD* | *opaJ* |
| [a]genocloud 1 | | 1 | 2 | 3 | A10 | 132 | 92 | 131 | 101 |
| genocloud 3 | | 1 | 2 | 3 | A4 | 132 | 92 | 131 | 101 |
| genocloud 5 | | 1 | 3 | 3 | A4 | 132 | 94 | 100 | 101 |
| genocloud 8 | | 55 | 2 | 19 | A7 | 132 | 92 | 131 | 101 |
| Moscow genocloud 2 | | | | | | | | | |
| 1969-1973 | 14 | 38 | 2 | 3 | A10 | 132 | 140 | 131 | 101 |
| 1970 | 1 | 38 | 2 | 3 | A10 | 132 | 113 | 131 | 101 |
| 1977 | 1 | 38 | 2 | 3 | A10 | 132 | 140 | 110 | 101 |
| 1977 | 1 | 38 | 2 | 3 | nd[b] | 132 | 140 | 109 | 101 |
| Moscow genocloud 8 | | | | | | | | | |
| 1994-1997 | 21 | 55 | 2 | 19 | A7 | 132 | 92 | 131 | 101 |

[a]Data for the genoclouds at the top of the table are from a separate manuscript (23). Sources of genoclouds: 1, China, 1966; 3, pre-Mecca isolates (1969-1984) from Europe, Brazil and China; 5, post-Mecca isolates (1987-1998) from Africa and pilgrims returning from Mecca in 1987 to various countries; and 8, China, Mongolia, and Africa, 1993-2000. All serogroup A isolates from Moscow that were assigned to subgroup III by random amplification of polymorphic DNA are included in this table. The *opaA* and *opaJ* alleles have not been tested in all bacteria from the various genoclouds because they are otherwise so uniform.
[b]nd = not determined because this strain yields an unusual 12-kb polymerase chain reaction product for the IS*1106*A region.

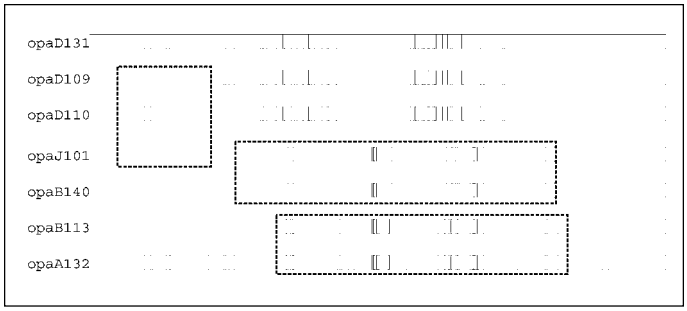


Figure 4. Sequence differences among *opa* alleles found in genocloud 2 subgroup III serogroup A meningococci from Moscow. Each vertical line represents a nucleotide different from the consensus sequence. Hatched rectangles indicate identical stretches that are inferred to represent gene conversion.

also found in all but two genocloud 2 strains from Moscow. The two exceptional strains contain the *opaD109* and *opaD110* alleles, which could easily have arisen by gene conversion of part of the *opaD131* locus with DNA from the *opaJ101* locus.

## Discussion

Since the 1920s, serogroup A meningococci have been responsible for a large proportion of the bacterial meningitis in Moscow (Figure 1). Serogroup A meningococci isolated in Moscow from 1969 to 1997 (103 isolates) were analyzed by RAPD. Strains representing the diversity revealed by the RAPD data were analyzed by MLST (44 isolates), and all 38 subgroup III strains were subjected to molecular fine typing by multilocus PCR-RFLP. The results illustrate the limitations of these methods and yield important information about the phylogenetic relationships among serogroup A meningococci. They also demonstrate that serogroup A meningococcal disease in Moscow since 1969 has been caused by four successive, independent waves of bacterial colonization, most recently by the spread of a third subgroup III pandemic wave from East Asia.

### Typing Methods

RAPD analysis was used to provide a quick overview of the genetic diversity in the 103 strains investigated (Figure 2). This information was used to select a smaller group of bacteria to be tested by more expensive and labor-intensive methods such as MLST. As in previous comparisons between RAPD and MLEE (7), the subgroup assignments were concordant between MLST and RAPD. The RAPD data allowed the initial recognition of a new subgroup, subgroup X, and the MLST data confirmed that subgroup IX is not genetically related to the epidemic serogroup A subgroups as originally demonstrated by RAPD data (7). Thus, RAPD analysis can be a highly reliable, quick method for recognizing genetically related bacterial groups.

Finer subdivisions in subgroups III and X, suggested by the RAPD data, were contradicted by MLST results. ST76 was distributed among both primary RAPD subdivisions of the subgroup X cluster, and the two primary RAPD subdivisions of the subgroup III cluster did not correlate with presence of *pgm3* versus *pgm19* (Figure 2) or with epidemiologic data. Such random noise will probably be most extreme for bacteria such as *N. meningitidis*, which frequently import DNA from unrelated neisseriae (15). Thus, putative subdivisions within phylogenetically related groups suggested by RAPD data should be confirmed by independent methods.

MLST is based on sequences of conserved housekeeping genes and sequence variants are rarer than with most other methods used in molecular epidemiology, thus facilitating global, long-term epidemiology (6). For subgroup III, the distinction between STs 5 and 7 based on *pgm* is a very useful molecular marker for recognizing bacteria from the third pandemic wave. However, the epidemiologic data do not suggest that other STs are useful markers for genetic variants that have spread extensively, and MLST does not necessarily provide the discrimination desired for fine typing.

Unfortunately, the fine structure of bacteria that frequently import foreign DNA is very difficult to resolve, and we know of no method that is eminently suitable for this task. Our data argue against the use of RAPD and MLST. Although pulsed-field gel electrophoresis is highly suitable for differentiating almost identical bacteria in limited numbers of strains (17,24,25), it is poorly suited for elucidating the relationships among large numbers of less strongly related bacteria. Fine typing was therefore attempted based on multilocus RFLP of PCR fragments of *pgm* plus the variable genes *opa*, *iga*, and *tbpB* and the IS*1106*A element. Multilocus PCR-RFLP of variable genes allowed molecular fine typing within subgroup III meningococci, but the data are also limited by considerable random noise, resulting in genoclouds of related variants (23). Alternative strategies involving IS

elements (26), amplified fragment-length polymorphism (27), or variable nucleotide repeat sequences (28) have been used successfully for fine typing of highly uniform microorganisms. Such methods have not yet been extensively tested with *N. meningitidis* but seem likely to be limited by at least as much random noise as the methods we used. Thus, reliable fine typing remains difficult to achieve.

### Phylogeny of Serogroup A Meningococci

The MLST data on 152 serogroup A strains allow inferences about the evolution of these bacteria. The STs in subgroups I through VIII and X (the epidemic subgroups) probably stem from a common ancestor. They form a monophyletic clade in phylogenetic trees (Figure 3), and many of these STs share the *fumC1*, *abcZ1*, *adk3*, *gdh1*, *pdhC1*, and *pgm3* alleles (Table 1). The epidemic subgroups include meningococci isolated since the beginning of this century (ST4, subgroup IV-2, 1917; ST57, subgroup II, 1930) (8) and must have evolved before then.

Older isolates of subgroups III (1960s), IV-1 (1960s), and IV-2 (1917) had identical or nearly identical *opa, iga*, and *tbpB* alleles (16,17), whereas more recent isolates have imported novel alleles at these loci. These data indicate that the common ancestor of these three subgroups existed approximately 100 years ago (17).

The branch lengths (Figure 3) and common alleles indicate that the epidemic subgroups are probably not much older than the split between subgroups III, IV-1, and IV-2. The epidemic serogroup A subgroups may therefore have descended from a common ancestor that existed within the last few hundred years. Epidemic meningitis was first observed in Geneva in 1805 (29), and that epidemic may have marked the evolution of serogroup A after the import of the *sacA-D* genes encoding the A polysaccharide. Horizontal exchange between serogroups B and C of genes encoding capsular polysaccharides has been documented (30), and more than one capsular polysaccharide serogroup is present in several of the otherwise uniform STs in the complete MLST database. Furthermore, the *sacA-D* genes are characterized by an unusually low GC content (31), as if these genes were recently imported from an unrelated species. If this speculation is correct, the other serogroup A bacteria (Figure 3, bottom) may represent unrelated *N. meningitidis* that have imported *sacA-D* genes from the epidemic subgroups. Alternatively, the *sacA-D* genes may have been imported from an unrelated species more than once, or the epidemic subgroups may have imported the *sacA-D* from one of the nonepidemic STs (Figure 3, bottom). Analysis of the sequence diversity of the *sacA-D* genes in various STs might resolve these alternative explanations.

### Molecular Epidemiology

Evaluation of the long-term molecular epidemiology of bacterial pathogens within a global context is complex, requiring large, representative strain collections and reliable, preferably phylogenetically based, typing schemes. Our results illustrate both strengths and weaknesses in our knowledge of serogroup A epidemiology.

The assignment of genocloud 8 bacteria isolated in the mid-1990s in Moscow to the third subgroup III pandemic wave is straightforward and indisputable. It rests on three unusual markers (*pgm19*, *tbpB55*, and IS*1106*A7), and no differences in these three markers were detected among recent subgroup III isolates from China, Mongolia, Africa, and Russia. The initial association of genocloud 8 disease in Moscow with the Vietnamese community remains unexplained.

The genocloud 2 subgroup III bacteria from the early 1970s differ at the *opaB* and *tbpB* loci from all known subgroup III meningococci. We argue that genocloud 2 is probably derived from genocloud 1 in China because of the shared IS*1106*A10 allele, which was not found in any other subgroup III bacteria. In support of this interpretation based on molecular data, this epidemic first broke out among Vietnamese immigrants who had traveled through China en route to Russia. However, few subgroup III strains exist that were isolated before 1969, and our knowledge about the diversity and patterns of serogroup A disease before 1969 is only fragmentary.

Subgroup VI bacteria of serogroups A, B, and C were occasionally isolated from endemic disease in East Germany in the 1980s (8). Our data show that most of the serogroup A meningococci isolated during 1986 to 1994 in Moscow were also subgroup VI, and these bacteria may have been widely distributed in eastern Europe in the 1980s. In contrast, subgroup X, common in Moscow during 1983 to 1985, has not been described previously. Various STs (Figure 3, bottom) have also been isolated only from single countries (subgroup IX in the Netherlands during 1989 to 1993 [7]; STs 299, 300, 388, and 400 in Czechoslovakia in 1972 [K. Jolley and P. Kriz, pers. comm.]; ST79 in Moscow in 1995; and ST103 in Greece in 1996 [R. Urwin, pers. comm.]). These bacteria have apparently not spread extensively, unlike the epidemic subgroups. In this respect, they resemble most endemic serogroup B meningococci, such as those identified by MLEE among endemic disease isolates from the Netherlands (32) and carrier isolates from Norway (33). Additional serogroup A STs specific to certain countries or regions probably remain to be identified. Current meningococcal strain collections containing a few thousand isolates are probably one or two orders of magnitude too small to yield reliable epidemiologic data for unusual STs that have not caused larger disease outbreaks.

Subgroup III has caused large disease outbreaks, and its molecular epidemiology has been extensively investigated (11,17,23). As a result, the available information on genetic changes during pandemic spread of these bacteria is among the most extensive for any bacterial pathogen. Our data show that the extensive pandemic spread of these bacteria is likely to continue. Although Moscow and western Europe differ somewhat in epidemiologic patterns, the recent extension of this third pandemic wave to Moscow warns us that we should be prepared for new, large serogroup A outbreaks in countries in both eastern and western Europe.

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Dr. Achtman is a scientific group leader at the Max-Planck Institut für Infektionsbiologie, Berlin. His group investigates the population structures of bacterial pathogens, including *N. meningitidis*, *Helicobacter pylori*, and *Yersinia pestis*. These analyses integrate results from epidemiologic studies of global disease patterns.

## References


1. Peltola H. Meningococcal disease: still with us. Rev Infect Dis 1983;5:71-91.
2. Crowe BA, Wall RA, Kusecek B, Neumann B, Olyhoek T, Abdillahi H, et al. Clonal and variable properties of *Neisseria meningitidis* isolated from cases and carriers during and after an epidemic in the Gambia, West Africa. J Infect Dis 1989;159:686-700.
3. Achtman M. Molecular epidemiology of epidemic bacterial meningitis. Rev Med Microbiol 1990;1:29-38.
4. Achtman M. Global epidemiology of meningococcal disease. In: Cartwright K, editor. Meningococcal disease. Chichester, England: John Wiley & Sons, Ltd; 1995:159-75.
5. Tikhomirov E, Santamaria M, Esteves K. Meningococcal disease: public health burden and control. World Health Statistical Quarterly 1997;50:170-6.
6. Maiden MCJ, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A 1998;95:3140-5.
7. Bart A, Schuurman IGA, Achtman M, Caugant DA, Dankert J, van der Ende A. Random amplified polymorphic DNA (RAPD) genotyping of serogroup A meningococci yields similar results to multilocus enzyme electrophoresis (MEE) and reveals new genotypes. J Clin Microbiol 1998;36:1746-9.
8. Wang J-F, Caugant DA, Li X, Hu X, Poolman JT, Crowe BA, et al. Clonal and antigenic analysis of serogroup A *Neisseria meningitidis* with particular reference to epidemiological features of epidemic meningitis in China. Infect Immun 1992;60:5267-82.
9. Hjetland R, Caugant DA, Hofstad T, Frøholm LO, Selander RK. Serogroup A *Neisseria meningitidis* of clone III-1 in western Norway, 1969-73. Scand J Infect Dis 1990;22:241-2.
10. Mäkelä PH, Peltola H, Käyhty H, Jousimies H, Pettay O, Ruoslahti E, et al. Polysaccharide vaccines to group A *Neisseria meningitidis* and *Haemophilus influenzae* type b: a field trial in Finland. J Infect Dis 1977;136:43-50.
11. Achtman M, Kusecek B, Morelli G, Eickmann K, Wang J, Crowe B, et al. A comparison of the variable antigens expressed by clone IV-1 and subgroup III of *Neisseria meningitidis* serogroup A. J Infect Dis 1992;165:53-68.
12. Moore PS, Reeves MW, Schwartz B, Gellin BG, Broome CV. Intercontinental spread of an epidemic group A *Neisseria meningitidis* strain. Lancet 1989;ii:260-3.
13. Jones DM, Sutcliffe EM. Group A meningococcal disease in England associated with the Haj. J Infect 1990;21:21-5.
14. Riou JY, Caugant DA, Selander RK, Poolman JT, Guibourdenche M, Collatz E. Characterization of *Neisseria meningitidis* serogroup A strains from an outbreak in France by serotype, serosubtype, multilocus enzyme genotype and outer membrane protein pattern. Eur J Clin Microbiol Infect Dis 1991;10:405-9.
15. Maiden MCJ, Malorny B, Achtman M. A global gene pool in the neisseriae. Mol Microbiol 1996;21:1297-8.
16. Linz B, Schenker M, Zhu P, Achtman M. Frequent interspecific genetic exchange between commensal neisseriae and *Neisseria meningitidis*. Mol Microbiol 2000;36:1049-58.
17. Morelli G, Malorny B, Müller K, Seiler A, Wang J, del Valle J, et al. Clonal descent and microevolution of *Neisseria meningitidis* during 30 years of epidemic spread. Mol Microbiol 1997;25:1047-64.
18. Bolshakov KN. Epidemiological and geographical aspects of meningococcal infection [dissertation]. Moscow: Moscow Univ.; 1973.
19. Kostyukova NN, Mironova TK, Leschinskaya EV. Microbiological characteristics of meningococcus infection in Moscow during a periodic rise in morbidity (1968-1971). Zh Mikrobiol Epidemiol Immunobiol 1974;5:29-32.
20. Demina AA, Martinov JV. An analysis of the epidemic cycle of meningococcal infection in Russia (1969-1993). Zh Mikrobiol Epidemiol Immunobiol 1996;4:57-61.
21. Sarkari J, Pandit N, Moxon ER, Achtman M. Variable expression of the Opc outer membrane protein in *Neisseria meningitidis* is caused by size variation of a promoter containing poly-cytidine. Mol Microbiol 1994;13:207-17.
22. Zhu P, Morelli G, Achtman M. The *opcA* and *opcB* regions in *Neisseria*: genes, pseudogenes, deletions, insertion elements and DNA islands. Mol Microbiol 1999;33:635-50.
23. Zhu P, van der Ende A, Falush D, Brieske N, Morelli G, Linz B, et al. Fit genotypes and escape variants of subgroup III *Neisseria meningitidis* during three pandemics of epidemic meningitis. Proc Natl Acad Sci U S A. In press 2001.
24. Kwara A, Adegbola RA, Corrah PT, Weber M, Achtman M, Morelli G, et al. Meningitis caused by a serogroup W135 clone of the ET-37 complex of *Neisseria meningitidis* in West Africa. Trop Med Int Health 1998;3:742-6.
25. Vogel U, Morelli G, Zurth K, Claus H, Kriener E, Achtman M, et al. Necessity of molecular techniques to distinguish between *Neisseria meningitidis* isolated from meningococcal disease and from their healthy contacts. J Clin Microbiol 1998;36:2465-70.
26. Van Embden JDA, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendation for a standardized methodology. J Clin Microbiol 1993;31:406-9.
27. Keim P, Kalif A, Schupp J, Hill K, Travis SE, Richmond K, et al. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. J Bacteriol 1997;179:818-24.
28. Keim P, Klevytska AM, Price LB, Schupp JM, Zinser G, Smith KL, et al. Molecular diversity in *Bacillus anthracis*. J Appl Microbiol 1999;87:215-7.
29. Vieusseux M. Mémoire sur le maladie qui a régné à Genève au printemps de 1805. J Med Chirurg Pharm 1806;11:163-82.
30. Swartley JS, Marfin AA, Edupuganti S, Liu LJ, Cieslak P, Perkins B, et al. Capsule switching of *Neisseria meningitidis*. Proc Natl Acad Sci U S A 1997;94:271-6.
31. Parkhill J, Achtman M, James KD, Bentley SD, Churcher C, Klee SR, et al. Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. Nature 2000;404:502-6.
32. Scholten RJPM, Bijlmer HA, Poolman JT, Kuipers B, Caugant DA, van Alphen L, et al. Meningococcal disease in the Netherlands, 1958-1990: a steady increase in the incidence since 1982 partially caused by new serotypes and subtypes of *Neisseria meningitidis*. J Infect Dis 1993;16:237-46.
33. Caugant DA, Høiby EA, Magnus P, Scheel O, Hoel T, Bjune G, et al. Asymptomatic carriage of *Neisseria meningitidis* in a randomly sampled population. J Clin Microbiol 1994;32:323-30.

# Melioidosis: An Emerging Infection in Taiwan?

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From January 1982 to May 2000, 17 infections caused by *Burkholderia pseudomallei* were diagnosed in 15 patients in Taiwan; almost all the infections were diagnosed from 1994 to May 2000. Of the 15 patients, 9 (60%) had underlying diseases, and 10 (67%) had bacteremic pneumonia. Thirteen (76%) episodes of infection were considered indigenous. Four patients died of melioidosis. Seventeen *B. pseudomallei* isolates, recovered from eight patients from November 1996 to May 2000, were analyzed to determine their in vitro susceptibilities to 14 antimicrobial agents, cellular fatty acid and biochemical reaction profiles, and randomly amplified polymorphic DNA patterns. Eight strains (highly related isolates) were identified. All isolates were arabinose non-assimilators and were susceptible to amoxicillin-clavulanate, piperacillin-tazobactam, imipenem, and meropenem. No spread of the strain was documented.

Melioidosis is an infectious disease of humans and animals caused by *Burkholderia pseudomallei* (1). This organism is widely distributed in rice field soil and in stagnant water throughout the tropics (1-8). Although a major disease in Southeast Asia and northern Australia, melioidosis occurs sporadically throughout the world (often in patients with a history of residence in these disease-endemic areas) (1). Humans are usually infected by traumatic inoculation of the organism from the soil or, rarely, by inhalation or ingestion (1,7). Clinical manifestations are protean, ranging from benign and localized abscess, to severe, community-acquired pneumonia to fatal septicemia (1,3,4,7). The two biotypes of *B. pseudomallei* are categorized by their ability to assimilate L-arabinose (9). The arabinose non-assimilators (Ara-) are virulent and can be isolated from both clinical specimens and the environment, whereas the arabinose assimilators (Ara+) are usually avirulent and mainly found in the environment (9,10). Relapse, recrudescence, or reinfection may occur in immunocompromised patients under inappropriate antimicrobial treatment or after resolution of primary infection (1,7,11,12).

In Taiwan in 1985, melioidosis was first reported in a previously healthy patient with multilobar pneumonia, which developed secondary to his near drowning in the Philippines (13). Since then, only six other cases have been reported (14-18). In our study, an additional eight patients with melioidosis found from 1996 to 2000 by National Taiwan University Hospital (NTUH) were reported and microbiologic characteristics (including biotyping, cellular fatty acid chromatograms, antimicrobial susceptibilities, and genotyping) of the strains isolated from these patients were evaluated.

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## Materials and Methods

### Patients and Bacterial Isolates

From January 1980 to May 2000, eight patients with melioidosis were treated at NTUH, a tertiary-care referral center with 2,000 beds in northern Taiwan, and seven patients reported to have melioidosis were treated at other hospitals in Taiwan. Data on demographics, underlying diseases, travel history, type of infection, antimicrobial treatment, and outcome of these patients were analyzed. Infections were considered indigenous if patients had no history of residence or travel to China, Australia, or Southeast Asia. A total of 17 isolates of *B. pseudomallei* from various clinical specimens of the eight patients treated at NTUH were studied. These isolates were identified by conventional biochemical methods and confirmed by the API 20NE identification system (Biomerieux, Basingstoke, UK) (19,20).

### Susceptibility and Antibiotypes

The following antimicrobial agents were provided by their manufacturers for use in this study: amoxicillin-clavulanate (SmithKline Beecham, Welwyn Garden City, UK); cefotaxime (Marion Merrell Dow, Cincinnati, OH); ceftazidime (Glaxo, Greenford, UK); amoxicillin, cefepime, aztreonam, and amikacin (Bristol-Meyer Squibb, Princeton, NJ); imipenem (Merck Sharp & Dohme, Rahway, NJ); meropenem (Sumitomo Pharmaceuticals, Osaka, Japan); piperacillin-tazobactam (Wyeth-Ayerst Laboratories, Pearl River, NY); flomoxef (Shionogi, Osaka, Japan); trovafloxacin (Pfizer Inc., New York, NY); and ciprofloxacin and moxifloxacin (Farbenfabriken Bayer GmbH, Leverkrusen, Germany). MICs of these antimicrobial agents were determined by the agar dilution method according to guidelines established by the National Committee for Clinical Laboratory Standards (21). Isolates were grown overnight on trypticase soy agar plates supplemented with 5% sheep blood (BBL Microbiology Systems, Cockeysville, MD) at 37°C.

Bacterial inocula were prepared by suspending the freshly grown bacteria in sterile normal saline and adjusted to a 0.5 McFarland standard. Mueller-Hinton agar (BBL Microbiology Systems) was used for susceptibility testing. With a Steers replicator, an organism density of $10^4$ CFU/spot was inoculated onto the appropriate plate with various concentrations of antimicrobial agents. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were included as control strains. Antibiotypes were considered identical if all MICs tested were identical or within a twofold dilution discrepancy (22).

### Cellular Fatty Acid Chromatogram and Biotypes

Biotypes of these isolates were identified according to the reaction profiles obtained by API 20NE. Arabinose utilization was determined by growth on minimal salt agar containing L-arabinose (0.2%) (9). Fatty acid methyl esters (FAMEs) of these isolates were analyzed by gas-liquid chromatography using a Hewlett-Packard 5890A (Hewlett-Packard; Avondale, PA) as described previously (22). The software library used to identify the *B. pseudomallei* was TSA, version 3.9 (Microbial ID Inc., Newark, DE).

### Strain Typing

Extraction of genomic DNA and polymerase chain reaction (PCR) for determining random amplified polymorphic DNA (RAPD) patterns generated by arbitrarily primed PCR of the 17 isolates of *B. pseudomallei* were performed as previously described (22). Three oligonucleotide primers used were: M13 (5'-TTATGTAAAACGACGGCCAG-3' (Gibco BRL products, Gaithersburg, MD), ERIC1 (5'-GTGAATCCCCAG-GAGCTTACAT-3' (Gibco Bethesda Research Laboratories Products), and OPH-03 (5'-AGACGTCCAC-3') (Operon Technologies, Inc., Alameda, CA). To interpret RAPD patterns, both faint and intense bands were included (22). The entire procedure, from bacterial growth to interpretation of RAPD pattern, was repeated three times for each isolate to confirm results. Patterns were considered identical only if they differed by no more than one band. Isolates were defined as being of the same strain (highly related isolates) if they had identical antibiotypes, biotypes, and RAPD patterns.

## Results

### Clinical Characteristics of Patients

From January 1982 to May 2000, 17 episodes of infection caused by *B. pseudomallei* were diagnosed in 15 patients in Taiwan. All but one episode occurred between 1994 and 2000 (Figure 1; Table 1), indicating that cases have increased substantially in recent years. In these 15 patients, 13 were male; mean age was 64 years (range, 40 to 76 years). Patient 1 acquired pneumonia secondary to his near drowning in the Philippines. Patient 4 had had a fever during his stay in mainland China. Patient 6 had fever and left upper abdominal pain on his arrival in Taiwan after a 4-day trip in Rangoon, Burma. Patient 8 had septicemic melioidosis 5 years after travel to Thailand. The 13 other episodes (76%) were considered indigenous. Of the 11 patients with indigenous melioidosis, occupation was known for 7 patients (patients 9 to 15); none were rice farmers. Of the 15 infected patients, 9 (60%) had underlying diseases (6 had diabetes mellitus, and 3 had chronic obstructive pulmonary disease),

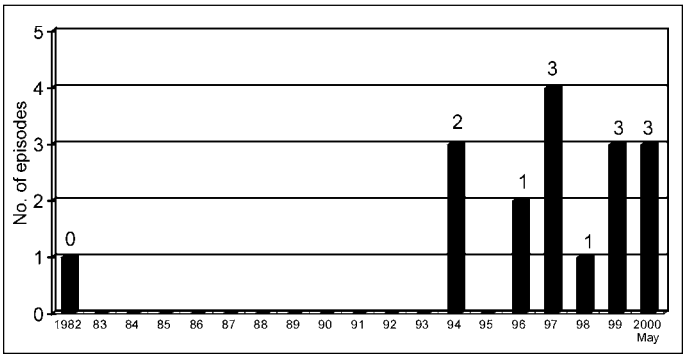


Figure 1. Cases of melioidosis in Taiwan, by year of diagnosis. Number above each bar indicates number of episodes with indigenous infection.

12 patients (80%) had pneumonia (including 10 with bacteremia and 1 with concomitant peritonitis), 2 (13%) had soft-tissue abscess, and 1 (7%) had mycotic aneurysm. Two patients each had two episodes of infection, separated by 8 and 10 months, respectively. Four patients (27%) died of melioidosis. One patient (no. 15), who had pneumonia caused by an organism initially identified as *P. aeruginosa* and treated at another hospital with ceftazidime and amikacin for 1 month, died on the third day in our hospital of refractory pneumonia complicated by empyema and acute respiratory distress syndrome. Two sets of blood cultures collected upon admission grew *B. pseudomallei*. Susceptibility results by the routine disk susceptibility test showed the organism was resistant to ceftazidime and amikacin. MICs of the organism to ceftazidime and amikacin were 32 µg/mL and >256 µg/mL, respectively (Table 2).

### Bacterial Isolates and Biotypes

All *B. pseudomallei* isolates had characteristic colonial morphology on trypticase soy agar supplemented with 5% sheep blood (BBL Becton Dickinson, Microbiology, Cockeysville, MD), positive oxidase reaction, and resistance to colistin and gentamicin. Of the 17 isolates (from eight patients), two biochemical profiles based on the results of identification by the API 20NE system were 1156576 (biotype I; citrate negative) and 1156577 (biotype II; citrate positive). All isolates tested were Ara-. The probability of identification of each *B. pseudomallei* biochemical profiles was 99.9%.

All 17 isolates (from eight patients) studied had similar FAME profiles, and all had five major FAMEs (each presenting >3% of the total): 14:0 (tetradecanoic acid), 3-OH-14:0 (3-hydroxytetradecanoic acid), 16:0 (hexadecanoic acid), 17:0cyc (-cis-9, 10-methylenehexadecanoic acid), 2-OH-16:0 (2-hydroxyhexadecanoic acid), 3-OH-16:0 (3-hydroxyhexadecanoic acid), 18:1w7c (cis-11-octadecenoic acid), and 19:0cyc (-cis-11, 12-methyleneoctadecanoic acid). FAME profiles of these isolates were consistent with the identification of *B. pseudomallei.*

### Antimicrobial Susceptibilities and Antibiotypes

MICs of 14 antimicrobial agents for the 17 isolates of *B. pseudomallei* were determined (Table 2). When MIC breakpoints for susceptibility and resistance used for non-*Enterobacteriaceae* were applied to *B. pseudomallei* (21), all isolates were susceptible to amoxicillin-clavulanate,

Table 1. Clinical characteristics of 15 patients in Taiwan with invasive infections caused by *Burkholderia pseudomallei*

| Patient no./ref | Age/ sex | Travel history | Underlying diseases/ associated conditions | Clinical diagnosis | Isolate source (date) | Treatment | Outcome |
|---|---|---|---|---|---|---|---|
| 1 (13) | 46/M | Philippines | Near drowning | Cavitary pneumonia | Blood (9/82) | Cephalothin, amikacin (30 d) | Surv |
| 2 (14) | 75/F | None | Liver cirrhosis, uremia, DM | Pneumonia, peritonitis | Blood, ascites (8/94) | Cefazolin, gentamicin (1 d) | Died |
| 3 (14) | 70/M | None | None | Pneumonia | Blood (8/94) | Ceftazidime (30 d), amoxicillin-clavulanate (3 mo) | Surv |
| 4 (15) | 70/M | China | None | Mycotic aneurysm | Blood (11/94); aortic tissue (12/94) | Ceftazidime (35 d); amoxicillin-clavulanate (6 mo) | Surv |
| 5 (16) | 75/M | None | DM | Pneumonia | Blood (11/96) | Ceftazidime (14 d); amoxicillin-clavulanate (2 mo) | Surv |
| 6 (17) | 51/M | Burma | DM | Pneumonia, adrenal gland abscess | Blood (7/97) | Ceftazidime (2 d); cotrimoxazole (60 d) | Surv |
| 7 (18) | 40/M | None | DM | Pneumonia, ARDS | Blood (7/97) | Moxalactam (9 d); netilmicin (9 d); erythromycin (9 d) | Died |
| 8 (PR) | 56/M | Thailand | None | Pneumonia | Blood-A (11/96) | Ceftazidime, gentamicin (1 d) | Died |
| 9 (PR) | 67/M | None | COPD | Pneumonia | Blood-B (1/97) | Ceftazidime (14 d); amoxicillin-clavulanate (2 d) | Surv |
| 10 (PR) | 73/M | None | Prostate cancer with bone metastasis; DM; cyproterone and leuprolide acetate use | Cavitary pneumonia | Lung aspirate-C1 7/97); RAPDa patterns sputum-C2 (7/97) | Ciprofloxacin (5 mo) | Surv |
| 11 (PR) | 76/F | None | None | Septic arthritis, subcutaneous abscess | Blood-D1; blood-D2; synovial fluid-D3; abscess fluid-D4; wound drainage-D5; all 9/98 | Ciprofloxacin (4 mo); surgical drainage | Surv |
| 12 (PR) | 58/M | None | None | Subcutaneous abscess | Abscess fluid-E1 (4/99); abscess fluid-E2 (2/2000) | Piperacillin (10 d); ciprofloxacin (14 d) | Surv |
| 13 (PR) | 66/M | None | DM | Pneumonia, arthritis, subcutaneous abscess | Lung aspirate-F1 (5/99); synovial fluid-F2 (5/99); abscess aspirate-F3 (1/2000) | Imipenem (10 d); ciprofloxacin (2 mo); meropenem (1 mo); amoxicillin-clavulanate (4 mo) | Surv |
| 14 (PR) | 74/M | None | COPD | Pneumonia | Blood-G (9/99) | Ceftazidime, amikacin (14 mo); ciprofloxacin (2 mo) | Surv |
| 15 (PR) | 70/M | None | COPD, TB | Pneumonia, empyema; ARDS | Blood-H1 Blood-H2 (1/2000) | Ceftazidime, amikacin (1 mo) | Died |

Abbreviations used in this table: ARDS, adult respiratory distress syndrome; COPD, chronic obstructive pulmonary disease; d, day; DM, diabetes mellitus; TB, tuberculosis; mo, month; PR, present report; surv, survived.

piperacillin-tazobactam, imipenem, and meropenem. Most isolates were intermediate or resistant to ampicillin, flomoxef, cefepime, aztreonam, amikacin, and ciprofloxacin. Ceftazidime had in vitro activity equal to or greater than that of cefotaxime against *B. pseudomallei*. Two isolates (both from patient 12) showed high resistance to ceftazidime and cefepime and intermediate resistance to cefotaxime. Five antibiotypes (antibiotypes I to V) were identified in the 17 isolates.

### RAPD Patterns and Identification

Eight RAPD patterns were identified by use of the three primers (Figure 2). RAPD patterns of multiple isolates from the same patients were identical. The two isolates (recovered 10 months apart) from patient 10 belonged to strain 5; two of the three isolates (recovered 8 months apart) from patient 11 were also identical (strain 6; Table 3). Isolates recovered from different patients had distinct RAPD patterns.

Table 2. Susceptibilities and antibiotypes of 17 isolates of *Burkholderia pseudomallei* isolated in Taiwan from November 1996 to January 2000

| Patient no./ isolate | MIC (mg/mL)[a] | | | | | | | | | | | | | | Antibiotic type |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AM | AMC | PZP | CTX | CAZ | FEP | FLO | ATM | IPM | MEM | AN | CIP | TRO | MOX | |
| 8/A | 64 | 8 | 0.5 | 4 | 4 | 16 | 128 | 16 | 0.5 | 1 | 64 | 2 | 2 | 2 | I |
| 9/B | 64 | 8 | 1 | 4 | 4 | 16 | 128 | 16 | 0.5 | 1 | 128 | 2 | 2 | 2 | I |
| 10/C1, 2 | 64 | 8 | 0.5 | 4 | 4 | 16 | 128 | 16 | 0.5 | 1 | 32 | 1 | 2 | 2 | II |
| 11/D1, 2, 3, 4, 5 | 64 | 8 | 0.5 | 2 | 1 | 16 | 128 | 16 | 0.5 | 1 | 32 | 2 | 4 | 1 | III |
| 12/E1, 2 | 64 | 8 | 0.5 | 2 | 1 | 8 | 128 | 16 | 0.5 | 1 | 32 | 2 | 4 | 1 | III |
| 13/F1, 2, 3 | 64 | 8 | 0.5 | 2 | 1 | 16 | 128 | 16 | 0.5 | 1 | 16 | 2 | 4 | 1 | III |
| 14/G | 16 | 2 | 0.12 | 2 | 1 | 8 | 64 | 8 | 0.25 | 0.5 | 64 | 1 | 1 | 1 | IV |
| 15/H1, 2 | 64 | 8 | 1 | 16 | 32 | 64 | 128 | 32 | 0.5 | 2 | >256 | 4 | 8 | 4 | V |

[a]AM, amoxicillin; AMC, amoxicillin-clavulanate; PZP, piperacillin-tazobactam; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; FLO, flomoxef; ATM, aztreonam; IPM, imipenem; MEM, meropenem; AN, amikacin; CIP, ciprofloxacin; TRO, trovafloxacin; MOX, moxifloxacin.

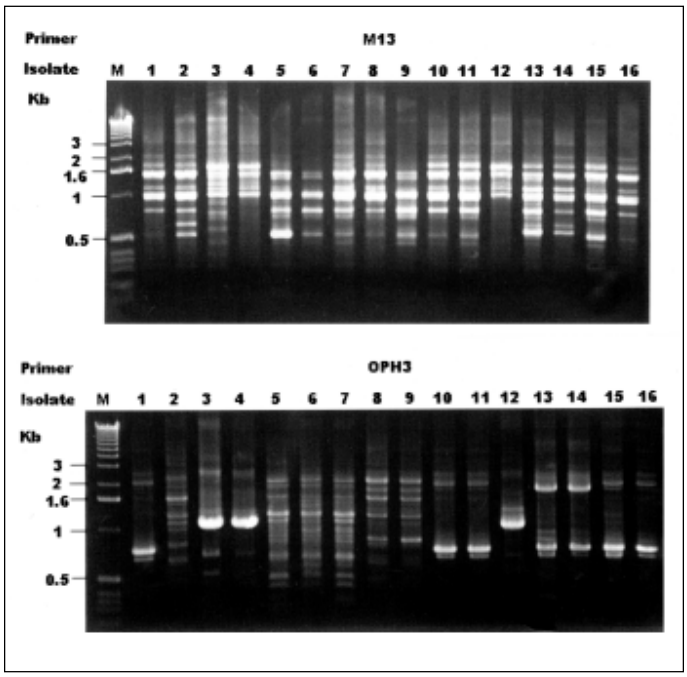


Figure 2. Random amplified polymorphic DNA (RAPD) patterns of 16 isolates of *Burkholderia pseudomallei* generated by arbitrarily primed polymerase chain reaction with the primers M13 (upper panel) and ERIC1 (lower panel). Lanes: M, molecular size marker; 1, isolate A; 2, isolate B; 3 and 4, isolates C1 and C2; respectively; 5 to 9, isolates D1 to D5; 10 and 11, isolates E1 and E2, respectively; 12, isolates G; 13 and 14, isolates F1 and F2, respectively; and 15 and 16, isolates H1 and H2, respectively. (See Table 3 for designation of isolates).

Table 3. Phenotypic and genotypic characteristics of 17 isolates of *Burkholderia pseudomallei*

| Isolate(s)/ patient no. | RAPD[a] patterns | | | |
|---|---|---|---|---|
| | Anti-biotype | Biotype | M13/ERIC1/ OPH-3 | Strain |
| A/8 | I | A | a | 1 |
| B/9 | I | A | b | 2 |
| C1, C2/10 | II | A | c | 3 |
| D1, D2, D3, D4, D5/11 | III | A | d | 4 |
| E1, E2/12 | III | A | e | 5 |
| F1, F2, F3/13 | III | A | f | 6 |
| G/14 | IV | B | g | 7 |
| H1, H2/15 | V | A | h | 8 |

[a]RAPD = Random amplified polymorphic DNA.

## Conclusion

Between January 1982 and 1994, one episode of melioidosis was identified in Taiwan. From 1994 to May 2000, 16 more cases occurred. Whether these figures represent an actual increase in *B. pseudomallei* infections in Taiwan or better recognition of this organism by microbiology laboratories is difficult to clarify. In NTUH, the first clinical isolate of *B. pseudomallei* was recognized in 1980 (the medical record and isolate are now unavailable). Since then, improved laboratory procedures and increasing alertness of laboratory staffs permit more accurate identification of this organism. However, no *B. pseudomallei* isolate was identified in our laboratory from 1981 to 1995. Thus, from our vantage point, the observed increase in cases of melioidosis is indeed an emerging problem of the last 5 years.

Most of these infections were indigenous. All strains, whether imported or indigenous, were genetically distinct. Different patients were infected with different strains, indicating that spread of strains (intercontinental or within this island), as with *Penicillium marneffei* (another emerging pathogen in Taiwan), did not occur (23). Our data suggest that Taiwan should be considered a melioidosis-endemic area, in addition to China, Australia, and Southeast Asia.

Melioidosis has been called the great mimicker because of its protean clinical features (1,7). The most common clinical sign is an acute pulmonary infection (as in our study), though its chronic pulmonary form often resembles tuberculosis (3,16). When localized, melioidosis may cause abscess formation in skin, soft tissue, joints, and visceral organs (1). Melioidosis can become a latent infection that later (as much as 26 years after initial exposure) reactivates into a full-blown illness (even with acute septicemia) (1,12). The content of Ara- *B. pseudomallei* in the soil of a geographic area has been documented to correlate directly with the incidence of melioidosis in that area (5,6). In our study, all but four patients had no prior exposure to well-known disease-endemic areas. Therefore, the strains of *B. pseudomallei* they acquired might have originated in Taiwanese soil. Unlike other reports (24), most (87%) of our patients were male. Also, all but one patient with indigenous infection were >65 years, and none were rice farmers or obviously had heavy exposure to soil. Environmental surveys for the presence of this organism in the soil of Taiwan, especially in rice fields, are ongoing; thus far, *B. pseudomallei* has been isolated from two soil samples (data not shown). Although Ara+ *B. pseudomallei* has been reported to cause severe infection (10), all isolates

causing melioidosis in our study were Ara-. Our findings support previous observations (1,5,6).

*B. pseudomallei* are frequently intrinsically resistant to many antibiotics, including aminoglycosides and first- or second-generation cephalosporins (25,26). Current recommendations for therapy of severe melioidosis include intravenous ceftazidime or imipenem for 10 days to 4 weeks (25,26), followed by maintenance therapy with oral amoxicillin-clavulanate or a combination of trimethoprim-sulfamethoxazole and doxycycline for 10 to 18 weeks (27-31). Cefotaxime and ceftriaxone are both less active than ceftazidime against *B. pseudomallei* in vivo and in vitro (28). However, the observation of ceftazidime resistance's emerging during treatment has been previously reported (30). Its occurrence in our patient 15 might be related to the presence of an empyema. In areas in which melioidosis is endemic, empirical regimens that contain cefotaxime or ceftriaxone for the treatment of severe community-acquired pneumonia or septicemia may not be appropriate.

Some investigators suggest that melioidosis is a facultative intracellular infection (25). The failure of beta-lactam antibiotics to penetrate intracellular sites and kill nonmultiplying dormant bacteria may explain the frequent relapses of melioidosis after treatment with these drugs (25). On the other hand, relapse is documented to be less common (10% versus 30%) in patients who complete a full course of antibiotic treatment (32). Our two patients who had recurrent infections both received oral ciprofloxacin for 2 or 8 weeks. Nearly all isolates had MICs ranging from 1 µg/mL to 4 µg/mL for the three newer fluoroquinolones tested. Further studies are needed to determine the clinical efficacy of these newer fluoroquinolones for treating melioidosis and their role in preventing future relapse.

Several molecular typing methods have been applied to *B. pseudomallei* to evaluate the strain relationship in isolates recovered from humans and environment (33-37). Among these methods, RAPD typing has also been documented to be useful for analyzing isolates that cause recurrent infection or reinfection (35). In our study, RAPD typing using three primers clearly demonstrated the genetic diversity of isolates from different patients (with either imported or indigenous infections). In addition, this method showed that multiple isolates from the same patient and isolates causing recurrent infections were genetically identical. The microbial identification system, based on cellular FAME analysis by use of gas chromatography, is an established method for identifying species of bacteria and fungi and showing clustering in bacterial and fungal strains (22,23). Although all our isolates of *B. pseudomallei* had identical FAME profiles, cluster analysis of these esters (data not shown) failed to provide acceptable discriminatory power for typing the isolates.

In conclusion, Taiwan should be included as an endemic area of melioidosis, and physicians managing patients in Taiwan should be alert to the possibility that this organism might cause community-acquired pneumonia and sepsis.

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## References

1. Dance DAB. Melioidosis: the tip of the iceberg? Clin Microbiol Rev 1991;4:52-60.
2. Kanaphun P, Thirawattanasuk N, Suputtamongkol Y, Naigowit P, Dance DAB, Smith MD, et al. Serology and carriage of *Pseudomonas pseudomallei*: a prospective study in 1000 hospitalized children in Northern Thailand. Clin Infect Dis 1993;167:230-3.
3. Everett ED, Nelson RA. Pulmonary melioidosis: observation in thirty-nine cases. Am Rev Respir Dis 1975;112:331-40.
4. Chaowagul W, White NJ, Dance DAB, Wattanagoon Y, Naigowit P, Davis TME, et al. Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. J Infect Dis 1989;159:890-9.
5. Smith MD, Wuthiekanun V, Walsh AL, White NJ. Quantitative recovery of *Burkholderia pseudomallei* from soil in Thailand. Trans R Soc Trop Med Hyg 1995;89:488-90.
6. Parry CM, Wuthiekanun V, Hoa NTT, Diep TS, Thao LTT, Loc PV, et al. Melioidosis in southern Vietnam: clinical surveillance and environmental sampling. Clin Infect Dis 1999;29:1323-6.
7. Yee KC, Lee MK, Chua CT, Puthucheary SD. Melioidosis, the great mimicker: a report of 10 cases from Malaysia. J Trop Med Hyg 1988;91:249-54.
8. Wilks D, Jacobson SK, Lever AML, Farrington M. Fatal melioidosis in a tourist returning from Thailand. J Infect 1994;29:87-90.
9. Smith MD, Angus BJ, Wuthiekanun V, White NJ. Arabinose assimilation defines a nonvirulent biotype of *Burkholderia pseudomallei*. Infect Immun 1997;65:4319-21.
10. Lertpatanauwun N, Sermsri K, Petkaseam A, Trakulsomboon S, Thamlikitkul V, Suputtamongkol Y. Arabinose-positive *Burkholderia pseudomallei* infection in humans. Clin Infect Dis 1999;28:927-8.
11. Silbermann MH, Gyssens IC, Endtz HP, Kuijper EJ, van der Meer JTM. Two patients with recurrent melioidosis after prolonged antibiotic therapy. Scand J Infect Dis 1997;29:199-201.
12. Mays EE, Ricketts EA. Melioidosis: recrudescence associated with bronchogenic carcinoma twenty-six years following initial geographic exposure. Chest 1975;68:261-3.
13. Lee N, Wu JL, Lee CH, Tsai WC. *Pseudomonas pseudomallei* infection from drowning: the first reported case in Taiwan. J Clin Microbiol 1985;23:352-4.
14. Lee SSJ, Liu YC, Chen YS, Wann SR, Wang JH, Yen MY, et al. Melioidosis: two indigenous cases in Taiwan. J Formos Med Assoc 1996;95:562-6.
15. Lee SSJ, Liu YC, Wang JH, Wann SR. Mycotic aneurysm due to *Burkholderia pseudomallei*. Clin Infect Dis 1998;26:1013-4.
16. Tsai WC, Liu YC, Yen MY, Wang JH, Chen YS, Wang JH, et al. Septicemic melioidosis in southern Taiwan: a case report. J Microbiol Immunol Infect 1998;31:137-40.
17. Lee SC, Ling TS, Chen JC, Huang BY, Sheih WB. Melioidosis with adrenal gland abscess. Am J Trop Med Hyg 1999;61:34-6.
18. Chen YH, Peng CF, Hwang KP, Tsai JJ, Lu PL, Chen TP. An indigenous melioidosis: a case report. Kaohsiung Journal of Medical Sciences 1999;15:292-6.
19. Dance DAB, Wuthiekanun V, Naigowit P, White NJ. Identification of *Pseudomonas pseudomallei* in clinical practice: use of simple screening tests and API 20NE. J Clin Pathol 1989;42:645-8.
20. Gilligan PH, Whittier S. *Burkholderia*, *Stenotrophomonas*, *Ralstonia*, *Brevundimonas*, *Comamonas*, and *Acidovorax*. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, editors. Manual of clinical microbiology. 7th ed. Washington: American Society for Microbiology; 1999.
21. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing: ninth informational supplement M100-S10. Wayne (PA): The Committee; 2000.
22. Hsueh PR, Teng LJ, Pan HJ, Chen YC, Sun CC, Ho SW, et al. Outbreak of *Pseudomonas fluorescens* bacteremia among oncology patients. J Clin Microbiol 1998;36:2914-7.

23. Hsueh PR, Teng LJ, Hung CC, Hsu JH, Yang PC, Ho SW, et al. Molecular evidence for strain dissemination of *Penicillium marneffei*: an emerging pathogen in Taiwan. J Infect Dis 2000;181:1706-12.
24. Suputtamongkol Y, Chaowagul P, Lertpatanauwun N, Intaranongpai S, Ruchutrakool T, Budhsarawong D, et al. Risk factors for melioidosis and bacteremic melioidosis. Clin Infect Dis 1999;29:408-13.
25. Mceniry DW, Gillespie SH, Felmingham D. Susceptibility of *Pseudomonas pseudomallei* to new ß-lactam and aminoglycoside antibiotics. J Antimicrob Chemother 1988;21:171-5.
26. Smith MD, Wuthiekanun V, Walsh AL, White NJ. In-vitro activity of carbapenem antibiotics against beta-lactam susceptible and resistant strains of *Burkholderia pseudomallei*. J Antimicrob Chemother 1996;37:611-5.
27. White NJ, Dance DAB, Chaowagul W, Wattanagoon Y, Wuthiekanun V, Pitakwatchara N. Halving of mortality of severe melioidosis by ceftazidime. Lancet 1989;2:697-701.
28. Chaowagul P, Simpson AJH, Suputtamongkol Y, White NJ. Empirical cephalosporin treatment of melioidosis. Clin Infect Dis 1999;28:1328.
29. Simpson AJH, Suputtamongkol Y, Smith MD, Angus BJ, Rajamuwong A, Wuthiekanun V, et al. Comparison of imipenem and ceftazidime as therapy for severe melioidosis. Clin Infect Dis 1999;29:381-7.
30. Dance DAB, Wuthiekanun V, Chaowagul W, Suputtamongkol Y, White NJ. Development of resistance to ceftazidime and co-amoxiclav in *Pseudomonas pseudomallei*. J Antimicrob Chemother 1991;29:408-13.
31. Chaowagul W, Simpson AJH, Suputtamongkol Y, Smith MD, Angus BJ, White NJ. A comparison of chloramphenicol, trimethoprim-sulfamethoxazole, and doxycycline with doxycycline alone as maintenance therapy for melioidosis. Clin Infect Dis 1999;29:375-80.
32. Chaowagul W, Suputtamongkol Y, Dance DA, Rajchanuvong A, Pattara-Arechachai J, White NJ. Relapse in melioidosis: incidence and risk factors. J Infect Dis 1993;168:1181-5.
33. Desmarchelier PM, Dance DAB, Chaowagul W, Suputtamongkol Y, White NJ, Pitt TL. Relationships among *Pseudomonas pseudomallei* isolates from patients with recurrent melioidosis. J Clin Microbiol 1993;31:1592-6.
34. Vadivelu J, Puthucheary SD, Drasar BS, Dance DAB, Pitt TL. Stability of strain genotypes of *Burkholderia pseudomallei* from patients with single and recurrent episodes of melioidosis. Trop Med Int Health 1998;3:518-21.
35. Haase A, Melder A, Smith-Vaughan H, Kemp D, Currie B. RAPD analysis of isolates of *Burkholderia pseudomallei* from patients with recurrent melioidosis. Epidemiol Infect 1995;115:115-21.
36. Trakulsomboon S, Dance DAB, Smith MD, White NJ, Pitt TL. Ribotype differences between clinical and environmental isolates of *Burkholderia pseudomallei.* J Med Microbiol 1997;46:565-70.
37. van Phung L, Chi TTB, Hotta H, Yabuuchi E, Yano I. Cellular lipid and fatty compositions of *Burkholderia pseudomallei* strains isolated from human and environment in Viet Nam. Microbiol Immunol 1995;39:105-16.

# Outbreak of Human Monkeypox, Democratic Republic of Congo, 1996–1997

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Human monkeypox is a zoonotic smallpox-like disease caused by an orthopoxvirus of interhuman transmissibility too low to sustain spread in susceptible populations. In February 1997, 88 cases of febrile pustular rash were identified for the previous 12 months in 12 villages of the Katako-Kombe Health Zone, Democratic Republic of Congo (attack rate = 22 per 1,000; case-fatality rate = 3.7%). Seven were active cases confirmed by virus isolation. Orthopoxvirus-neutralizing antibodies were detected in 54% of 72 patients who provided serum and 25% of 59 wild-caught animals, mainly squirrels. Hemagglutination-inhibition assays and Western blotting detected antibodies in 68% and 73% of patients, respectively. Vaccinia vaccination, which protects against monkeypox, ceased by 1983 after global smallpox eradication, leading to an increase in the proportion of susceptible people.

Human monkeypox, a sporadic smallpox-like zoonotic viral exanthema that occurs in the rain forests of Central and West Africa, was discovered in 1970 (1-3). The illness is caused by an orthopoxvirus, monkeypox virus, which was first isolated from primate tissues (4). Animal antibody surveys in the Democratic Republic of Congo (DRC; former Zaire) suggested that squirrels play a major role as a reservoir of the virus and that humans are sporadically infected (3,5,6). Human-to-human transmission occurs with an incubation period of 12 days (range 7-21 days) (3).

After smallpox eradication, surveillance for human monkeypox from 1981 to 1986 in the DRC identified 338 cases (67% confirmed by virus culture). The case-fatality rate was 9.8% for persons not vaccinated with vaccinia (smallpox) vaccine, which was about 85% efficacious in preventing human monkeypox (3,7). The secondary attack rate in unvaccinated household members was 9.3%, and 28% of case-patients reported an exposure to another case-patient during the incubation period. Transmission chains beyond secondary were rare (8,9). A mathematical model to assess the potential for monkeypox to spread in susceptible populations after cessation of vaccinia vaccination indicated that person-to-person transmission would not sustain monkeypox in humans without repeated reintroduction of the virus from the wild (7).

In 1996, 71 suspected human monkeypox cases were reported from the Katako-Kombe Health Zone, Kasai Oriental, DRC. These initial reports suggested predominant person-to-person transmission and prolonged chains of transmission. Two cases were confirmed by monkeypox virus isolation from lesion material (10). In February 1997, an investigation was initiated (11). Our report describes epidemiologic observations and laboratory results supporting the conclusion that repeated animal reintroduction of monkeypox virus is needed to sustain the disease in the local human population.

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## Methods

### Epidemiologic and Clinical Studies

Before civil unrest in the area curtailed the study, a dwelling-to-dwelling search was conducted for cases that occurred from February 1996 and February 1997 in 12 villages (total population 4,057) in the Katako-Kombe Health Zone, located around Akungula, a village reported to be the epicenter of the current outbreak. A clinical case of monkeypox was defined as the occurrence of fever with a rash recognized as being similar to that in a reference photo provided by the World Health Organization. Monkeypox cases were classified as active until desquamation of the rash. After desquamation, cases were identified retrospectively by interview and examination for residual scars. Onset dates were estimated by using local event calendars.

Patients (or their adult respondent) who agreed to participate were queried by using a standardized data collection instrument to obtain information on demographics, signs and symptoms of disease, exposures to wild animals, presence of a smallpox vaccination scar, and exposure to another patient. Consenting participants underwent a physical examination, and a blood sample for serum was obtained.

### Animal Studies

Local trappers were asked to capture and bring to the study veterinarian wild animals, especially rodents, squirrels, and nonhuman primates, for which the trappers were paid as an incentive. Animals were processed in a field laboratory (12), identified to genus and species, and bled for serum. Representative voucher specimens of each animal were preserved in 10% formalin for definitive identification.

### Laboratory Studies

Human and animal sera were clarified by low-speed centrifugation, immediately stored in liquid nitrogen, and shipped to the Centers for Disease Control and Prevention (CDC) in Atlanta. At CDC, an aliquot of each serum was heated at 56°C for 30 minutes, the following tests were performed: 1) vaccinia virus hemagglutination-inhibition (HAI) assay (13); 2) monkeypox 50% plaque-reduction neutralization assay (13); and 3) Western blot assays for immunoglobulin G (IgG) against monkeypox antigens essentially by using Towbin's and colleagues' methods adapted to mini-transblot and multiscreen apparatuses (Bio-Rad, Hercules, CA) (14). Western blotting used selected human sera and antigen preparations that consisted of a soluble antigen (20x culture medium concentrate) from monkeypox virus-infected Vero cells (15). Positive controls consisted of sera collected in the 1980s from convalescent-phase monkeypox cases from the prospective study in the DRC and from vaccinia-vaccinated persons; negative controls consisted of sera collected in the 1980s from DRC inhabitants with no history of vaccinia vaccination or monkeypox. Human sera were also tested for antibodies against varicella virus by using kits to detect human IgM or IgG by enzyme-linked immunosorbent assays (ELISA; Wampole Laboratories, Cranberry, NJ). Samples of crusted scab or pustule lesion were cultured for monkeypox virus using the monkey cell lines Vero, LLCMK-2, or OMK (13,16), and assayed by polymerase chain reaction (PCR) amplification for monkeypox virus-specific DNA (16,17) and for varicella virus gene 1 (P. Pellett, pers. comm.). In addition, the gene encoding the hemagglutinin (HA) protein of selected monkeypox isolates was sequenced by fluorescence-based methods (Applied Biosystems, Inc., Foster City, CA). Available duplicate coded sera were tested anonymously in Kinshasa, DRC, for antibodies against HIV (Vironostika Human Form II ELISA, Organon Teknika, Denmark).

The relatedness of isolates was examined by comparing DNA restriction endonuclease patterns with patterns of previously mapped monkeypox virus isolates (18) and by comparing the hemagglutinin gene sequences with cognate sequences of other monkeypox isolates.

### Statistical Methods

Attack rates were calculated by using a census conducted during the dwelling-to-dwelling case search; information on the age and sex of each person living in Akungula was also obtained. Secondary attack rates within households were calculated by dividing the number of cases that occurred 7 to 21 days following one or more index cases in a household (first-generation secondary cases) by the total number of household members, excluding index cases. Confidence intervals (CI) for proportions were calculated with exact methods and compared with Fisher's exact tests as appropriate by using Epi-Info software (19).

## Results

### Epidemiologic and Clinical Studies

Eighty-eight clinical cases (7 active and 81 retrospectively identified) were discovered in 9 of the 12 villages (attack rate 22 per 1,000). Fifty (56.8%) of 88 case-patients were male; the median age at onset was 10 years (quartiles 5-19; range 1 month to 62 years).

The number of clinical cases reported per month increased from February to August 1996, followed by a decline until January 1997 and a resurgence of cases in February 1997 (Figure 1). Attack rates in the villages ranged from zero to 105 per 1,000 population in Akungula, where 42 cases were identified in 14 of the 62 village households.

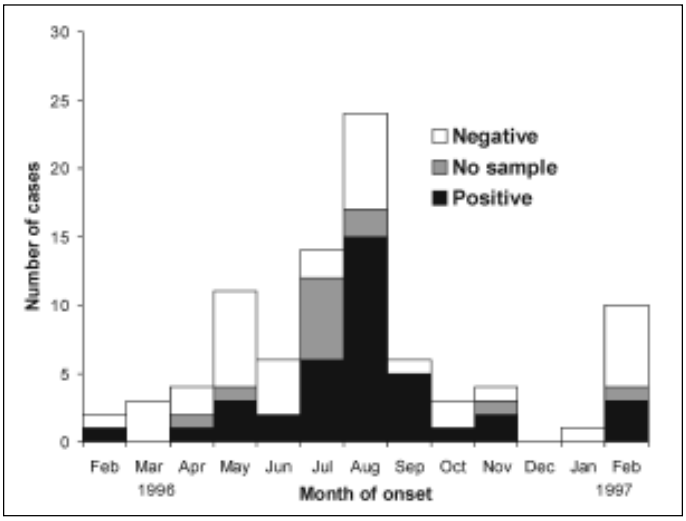


Figure 1. Human monkeypox cases by month of onset in 12 villages, according to results of neutralization assay, Katako-Kombe Health Zone, February 1996 to February 1997.

In Akungula, where the age and sex distributions of the population were available, the attack rate was identical for males and females, but was higher (30 of 206 = 146/1,000) in children <15 years of age than in persons 15 to 24 years old (4 of 85 = 47 /1,000) and ≥25 years of age (8 of 109 = 73/1,000).

Of 78 retrospectively identified case-patients for whom information was available, 40 (51.3%) reported swelling consistent with lymphadenopathy. All 75 examined patients had scars from a rash (median 50; range 4 to 830; standard deviation 179). Thirteen (15.5%) of 84 patients for whom information was available had a vaccination scar on the upper left arm compatible with prior vaccinia vaccination; all patients were ≥20 years of age. Alopecia was seen in three cases with acute rash illness. Three deaths occurred among 81 of the 88 cases for which follow-up information was available (3.7% case-fatality rate), all in children <3 years of age who died within 3 weeks of rash onset. No information was available to attribute the deaths to monkeypox, superinfection, or other cause.

Seven case-patients (six in a single household) had active disease at the time of the investigation. Each had lymphadenopathy and more than 100 crusty skin lesions. Five had a rash on the soles and palms. Sixty-two (73%) of 85 case-patients for whom information was available reported exposure to another patient 7 to 21 days before onset of their

illness. The remaining 23 (27%) patients reported either no exposure to other cases (n=13; 15%) or an exposure to another case from 0 to 6 days before onset of illness (n=10; 12%). Dates of onset for patients who reported no exposure to other cases during the incubation period were distributed throughout the study period (11). Exposures during the incubation period that were reported by all six patients who resided in a single household and who had active disease at the time of this investigation included exposure to a patient within the household and eating monkey, gazelle, pig, and rat.

The 88 clinical cases were identified for 39 households, in which 297 persons resided (overall attack rate in affected households = 30%). For 240 household members for whom information was available, rash that met the clinical case definition developed in 20 in 7 to 21 days after exposure to ≥1 index cases in a household (household secondary attack rate 8.3 per 100; 95% CI 5.2% to 12.6%).

Eating wild animals was common for all patients. However, patients who had no exposure to other case-patients during the incubation period were more likely to eat porcupine at least once a month (Table 1). All queried participants reported trapping animals less frequently.

### Laboratory Studies

Human monkeypox was confirmed in all seven active cases by virus isolation and monkeypox virus-specific DNA amplification from skin lesion samples; antibodies against orthopoxvirus were detected in two by neutralization assay, in three by HAI assay, and in six by Western blotting. In addition, IgM antibodies against varicella virus were detected in five active cases in patients who lived in the same household. Varicella virus DNA was detected in lesion material from two of these patients.

Table 1. Exposure to wild animals reported by monkeypox case-patients according to history of exposure to another case-patient, Katako-Kombe Health zone, February 1996 to February 1997

| Exposures reported to occur usually on a monthly basis | Cases without exposure to other case-patients during incubation (N=23) (#) | (%) | Cases with exposure to other case-patients during incubation (N=62) (#) | (%) | p value |
|---|---|---|---|---|---|
| Exposure to squirrels | | | | | |
| Trapping | 2 | 8.7 | 9 | 14.8 | 0.79 |
| Exposure to raw meat | 11 | 47.8 | 33 | 54.1 | 0.78 |
| Eating | 19 | 86.4 | 52 | 85.2 | 1.00 |
| Exposure to monkeys | | | | | |
| Trapping | 1 | 4.3 | 4 | 6.6 | 1.00 |
| Exposure to raw meat | 16 | 69.6 | 34 | 55.7 | 0.36 |
| Eating | 21 | 91.3 | 58 | 95.1 | 0.61 |
| Exposure to rats | | | | | |
| Trapping | 5 | 21.7 | 18 | 29.5 | 0.66 |
| Exposure to raw meat | 13 | 56.5 | 40 | 65.6 | 0.60 |
| Eating | 22 | 95.7 | 55 | 90.2 | 0.66 |
| Exposure to porcupines | | | | | |
| Trapping | 1 | 4.3 | 3 | 4.9 | 1.00 |
| Exposure to raw meat | 12 | 52.2 | 24 | 39.3 | 0.41 |
| Eating | 22 | 95.7 | 42 | 68.9 | 0.02 |
| Exposure to gazelles | | | | | |
| Trapping | 1 | 4.3 | 2 | 3.3 | 1.00 |
| Exposure to raw meat | 14 | 56.5 | 28 | 45.9 | 0.53 |
| Eating | 21 | 91.3 | 47 | 77.0 | 0.21 |

Seventy-two of the 81 retrospectively identified cases provided a serum sample, although 2 were low in volume. Orthopoxvirus antibodies were detected by neutralization assay in 39 (54%, Figure 1) of the 72 sera; by HAI in 51 (73%) of 70 sera, and by a new, nonvalidated Western blot test in 49 (68%) of 72 sera. Thirty-eight (54%) of 70 available sera were positive by all three tests, 45 (64%) were positive by two tests, and 62 (89%) were positive by at least one test. Western blotting and HAI test results were concordant for 79% of the sera; Western blotting and neutralization test results agreed for 83% of the sera; and HAI and neutralization test results agreed for 79% of the sera. Fifty-five (76%) of the 72 patients who provided serum had detectable varicella IgG antibodies, 1 had varicella IgM, and none showed detectable HIV antibodies.

Based on DNA assays (16-18), monkeypox virus isolates from the seven active cases appeared virtually identical to each other and very closely related to two isolates from humans in 1996 and an isolate from a squirrel trapped in the Equateur Province of the DRC in 1985. Analysis of sequences coding for the viral HA protein (Figure 2) indicated that viruses isolated in the DRC since 1970 comprised a clade distinguishable from a clade comprising isolates from other African countries and from outbreaks in primate-holding facilities in Europe and the United States. The group of nine isolates from the 1996-97 outbreak, which showed identical HA sequences, and from the squirrel from 1985 constituted a subset of the clade of examined viruses previously isolated in the DRC.

### Animal Studies

Fifty-nine captured animals representing 14 species were tested. Sera from 42% of the various squirrel species, from 16% of Gambian rats, and from an elephant shrew and a domestic pig showed orthopoxvirus neutralizing antibodies (Table 2).

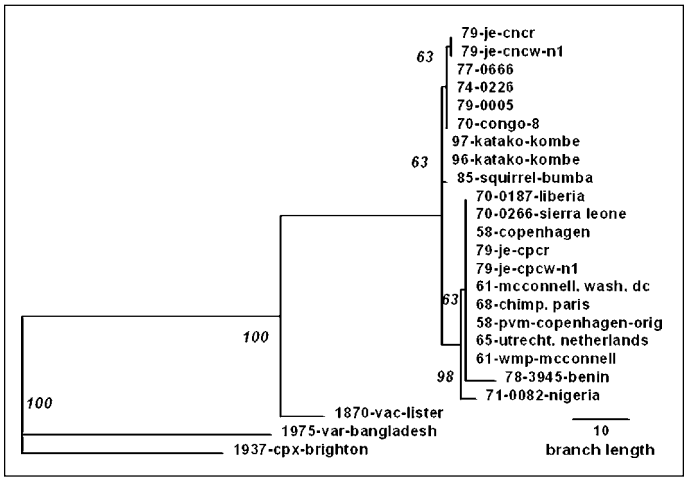


Figure 2. Phylogenetic inference relationships of the open reading frames encoding the viral hemagglutinin protein of various monkeypox virus isolates and selected strains of vaccinia, variola, and cowpox viruses. Nucleotide sequences of polymerase chain reaction-generated amplicons were analyzed using PAUP parsimony analysis software version 3.1.1, as described (10). Parsimony analysis used 5,000 bootstraps and weighted the sequences for a transition-transversion ratio of 2 (bootstrap confidence intervals shown on branches).

Table 2. Species of animals caught in the wild and monkeypox virus plaque reduction neutralization antibody assay results, Katako-Kombe Health Zone, February 23-27, 1997

| Animal species | Common name | No. Tested | Pos. | Proportion pos. |
|---|---|---|---|---|
| *Cercopithecus ascanius* | Spot-nosed monkey | 2 | 0 | 0.0% |
| *Cercopithecus neglectus* | Debrazza monkey | 1 | 0 | 0.0% |
| *Perodicticus potto* | Bosman's potto | 1 | 0 | 0.0% |
| *Galogoides demidovi* | Demidoff galago | 2 | 0 | 0.0% |
| *Genetta rubiginosa* | Genet | 1 | 0 | 0.0% |
| *Sus scrofa* | Domestic pig | 1 | 1 | 100.0% |
| *Praomys jacksoni* | Forest rat | 1 | 0 | 0.0% |
| *Cricetomys emini* | Gambian rat | 19 | 3 | 15.8% |
| *Cavia sp.* | Guinea pig | 3 | 0 | 0.0% |
| *Petrodromus tetradactylus* | Elephant shrew | 3 | 1 | 33.3% |
| *Dendrohyrax arboreus* | Tree hyrax | 1 | 0 | 0.0% |
| *Funisciurus anerythrus* | Thomas's tree squirrel | 4 | 2 | 50.0% |
| *Funisciurus congicus* | Kuhl's tree squirrel | 18 | 7 | 38.9% |
| *Heliosciurus rufobrachium* | Sun squirrel | 2 | 1 | 50.0% |
| Total | | 59 | 15 | 25.4% |

## Conclusion

The epidemiologic and laboratory evidence presented here suggests that this monkeypox outbreak is the largest reported. Epidemiologic features of monkeypox that differed from those described during an intensified prospective study in the DRC in the 1980s included an increase in the proportion of patients reporting an exposure to another case during the incubation period (28% in the 1980s, 73% this study) and the clustering of successive cases within households, with as many as eight consecutive cases in one instance (3,10).

Prospective surveillance in the 1980s in the DRC provided opportunities to observe most patients with active-stage monkeypox; thus, skin lesion samples were available for virus isolation to confirm clinical diagnosis. In contrast, the present study identified most patients retrospectively and relied on serologic testing for confirmation of the diagnosis; only seven clinically active cases could be confirmed by virus isolation and PCR (17) for monkeypox virus. Because monkeypox is the only known indigenous orthopoxvirus of Africa that infects humans systemically, seropositive cases showing genus-specific antibodies can be reasonably interpreted as cases that had a monkeypox virus infection (20). Of 72 retrospectively identified cases tested, 54% had antibodies by plaque-reduction assay, a preferred test for verifying orthopoxvirus infections during smallpox eradication (20,21). However, this neutralization test was only 83% sensitive for the detection of vaccinia vaccine-induced antibodies and may have been negative (3) in some patients with monkeypox virus infection during this study interval. A higher proportion of sera from retrospectively identified cases (73%) tested positive by the HAI test, which had a higher sensitivity (96%) than the neutralization test for the identification of vaccinia-induced antibodies (22). However, the HAI test may be less specific, and antibody titers detected by it decrease rapidly after infection and would be unlikely to be residual from prior vaccination in the small percentage of patients who had been vaccinated (3,21,23). Finally, 72% of sera from retrospectively identified cases tested positive in a new, nonvalidated Western blot orthopoxvirus antibody test for which sensitivity and specificity have not been determined. Chickenpox, which can be mistaken for monkeypox (24), was reported in the area during the study period. Some patients may have had dual infection by monkeypox and varicella viruses. Within one household, five patients from whom monkeypox virus was isolated showed serologic evidence of recent varicella virus infection, and two of those had detectable varicella virus DNA in skin lesion samples. Thus, a substantial percentage of cases may have been chickenpox, thereby explaining why the case-fatality rate from this study (3.7%) was lower than the 9.6% previously reported (3,24). However, the presence of antibodies against orthopoxviruses by three different assays in 54% of patients suggests that varicella virus was not responsible for most cases during the study period.

The 1996-97 crude secondary attack rate of about 8% in households cannot be compared with the 3.7% risk of attack for household contacts from the 1981-1986 study (3) because the proportion of susceptible (unvaccinated) persons in household members increased markedly from 1981-1986 to 1996-97. However, the 1996-97 secondary attack rate within households was similar to the 1981-1986 rates (3) of 9.3% for unvaccinated household members. This similarity is consistent with the high degree of similarity noted in comparing current monkeypox virus isolate DNAs with DNAs of isolates from the DRC in the 1970s, including the HA sequences (Figure 2). While the virus and its person-to-person transmission potential have not changed substantially, the proportion of susceptible persons among exposed household members increased most likely because vaccinia vaccination ceased after smallpox eradication. The reduced herd immunity probably also caused a higher proportion of cases to be attributable to person-to-person transmission, as the increase in the proportion of patients who reported exposure to another patient during the incubation interval would suggest. HIV was not a cofactor of this outbreak because no HIV antibodies were detected in patients' sera.

Exposure to an animal reservoir might have been the source of infection for at least 27% of patients who reported no exposure to other patients during the incubation period. Past animal studies pointed to several species of squirrels as animal reservoirs (5,6). Although the possibility of infection of animals with another unknown indigenous orthopoxvirus cannot be ruled out in animals with neutralizing antibodies, the results of our investigation suggest that, in addition to squirrels, Gambian rats may play a role in monkeypox virus circulation. Villagers ate a number of different animals, and it was impossible to draw any conclusions as to whether any one species was a greater risk factor than any other. However, antibody surveys could be used to evaluate porcupines as a possible reservoir (Table 1), which was not done during our investigation because no porcupines were captured by trappers.

Our study had several limitations, some of which related to the brevity imposed by the civil unrest. Most cases were identified retrospectively and without a case-control group; thus, they could not be confirmed by a serologic test for which

the sensitivity and the specificity had been measured using a standard technique and case-control sera. Because information was not available regarding the vaccination status of other members of the patients' households, secondary attack rates within households could not be calculated according to the vaccination status. We did not assess the environmental changes that could have facilitated this outbreak, including increases in household sizes, rates of monkeypox virus infection in the animal population, and a change in rate and type of exposure to wild animals among residents. We were unable to attribute infections in patients who reported an exposure to another patient during the incubation period to person-to-person transmission because some of these patients may also have been exposed to infected animals. Finally, patients with a history of exposure to a patient during the incubation period were the only controls available to generate hypotheses regarding the type of animal exposure possibly associated with infection in patients not reporting an exposure to other patients during the incubation period. This aspect may have caused bias because cases clustered within households where exposures to animals may have been identical and because cases may have been misclassified as to their purported source of infection.

There is no evidence to date that person-to-person transmission alone can sustain monkeypox in the local population. However, our study suggests that in a population with low herd immunity, person-to-person transmission and repeated introduction of virus from the animal reservoir may lead to more and larger clusters of human monkeypox cases in African rain forest areas (25). Further studies are needed to measure the sensitivity and the specificity of the serologic tests currently available so they can be used in the future to better confirm retrospectively identified cases, identify the type of animal or patient exposures associated with acquisition of illness, and evaluate the respective roles of person-to-person and animal-to-human transmission. However, access to the Kasai region has been limited since our study, and little information has become available to better document the current dynamics of monkeypox occurrences or improve World Health Organization recommendations to prevent human monkeypox more substantially.

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Dr. Yvan Hutin was an Epidemic Intelligence Service officer with the Centers for Disease Control and Prevention at the time of this investigation. He is now at the World Health Organization.

## References

1. Marennikova SS, Seluhina EM, Mal'ceva NN, Cimiskjan KL, Macevic GR. Isolation and properties of the causal agent of a new variola-like disease (monkeypox) in man. Bull World Health Organ 1972;46:599-611.
2. Arita I, Jezek Z, Khodakevich L, Ruti K. Human monkeypox: a newly emerged orthopoxvirus zoonosis in the tropical rain forests of Africa. Am J Trop Med Hyg 1985;34:781-9.
3. Jezek Z, Fenner F. Human monkeypox. In: JL Melnick, editor. Monographs in virology. Volume 17. Basel: Karger; 1988.
4. von Magnus P, Andersen EK, Petersen KB, Birch-Andersen A. A pox-like disease in cynomolgus monkeys. Acta Pathol Microbiol Scand 1959;46:156-76.
5. Khodakevich L, Jezek Z, Messinger D. Monkeypox virus: ecology and public health significance. Bull World Health Organ 1988;66:747-52.
6. Khodakevich L, Szczeniowski M, Manbu-ma-Disu, Jezek Z, Marennikova S, Nakano J, et al. The role of squirrels in sustaining monkeypox virus transmission. Trop Geogr Med 1987;39:115-22.
7. Fine PE, Jezek Z, Grab B, Dixon H. The transmission potential of monkeypox virus in human populations. Int J Epidemiol 1988;17:643-50.
8. Jezek Z, Grab B, Szczeniowski MV, Paluku KM, Mutombo M. Human monkeypox: secondary attack rates. Bull World Health Organ 1988;66:465-70.
9. Jezek Z, Arita I, Mutombo M, Dunn C, Nakano JH, Szezeniowski M. Four generations of probable person-to-person transmission of human monkeypox. Am J Epidemiol 1986;123:1004-12.
10. Mukinda VB, Mwema G, Kilundu M, Heymann DL, Khan AS, Esposito JJ. Re-emergence of human monkeypox in Zaire in 1996. Lancet 1997;349:1449-50.
11. Centers for Disease Control and Prevention. Human monkeypox-Zaire, 1996-1997. MMWR Morb Mortal Wkly Rep 1997;46:304-7.
12. Mills JN, Childs JE, Ksiazek TG, Peters CJ, Velleca WM. Methods for trapping and sampling small mammals for virologic testing. Atlanta: Centers for Disease Control and Prevention; 1995.
13. Esposito JJ, Massung RF. Poxvirus infections in humans. In: Murray PR, Tenover F, Baron EJ, Pfaller MA, Yolken RH, editors. Manual of clinical microbiology. 6th ed. Washington: ASM Press;1995. p. 1131-8.
14. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979;76:4350-4.
15. Loparev VN, Parsons JM, Knight JC, Panus JF, Ray CA, Buller RM, et al. A third distinct tumor necrosis factor receptor of orthopoxviruses. Proc Natl Acad Sci U S A 1998;95:3786-91.
16. Ropp SL, Esposito JJ, Loparev VN, Palumbo G. Poxviruses infecting humans. In: Murray PR, Barron CJ, Pfaller MA, Tenover FC, Yolken RH, editors. Manual of clinical microbiology. 7th ed. Washington: ASM Press; 1999. p. 1137-44.
17. Ropp SL, Jin Q, Knight JC, Massung RF, Esposito JJ. PCR strategy for identification and differentiation of smallpox and other orthopoxviruses. J Clin Microbiol 1995;33:2069-76.
18. Esposito JJ, Knight JC. Orthopoxvirus DNA: A comparison of restriction profiles and maps. Virology 1985;143:230-51.
19. Dean AG, Dean JA, Coulombier D, Burton AH, Brendel KA, Smith DC. Epi Info, version 6: A word-processing, database, and statistics program for public health on IBM-compatible microcomputers. Atlanta: Centers for Disease Control and Prevention; 1994.
20. Fenner F, Wittek R, Dumbell KR. The Orthopoxviruses. San Diego: Academic Press; 1989. p. 162-5, 312-15.
21. Ziegler DW, Hutchinson HD, Koplan JP, Nakano JH. Detection by radioimmunoassay of antibodies in human smallpox patients and vaccinees. J Clin Microbiol 1975;1:311-7.
22. Cherry JD, McIntosh K, Connor JD, Benenson AS, Alling DW, Rolfe UT, et al. Primary percutaneous vaccination. J Infect Dis 1977;135:145-54.
23. McIntosh K, Cherry JD, Benenson AS, Connor JD, Alling DW, Rolfe UT, et al. Clinical and serologic study of four smallpox vaccines comparing variations of dose and route of administration. Standard percutaneous revaccination of children who receive primary percutaneous vaccination. J Infect Dis 1977;135:155-66.
24. Jezek Z, Szczeniowski M, Paluku KM, Mutombo M, Grab B. Human monkeypox: confusion with chickenpox. Acta Trop 1988;45:297-307.
25. Centers for Disease Control and Prevention. Human monkeypox — Kasai Oriental, Democratic Republic of Congo, February 1996-

# Nipah Virus Infection in Bats (Order Chiroptera) in Peninsular Malaysia

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Nipah virus, family Paramyxoviridae, caused disease in pigs and humans in peninsular Malaysia in 1998-99. Because Nipah virus appears closely related to Hendra virus, wildlife surveillance focused primarily on pteropid bats (suborder Megachiroptera), a natural host of Hendra virus in Australia. We collected 324 bats from 14 species on peninsular Malaysia. Neutralizing antibodies to Nipah virus were demonstrated in five species, suggesting widespread infection in bat populations in peninsular Malaysia.

From September 1998 to April 1999, a major outbreak of disease in peninsular Malaysia resulted in the deaths of 105 persons and the slaughter of approximately 1.1 million pigs. The primary causal agent in both pigs and humans, first thought to be endemic Japanese encephalitis virus, was shown to be a previously undescribed member of the Paramyxoviridae family. Preliminary characterization of a human isolate of the new virus, subsequently named Nipah virus, showed it to have ultrastructural, serologic, antigenic, and molecular similarities to Hendra virus (1-3).

This apparently close phylogenetic relationship focused initial wildlife surveillance on bats (order Chiroptera), particularly pteropid bats (flying foxes), species of which are the probable natural host of Hendra virus in Australia (4-6). Additional support for this targeted approach was provided by the findings of earlier serologic surveillance of flying foxes in Papua New Guinea, in which antibodies neutralizing Hendra virus were found in five of six species tested (Field et al., unpub. data). Malaysia has diverse bat fauna, with at least 13 species of fruit bats (including two species of flying fox) and >60 species of insectivorous bats (7).

We investigated fruit bats (suborder Megachiroptera) and insectivorous bats (suborder Microchiroptera) in peninsular Malaysia for evidence of infection with Nipah virus. Wild boar (*Sus scrofa*), domestic dogs (*Canis lupus*) used to hunt wild boar, and rodents (*Rattus rattus*) trapped on farms with infected pigs were a secondary focus. A parallel study undertook the primary surveillance of rodents, domestic dogs, and other peridomestic species (Mills et al., unpub. data).

From April 1 to May 7, bats were sampled in 11 primary locations in the states of Perak (n = 6), Selangor (n = 1), Negeri Sembilan (n = 1), and Johore (n = 3) (Figure 1). Most primary locations had more than one sampling site. Locations included but were not restricted to places where Nipah virus-associated disease was reported in pigs. Populations of flying foxes were nonrandomly sampled by shooting foraging or roosting animals. Populations of smaller fruit bats and insectivorous bats were nonrandomly sampled by using mist nets in orchards, oil palm plantations, secondary native

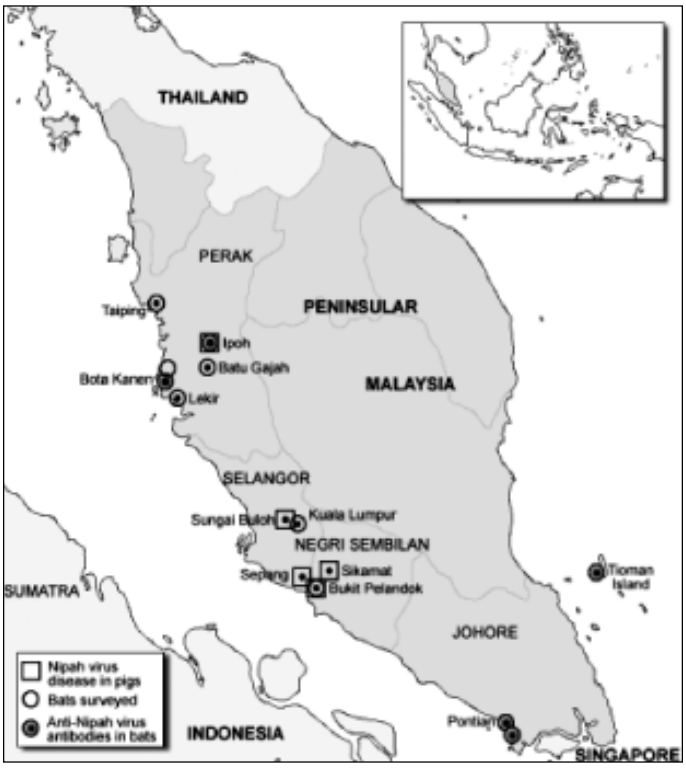


Figure 1. Primary sampling locations of bats, Malaysia.

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vegetation, and residential areas, where bats were reported or observed, where flowering or fruiting trees were observed, and near known or possible roosts. A target of 30 animals per species was set, providing 95% statistical confidence of detecting infection at a minimum population prevalence of 10%, assuming homogeneity of infection across overlapping populations and a test sensitivity and specificity of 100% (8). Blood for serologic examination was also collected from two captive colonies of flying foxes in zoos. In addition to blood, fresh tissue samples of liver, lung, kidney, spleen, heart, and fetus were taken from wild-caught animals, and the carcasses were stored in 10% buffered formalin for reference. Virus isolation was attempted by using Vero E6 cells in a biosafety level 4 laboratory as described (9). All cell harvests were checked for Nipah virus antigens by indirect fluorescence with Nipah hyperimmune ascitic fluid. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on the same tissues, from which RNA was extracted. Virus isolation and RT-PCR were performed on both kidney and spleen from each animal from which tissues had been collected. Additionally, all tissues were blind passaged twice more, and each harvest was tested for viral antigen. RT-PCR used forward and reverse primers designed to amplify a 228-bp region of the N gene.

A total of 324 animals from 14 species of bat were sampled, with the target sample size being achieved for five species. Sera were either forwarded directly to the Australian Animal Health Laboratory (AAHL) in Geelong, Australia, or screened by indirect enzyme-linked immunofluorescent assay (ELISA) using Nipah virus antigen at the Veterinary Research Institute in Ipoh, Malaysia, before being forwarded to AAHL. At AAHL, sera were tested by indirect ELISA using Nipah virus antigen and by serum neutralization test (SNT). As the latter is the recognized standard, we used these data in our analysis. Serum neutralization results were obtained for 265 sera, the balance producing toxic reactions (attributed to poor serum sample quality) at a dilution of ≥1:10. Neutralizing antibodies to Nipah virus were detected in the sera of 21 wild-caught animals from five species (Table): *Pteropus hypomelanus* (island flying fox), *P. vampyrus* (Malayan flying fox), *Eonycteris spelaea* (cave bat), *Cynopterus brachyotis* (lesser dog-faced fruit bat), and *Scotophilus kuhli* (house bat). Antibody prevalence among these species was 31%, 17%, 5%, 4%, and 3%, respectively. Titers ranged from 1:5 (n = 2), the lowest dilution tested, to 1:40 (n = 1), median 1:10 (Figure 2). The Nipah virus neutralization titer of the positive control rabbit anti-Hendra virus serum was 1:20. Of the 21 sera neutralizing Nipah virus, only one neutralized Hendra virus, with a titer fourfold less than the corresponding Nipah virus titer. The Hendra virus neutralization titer of the positive control anti-Hendra virus serum was 1:160. All culture harvests were negative for Nipah virus antigen. Two of the tissues yielded cytopathic agents that do not react with either Nipah or Hendra antibodies; these agents are being characterized. All attempts to amplify Nipah virus RNA were also negative.

Wild boar, hunting dogs, and rodents were sampled in Perak state from April 1 to May 7. Wild boar (n = 18) were nonrandomly sampled by shooting in oil palm plantations, secondary native vegetation, national parks, and rural residential areas. Blood samples were also collected from dogs (n = 16) used to hunt wild boar. Rodents (n = 25) were trapped on several farms where pigs were infected. None of the sera from wild boar, hunting dogs, or rodents were positive by indirect ELISA using Nipah virus antigen.

We interpret the presence of neutralizing antibodies to Nipah virus in the identified bat species as evidence of infection with this virus or a cross-neutralizing virus. Cross-neutralization of Nipah antigen by antibodies to Hendra virus was excluded as the cause of the reactivity, and other paramyxoviruses have not demonstrated cross-neutralization with either Hendra (10) or Nipah virus (2). We believe that the presence of anti-Nipah antibodies in a population of *P. hypomelanus* on the east coast island of Tioman (Figure 1), geographically remote from the west coast foci of Nipah viral disease in pigs, indicates that Nipah virus infection is widespread in flying fox populations in peninsular Malaysia. Ecologically, *P. hypomelanus* is an island specialist whose mainland foraging is limited to nearby coastal areas (11).

The low neutralizing antibody titers in the positive Malaysian bats were unexpected. In Australia, anti-Hendra virus titers of >1:640 in wild-caught flying foxes (Field et al., unpub. data) and 1:80 in experimentally infected flying foxes (12) have been observed. The absence of high titers in the sampled animals could be explained in several ways: the sample may not be representative of the population; Nipah virus may bind inefficiently to Vero cells used in the

Table. Species description of 237 wild-caught Malaysian bats of known neutralizing antibody status to Nipah virus[a] surveyed April 1–May 7, 1999

| Species | No. of bats | No. positive (%) |
|---|---|---|
| Megachiroptera (fruit bats) | | |
| *Cynopterus brachyotis* | 56 | 2 (4) |
| *Eonycteris spelaea* | 38 | 2 (5) |
| *Pteropus hypomelanus* | 35 | 11 (31) |
| *Pteropus vampyrus* | 29 | 5 (17) |
| *Cynopterus horsfieldi* | 24 | 0 |
| *Ballionycterus maculata* | 4 | 0 |
| *Macroglossus sobrinus* | 4 | 0 |
| *Megaerops ecaudatus* | 1 | 0 |
| Microchiroptera (Insectivorous bats) | | |
| *Scotophilus kuhlii* | 33 | 1 (3) |
| *Rhinolophus affinis* | 6 | 0 |
| *Taphozous melanopogon* | 4 | 0 |
| *Taphozous saccolaimus* | 1 | 0 |
| *Hipperosiderus bicolor* | 1 | 0 |
| *Rhinolophus refulgens* | 1 | 0 |
| Total | 237 | 21 |

[a]Sera from 324 bats were tested: 59 sera that gave toxic results at dilutions 1:10 were excluded from analysis, as were sera from 28 captive *P. vampyrus*.

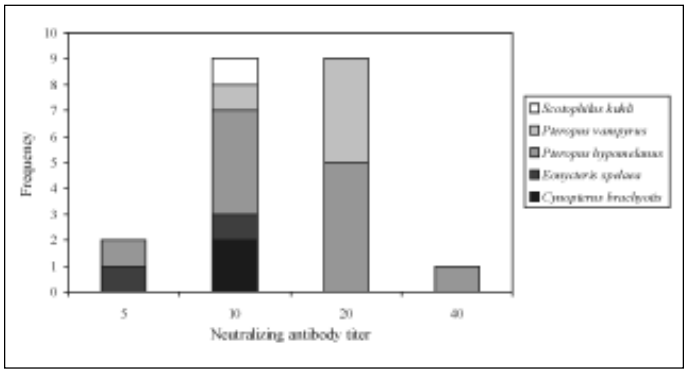


Figure 2. Frequency distribution of antibody titers to Nipah virus in 21 Malaysian bats testing positive by serum neutralization test.

neutralization assays; bats' immune response to Nipah virus may be muted as a result of high-level adaptation of the virus to these species; or the antigenic structure of the virus in pigs and humans may differ from that in bats, resulting in less effective neutralization of a test antigen derived from a human isolate. Alternatively, the antibodies detected may be cross-neutralizing antibodies to another related but as yet unidentified virus in bats.

The detection of anti-Nipah virus antibodies in non-pteropid species is notable, although the significance of the finding remains unclear. Limited surveillance of non-pteropid species in Australia for anti-Hendra virus antibodies has not found evidence of infection in these species. Further work is needed to clarify any role of non-pteropid species in the natural history of both viruses.

Isolation of Nipah virus from bats is essential to corroborate the serologic findings and enable comparison of bat isolates with human and pig isolates. However, cell culture of fresh tissue samples from antibody-positive and -negative bat species forwarded to the Centers for Disease Control and Prevention did not produce an isolate reactive with anti-Nipah virus antibodies. All PCR attempts on these tissues were also negative. The tissues submitted (heart, liver, lung, kidney, spleen, fetal) were considered appropriate, as these tissues, as well as white cells, have yielded Hendra virus isolates in naturally infected (13) or experimentally infected (12) flying foxes in Australia. In Malaysia, the period of sampling did not overlap the seasonal gestation of either *P. vampyrus or P. hypomelanus*. Fetal tissues submitted were from *S. kuhli*, *E. spelaea*, *C. brachyotis*, *Taphozous melanopogon*, *T. saccolaimus*, and *Rhinolophus affinis*.

The wild boar and hunting dog serologic results need to be interpreted in light of the limited sample size, nonrandom sampling, and test methods. Nonetheless, as the behavioral and foraging patterns of wild boar promote contact within and between neighboring populations, the absence of anti-Nipah virus antibodies in the sample supports the absence of established infection in wild boar populations in the areas surveyed. The absence of anti-Nipah virus antibodies in hunting dogs is also consistent with lack of exposure to Nipah virus. A Nipah virus antibody prevalence of 42 (46%) of 92 was identified in domestic dogs sampled near infected pig farms (Mills et al., unpub. data), and if hunting dogs, which have regular contact with the blood, urine, and oronasal secretions of wild boar, were exposed, similar antibody prevalences could reasonably be expected. The negative findings in the rodent sample are consistent with those of the comprehensive parallel survey of rodents (Mills et al., unpub. data).

We report evidence of infection with Nipah virus in four fruit bat species and one insectivorous bat species in peninsular Malaysia. A proposed second phase will describe the occurrence and frequency of infection in the identified Nipah antibody-positive species at additional locations in peninsular Malaysia and in Sabah and Sarawak, Borneo. In addition to successful virus isolation from bats, other proposed research includes retrospective studies of archival specimens, experimental infections of fruit bats, and serologic surveys of other arboreal mammalian species.

## Acknowledgments

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Dr. Johara is a senior veterinarian with the Malaysian Department of Veterinary Services. Her research interests include infectious diseases of livestock, infectious zoonotic diseases, animal welfare and wildlife conservation. During the Nipah virus outbreak, she played a key role in the investigation of wildlife species for the origin of Nipah virus.

## References

1. Centers for Disease Control and Prevention. Outbreak of Hendra-like virus—Malaysia and Singapore, 1998-1999. MMWR Morb Mortal Wkly Rep 1999;48:265-9.
2. Chua KB, Bellini WJ, Rota PA, Harcourt BH, Tamin A, Lam SK, et al. Nipah virus: a recently emergent deadly paramyxovirus. Science 2000;288:1432-5.
3. Mohd Nor M, Gan C, Ong B. Nipah virus infection of pigs in peninsular Malaysia. Rev Sci Tech 2000;19:160-5.
4. Young PL, Halpin K, Selleck PW, Field HE, Gravel JL, Kelly MA, et al. Serologic evidence for the presence in Pteropus bats of a paramyxovirus related to equine morbillivirus. Emerg Infect Dis 1996;2:239-40.
5. Field H, Halpin K, Young P. An overview of equine morbillivirus and bat paramyxovirus in Australia. The OIE/FAVA Epidemiology Programme: special session on emerging diseases, Cairns, Australia; 1997 Aug 28; Office International des Epizooties (OIE).
6. Williamson MM, Hooper PT, Selleck PW, Gleeson LJ, Daniels PW, Westbury HA, et al. Transmission studies of Hendra virus (equine morbillivirus) in fruit bats, horses and cats. Aust Vet J 1998;76:813-8.
7. Medway L. The wild mammals of Malaya (Peninsular Malaysia) and Singapore. Kuala Lumpur: Oxford University Press; 1978.
8. Cannon R. Livestock disease surveys: a field manual for veterinarians. Canberra (Australia): Australian Bureau of Animal Health; 1982.
9. Leirs H, Mills JN, Krebs JW, Childs JE, Dudu A, Woollen N, et al. Search for the Ebola virus reservoir in Kikwit, Democratic Republic of the Congo: reflections on a vertebrate collection. J Infect Dis 1999;179:155-63.
10. Murray K, Selleck P, Hooper P, Hyatt A, Gould A, Gleeson L, et al. A morbillivirus that caused fatal disease in horses and humans. Science 1995;268:94-7.
11. Mickleburg S, Hutson A, Racey P. Old world fruit bats: an action plan for their conservation. Gland (Switzerland): International Union for the Conservation of Nature and Natural Resources; 1992.
12. Williamson M, Hooper P, Selleck P, Westbury H, Slocombe R. The effect of Hendra virus on pregnancy in fruit bats and guinea pigs. J Comp Pathol 2000;122:201-7.
13. Halpin K, Young P, Field H, Mackenzie J. Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. J Gen Virol 2000;81:1927-32.

# Third-Generation Cephalosporin Resistance in *Shigella sonnei*, Argentina

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*Shigella sonnei* resistant to cefotaxime (but not to ceftazidime) was isolated for the first time in stool samples from a pediatric patient with vomiting and bloody diarrhea in northern Argentina. Microbiologic and biochemical tests confirmed the presence of an extended spectrum beta-lactamase displaying an apparent isoelectric point value of 8.2.

Shigellosis is an important public health problem, especially in developing countries. Antibiotic treatment of bacterial dysentery, aimed at resolving diarrhea or reducing its duration, is especially indicated whenever malnutrition is involved. First-line drugs include ampicillin and trimethoprim-sulfamethoxazole (TMP-SMX); however, multidrug-resistant (i.e., resistant to ampicillin, TMP-SMX, and chloramphenicol) *Shigella* isolates are ubiquitous (1-3). When epidemiologic data indicate a rise in resistance, fluoroquinolones may be used in adults and oral third-generation cephalosporins in children. Except for a single strain in Calcutta (4), *Shigella* have not obtained any extended-spectrum beta-lactamase (ESBL)(4), even though multidrug-resistance plasmids were described in this genus as early as the late 1950s (5,6).

A new family of ESBLs, displaying greater affinity for cefotaxime than ceftazidime (CTX-M), has appeared in various countries. In Argentina, CTX-M-2 is the most prevalent ESBL (7) and has been reported in several enterobacteria (8-10). Although very common in nosocomial pathogens, it has also been recovered from other enteric microorganisms (11). We describe our analysis of the first *Shigella sonnei* isolate resistant to cefotaxime (CTX) but not to ceftazidime.

## The Study

The isolate was from stool samples from a 6-month-old girl, seen in the outpatient clinic of Hospital SAMIC Oberá in northern Argentina. The child had vomiting and laboratory-confirmed bloody diarrhea approximately 20 days after a 1-week hospital stay for diarrhea and primary malnutrition. During the hospital stay, she received gentamicin for 1 week, but no bacterial enteropathogen was isolated.

The child lived in Oberá, a subtropical area in Misiones that has no running water. In this region, *Shigella* spp. is three times more prevalent than *Salmonella* spp. and other enteropathogens as the cause of pediatric diarrhea, and *S. sonnei* resistance to ampicillin and TMP-SMX is approximately 43% and 74%, respectively.

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*S. sonnei* was isolated on eosin-methylene blue agar plates. The isolate did not produce gas and was hydrogen sulfide-negative and nonmotile. In triple sugar iron agar, it was negative for citrate, phenylalanine deaminase, and indol production. It was methyl-red positive, Voges-Proskauer negative, ornithine decarboxylase positive, arginine dihydrolase negative, lysine decarboxylase negative, and urease (Christensen) negative. (All media were from Britania, Argentina.) Confirmatory serotyping was carried out with antisera from the Instituto Nacional de Microbiología Dr. Carlos Malbran.

Confirmatory susceptibility tests followed conventional methods (12,13). Briefly, MICs were determined by the agar dilution method, using Mueller-Hinton agar (Britania) and inoculums of $10^4$ CFU per spot; plates were incubated 18 hours at 35°C. *Escherichia coli* ATCC 25922 and *E. coli* ATCC 35218 were included as quality controls. Antibiotics tested were ampicillin, ampicillin + clavulanate (CLA), cefoxitin, cefotaxime (CTX), CTX + CLA (CTX/CLA), ceftazidime, and ceftazidime + CLA (ceftazidime/CLA); a fixed concentration of 4 µg/mL lithium CLA was used when combined with beta-lactam drugs. Antimicrobial drugs were provided by Argentia, Argentina (Ampicillin, CTX), Sigma Chemical Co., St. Louis, MO, USA (ceftazidime), Roemmers, Argentina (CLA) and Merck Sharp & Dohme, Argentina (cefoxitin). MICs were as follows: ampicillin, >1,024 µg/mL; ampicillin + CLA 16 µg/mL; cefoxitin, 2 µg/mL; CTX, 16 µg/mL; CTX/CLA, ≤ 0.063 µg/mL; ceftazidime, 1 µg/mL; and ceftazidime/CLA, ≤ 0.063 µg/mL.

Resistance to gentamicin, amikacin, and TMP-SMX was detected by agar diffusion (13); quality controls also included *E. faecalis* ATCC 29212.

## Conclusions

The presence of an ESBL was confirmed by microbiologic and biochemical tests using different third-generation cephalosporins as substrates for the enzymes present in bacterial sonicates and an iodometric detection system (9). A microbiologic confirmation test for ESBLs was performed according to recommendations of the National Committee for Clinical Laboratory Standards for *E. coli* and *Klebsiella* spp. (13). After isoelectric focusing (9), crude bacterial extracts rendered two bands that hydrolyzed 500 µg/mL ampicillin

(isoelectric points 5.4 and 8.2), the latter also active on 1,000 µg/mL ceftriaxone. As this enzyme was likely CTX-M-2 (the band comigrated with authentic CTX-M-2 samples) and the first probably corresponded to TEM-1 (or another related enzyme), plasmid DNA obtained by the method of Birnboin and Doly (14) was used as the template for polymerase chain reaction amplification, with specific primers for CTX-M-2 ($bla_{\text{CTX-M-2}}$ I: 5'-TTAATGATGACTCAGAGCATTC-3'; $bla_{\text{CTX-M-2}}$ II: 5'-GATACCTCGCTCCATTTATTG-3') and TEM-1 ($bla_{\text{TEM-1}}$ I: 5'-ATAAAATTCTTGAAGACGAAA-3'; $bla_{\text{TEM-1}}$ II 5'-GACAGTTACCAATGCTTAATCA-3'). Two fragments of 0.9 kbp and 1.2 kbp were obtained; they showed 100% agreement with theoretical and experimentally obtained fragments from bona fide CTX-M-2 and TEM-1 producing strains, respectively (8,10).

No resistant *Shigella* was isolated from the other 124 pediatric patients admitted that month for nonsurgical (85 patients) or surgical reasons (29) nor was a nosocomial outbreak caused by a third-generation cephalosporin-resistant enterobacteria detected. The patient recovered. The isolated microorganism was not the likely cause for the patient's first hospitalization as it could not be isolated at that time. Likely alternatives for its acquisition are 1) intestinal selection or acquisition of a resistant enterobacterium and in vivo transference to a freshly acquired *Shigella* or 2) direct acquisition of the resistant strain from contaminated water (not sustained, as no other resistant *Shigella* was obtained from the same community in the following year).

These findings, which may portend the spread of serious resistance in *Shigella* throughout Argentina and beyond, suggest the need for susceptibility testing of all *Shigella* spp. whenever financially feasible.

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## References

1. Mates A, Eyny D, Philo S. Antimicrobial resistance trends in Shigella serogroups isolated in Israel, 1990-1995. Eur J Clin Microbiol Infect Dis 2000;19:108-11.
2. Prats G, Mirelis B, Llovet T, Muñoz C, Miro E, Navarro F. Antibiotics resistance trends in enteropathogenic bacteria isolated in 1985-1987 and 1995-1998 in Barcelona. Antimicrob Agents Chemother 2000;44:1140-5.
3. Replogle ML, Fleming DW, Cieslak PR. Emergence of antimicrobial-resistant shigellosis in Oregon. Clin Infect Dis 2000;30:515-9.
4. Ahamed J, Kundu M. Molecular characterization of the SHV-11 ß-lactamase of *Shigella dysenteriae.* Antimicrob Agents Chemother 1999;43:2081-3.
5. Watanabe T, Fukasawa T. Episome-mediated transfer of drug resistance in *Enterobacteriaeceae*. J Bacteriol 1960;81:669-78.
6. Akiba T, Koyama T, Isshiki Y, Kimura S, Fukushima T. Studies on the mechanism of development of multiple drug-resistant *Shigella* strains. Nihon Iji Shimpo 1960;1866:45-50.
7. Radice M. Mecanismos de resistencia a antibióticos ß-lactámicos: Análisis de las ß-lactamasas de espectro expandido en *Escherichia coli* [dissertation]. Buenos Aires: University of Buenos Aires; 1998.
8. Bauernfeind A, Casellas JM, Goldberg M, Holley M, Jungwirth R, Mangold P, et al. A new plasmidic cefotaximase from patients infected with *Salmonella typhimurium*. Infection 1992;20:158-63.
9. Rossi A, Lopardo H, Woloj M, Picandet AM, Mariño M, Galas M, et al. Non-typhoid *Salmonella* spp. resistant to cefotaxime. J Antimicrob Chemother 1995;36:697-702.
10. Power P, Radice M, Barberis C, de Mier C, Mollerach M, Maltagliatti M, et al. Cefotaxime-hydrolysing ß-lactamases in *Morganella morganii*. Eur J Clin Microbiol Infect Dis 1999;18:743-7.
11. Rossi A, Galas M, Binztein N, Rivas M, Caffer MI, Corso A, et al. Unusual multiresistant *Vibrio cholerae* O1 El Tor in Argentina. Lancet 1993;342:1172-3.
12. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard M7-A4, 4th edition, Vol. 19, No. 1. Wayne (PA): NCCLS; 1999.
13. National Committee for Clinical Laboratory Standards. Zone diameter interpretative standards and equivalent minimum inhibitory concentration (MIC) breakpoints for *Enterobacteriaceae*. Approved Standard M-2-A6, 6th edition, Vol. 19, No. 1. Wayne (PA): NCCLS; 1999.
14. Birnboin HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 1979;7:1513.

# Expanding Drug Resistance through Integron Acquisition by IncFI Plasmids of *Salmonella enterica* Typhimurium

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We conducted a 30-year retrospective analysis of IncFI plasmids from *Salmonella enterica* serotype Typhimurium. These plasmids have been associated with the emergence of epidemic clones of multidrug-resistant *Salmonella*. Molecular and genetic evidence indicates that IncFI plasmids are evolving through sequential acquisition of integrons carrying different arrays of antibiotic-resistance genes.

Multidrug resistance (MDR) in *Salmonella enterica* serotype Typhimurium has substantial public health and economic impact. Recent epidemiologic data highlighted broadening of antibiotic resistance spectra in this serotype (1), along with worldwide distribution of MDR *S.* Typhimurium clones (2). These clones may have been selected by indiscriminate use of antimicrobial agents in clinical practice and animal husbandry. Understanding the genetic mechanism(s) responsible for acquisition and spread of antibiotic resistance could facilitate development of effective prevention and control strategies.

Acquisition of mobile genetic elements encoding multiple antibiotic resistance genes is the main mechanism for short-term accumulation of resistance determinants in bacterial genomes. Resistance (R-) plasmids of the IncFI incompatibility group have been associated with emergence of MDR *S. enterica* serotypes (3). IncFI plasmids were found most often in Middle Eastern isolates of *S.* Typhimurium, but similar plasmids were also identified in *Salmonella* serotypes from Africa, Europe, and North America (3). Convincing epidemiologic and genetic data support the view that acquisition of IncFI plasmids contributed in the mid-1970s to epidemic spread of *S. enterica* serotype Wien. The IncFI-carrying salmonellae were responsible for protracted outbreaks of severe pediatric infections that were difficult to treat because of the wide spectrum of antimicrobial resistance (4). Most of IncFI plasmids isolated in the early 1970s from epidemic *S.* Typhimurium and *S.* Wien strains conferred resistance to ampicillin (Ap), chloramphenicol (Cm), streptomycin (Sm), spectinomycin (Sp), sulphonamides (Su), tetracycline (Tc), mercuric ions (Hg), and occasionally kanamicin (Km) (3). A few IncFI plasmids, particularly those from Mediterranean *S.* Wien isolates, lacked R-SmSpSu determinants (5). After the *S.* Wien epidemics waned in the early 1980s, outbreaks linked to IncFI-carrying *Salmonella* strains have occasionally been reported (6).

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We have recently shown that 37 MDR *S.* Typhimurium strains isolated from 1996 to 1997 from sporadic cases of pediatric gastroenteritis harbored conjugative IncFI plasmids of around 120 kilobases (kb) with the common profile R-ApCmKmSmSuTp (7). Conjugal transfer of a prototypic IncFI plasmid (designated IncFI/97) from the type strain ST366 to *Escherichia coli* K-12 was associated with transmission of the entire resistance cluster. We demonstrated that most of the antibiotic-resistance genes in this plasmid were located in integrons (7). These genetic elements encode a site-specific recombinase (integrase) capable of capturing resistance genes, which are assembled as gene cassettes controlled by the strong promoter *P*ant. Gene cassettes are flanked by two conserved segments, the 5'CS and 3'CS, encoding the integrase (*intI1*) and the sulphonamide resistance determinant (*sul1*), respectively (8).

Two integrons were identified in IncFI/97: In-t1 (R-CmKmSu), carrying the *aadB* and *catB3* genes, and In-t2 (R-ApSmSu), carrying the *oxa1* and *aadA1* genes (7). Integrons are now recognized as the main genetic vehicles of antibiotic resistance in gram-negative bacteria (9), but it remains unclear to what extent these elements contributed to the expansion of the antibiotic resistance repertoire of IncFI plasmids.

## The Study

To gain insight into the evolution of the resistance determinants on IncFI plasmids, we compared the R-profile and the integron content of IncFI/97 with that of five IncFI plasmids representative of those identified in epidemic *S.* Typhimurium and *S.* Wien from human sources during 1970-1978 (Table). Prototypic plasmids were transferred to *E. coli* K-12 either by conjugation (11) or conventional electrotransformation before antibiotic-susceptibility testing by the disc-diffusion method (12). All but one plasmid showed a common core of antibiotic resistance (R-ApCmTcHgKm), while additional resistance to Sm, Sp, and Su was observed in plasmids IncFI/97, NTP101, pZM3, and TP181.

The IncFI collection was screened for the presence of the integrase gene *intI1*. Six IncFI plasmids were purified from

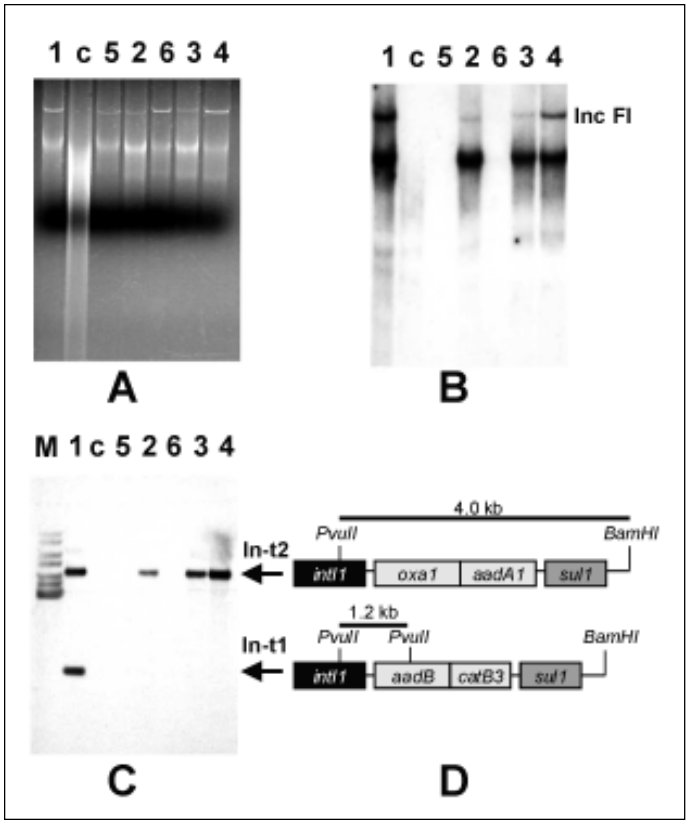
Table. Designation and relevant characteristics of representative IncFI plasmids used for genetic analysis of integrons

| No. | Plasmid | Host strain | Origin, year[a] | Resistance pattern[b] | Tra[c] | kb | Reference |
|---|---|---|---|---|---|---|---|
| 1 | IncFI/97 | ST 366 | Albania, 1997 | Ap Cm Sm Sp Su Tc Hg Km Tp | + | 120 | 7 |
| 2 | NTP101 | ST DT 208 | England, 1974 | Ap Cm Sm Sp Su Tc Hg | - | 135 | 3 |
| 3 | pZM3 | SW 20 | Algeria, 1970 | Ap Cm Sm Sp Su Tc Hg Km | + | 165 | 10 |
| 4 | TP181 | ST DT 208 | Iran, 1975 | Ap Cm Sm Sp Su Tc Hg Km | + | 165 | 3 |
| 5 | pZM111 | SW WZM111 | Italy, 1978 | Ap Cm Hg Km Gm | + | 130 | 5 |
| 6 | PZM61 | SW WZM6 | Italy, 1974 | Ap Cm Tc Hg Km | - | 145 | 11 |

[a] Year of isolation.
[b] Abbreviations: Ap = ampicillin; Cm = chloramphenicol; Sm = streptomycin; Sp = spectinomycin; Su = sulfonamides; Tc = tetracycline; Hg = mercuric ions; Km = kanamycin; Tp = trimethoprim; Gm = gentamicin.
[c] Autotransferring properties.

*E. coli* K-12 recipients (13), analyzed by agarose gel electrophoresis (Figure 1A), and hybridized with a Tn*21*-derived *intI1* probe. The class 1 integrase gene(s) was located on IncFI plasmids carrying the R-SmSpSu determinants, including isolates from the 1970s as well as more recent ones (Figure 1B). This is the first demonstration of integron presence in MDR *Salmonella* isolates traced to the early 1970s.

To define the structure of integrons carried by IncFI plasmids, Southern blot hybridization of *PvuII-BamHI*-digested DNA with the *intI1* probe was performed. The In-t2 integron was conserved in all the integrase-positive plasmids, including IncFI/97, NTP101, pZM3, and TP181 (Figure 1C). This observation indicates that In-t2 has been maintained unaltered in IncFI plasmids for nearly 30 years. IncFI/97 is likely to be a new plasmid variant, since it contains a second integron, designated In-t1 (7), which appears as a smaller band on the hybridization profile (Figure 1C). Sequence data confirmed the structural organization of the integrons (Figure 1D).




To further explore the structural identity between recent and ancestral IncFI plasmids, restriction enzyme analysis of plasmid DNAs was performed with *Sal*I and *Sph*I endonucleases. Based on electrophoretic patterns (Figure 2A), moderate similarity between IncFI/97 and IncFI plasmids isolated in the early 1970s can be recognized. The presence of similar-sized restriction fragments is particularly evident for plasmids carrying the In-t2 integron, including IncFI/97, NTP101, pZM3, and TP181. This finding suggests that the evolution of MDR-IncFI plasmids may have occurred through the sequential acquisition of integrons.

To investigate the potential inheritance of genetic elements from ancestral to recent IncFI plasmids, we probed our collection for the presence of additional resistance determinants. Plasmid pZM3 was previously described to contain an IS*15*-like composite element (IS*1936*-Km$^r$) conferring Km-resistance (10). This structure is located within a Tn*21* derivative (Tn*1935*), which also carries an Ap-resistance (*oxa-1*) gene upstream of the resident Sm-resistance (*aadA1*) gene of Tn*21*, in addition to the distal Hg-resistance determinant (10). Although the manner by which new antibiotic resistance genes are acquired by Tn*21*-like transposons was unclear at the time pZM3 was characterized (10), acquisition of *oxa-1* likely occurred by integrase-mediated recombination. This event led to the assembly of In-t2 from its putative precursor, the Tn*21*-borne In2 integron

Figure 1. Structural organization of integrons carried by IncFI plasmids. Numbers above each lane indicate plasmid reference numbers as defined (Table). Lane C shows the plasmidless *E. coli* K-12 strain CSH26 (11). A: agarose (0.8%) gel electrophoresis in 1x Tris-borate-EDTA buffer of plasmid DNA extracted from *E. coli* K-12 exconjugants. DNA was stained with ethidium bromide and visualized under UV light. B: Southern blot hybridization of plasmids shown in panel A with the *intI1* probe. C: Southern blot hybridization with the *intI1* probe of *PvuII-BamHI* double-digested DNA from *E. coli* K-12 exconjugants. The DNA was extracted as described (7). The *intI1* probe was amplified with primers corresponding to nt 4680-4700 and nt 5252-5232 of the released Tn*21* sequence (GenEMBL accession no. AF071413). Plasmid pACYC184::Tn*21* (10) was used as the template. The probe was labeled with [α-$^{32}$P]dCTP by using a random priming kit (GibcoBRL). Chromosomal DNA fluoresced because of weak trapping of partially degraded plasmids. D: schematic representation of In-t2 and In-t1 integrons. The thick bars above each structure represent the DNA fragments recognized by the *intI1* probe. The 4.0-kb *PvuII-BamHI* fragment (In-t2) encompasses the *intI1* gene segment downstream of the conserved *PvuII* site (0.6 kb), the *oxa1* and *aadA1* gene cassettes (2.2 kb), and the 3'-CS comprising the *sul1* gene upstream of the *BamHI* site (1.2 kb) (7,8). The 1.2-kb *PvuII-BamHI* fragment (In-t1) encompasses the *intI1* gene segment and part of the *aadB* gene cassette (7).

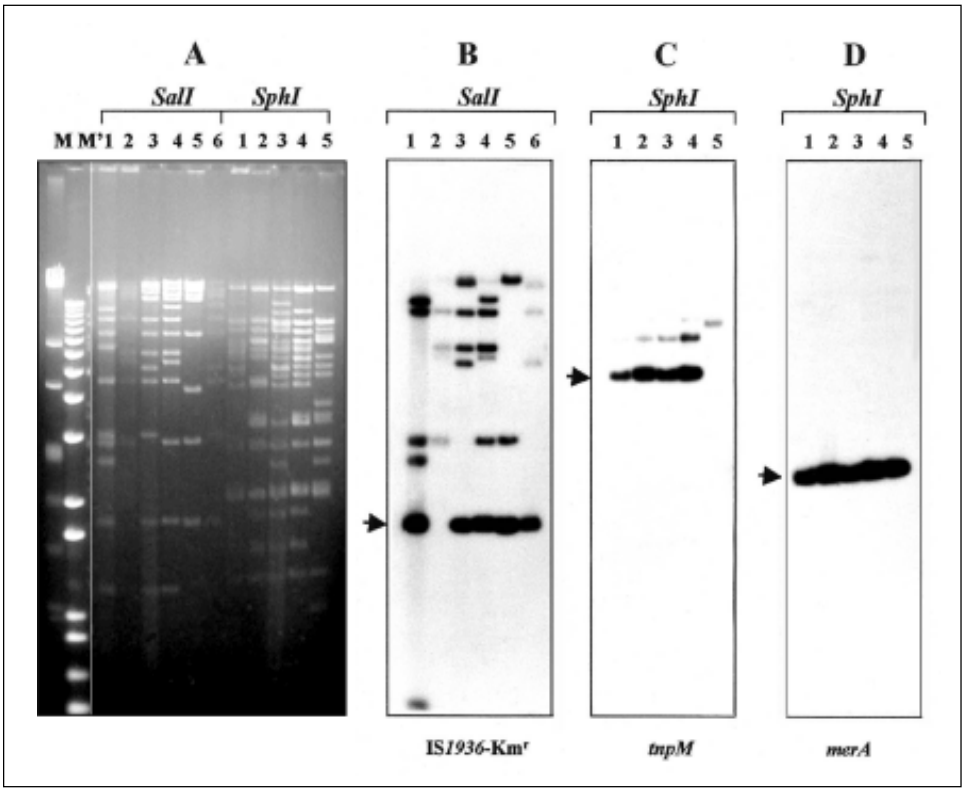



Figure 2. Restriction enzyme analysis and detection of common genetic elements in IncFI plasmids. Numbers above each lane indicate plasmid reference numbers as defined (Table). M and M' are *Hin*dIII-digested lambda DNA and 1-kb plus DNA ladder (GibcoBRL), respectively. A: agarose (0.8%) gel electrophoresis in 1x Tris-borate-EDTA buffer of plasmids digested with restriction endonucleases *SalI* and *SphI*. DNA was stained with ethidium bromide and visualized under UV light. B: Southern blot hybridization of *SalI*-digested plasmids with the IS*1936*-Km$^r$ probe purified as 1.8-kb *SalI* fragment from plasmid pACYC184::Tn*1935* (10). C and D: Southern blot hybridizations of *SphI*-digested plasmids with the *tnpM* and *merA* probes, respectively. The *tnpM* probe was amplified with primers corresponding to nt 3689-3709 and nt 4070-4050 of the released Tn*21* sequence (GenEMBL accession no. AF071413). For synthesis of the *merA* probe, primers annealing to nt 17224-17249 and nt 17931-17907 of the Tn*21* sequence were used. Plasmid pACYC184::Tn*21* (10) was used as the template. Probes were labeled with [α-$^{32}$P]dCTP by using a random priming kit (GibcoBRL, Rockville, MD). The arrows point to predicted hybridization fragments.

(9). Therefore, we tested IncFI plasmids for the presence of the IS*1936*-Km$^r$ element, the transposase (*tnp*) machinery, and the Hg-resistance (*mer*) locus. All but one of the IncFI plasmids carry the IS*1936*-Km$^r$ element (Figure 2B). NTP101 is the only exception, consistent with the Km-susceptible phenotype conferred by this plasmid. Additional bands, some of which were shared by different IncFI plasmids, were also detected, likely as a result of cross-hybridization of the probe with IS*15*-like elements located on these replicons. Plasmids IncFI/97, NTP101, PZM3, and TP181 were also positive for the presence of the 4.4-kb *SphI* band (Figure 2C), which is predicted to contain the *tnpM* (regulator), the *tnpR* (resolvase), and part of the *tnpA* (transposase) genes of Tn*21*-like transposons (GenEMBL accession no. AF071413).

Furthermore, the IncFI plasmids recognized by the *merA* probe also showed a common *SphI* band of 2.1 kb, providing evidence for a structural conservation of the *mer* operon (Figure 2D). Plasmid pZM61 showed hybridization patterns indistinguishable from those of pZM111 (data not shown).

Taken together, the above data indicate that IncFI/97 shares antibiotic resistance determinants with the ancestor plasmids pZM3, NTP101, and TP181, suggesting that MDR developed through independent acquisition of integrons within the structurally conserved IncFI plasmid scaffold. The evolutionary story of these plasmids combines the maintenance of indigenous antibiotic resistance genes with the acquisition of new determinants, leading to accumulation of multiple resistance mechanisms. For example, the *oxa1* gene cassette was acquired despite the presence of the TEM-type beta-lactamase encoded by the preexisting Tn*A* transposon (11). Likewise, acquisition of In-t1 introduced a third aminoglycoside-resistance determinant (*aadB*) in addition to the resident IS*1936*-Km$^r$ (*aph*) element (10) and the In-t2-encoded *aadA1* gene. Along the same lines, resistance to chloramphenicol was determined by both the *catB3* gene cassette of In-t1 and by the resident *cmlA* gene. Besides increasing the level of antibiotic resistance due to the expression of multiple detoxification mechanisms, acquisition of new resistance genes is also expected to enlarge the spectrum of resistance. Our results (not shown) indicate that In-t2 serves as a vehicle for the *oxa-1* gene, extending beta-lactam resistance to ureidopenicillins, and of the *aadA1* gene, conferring resistance to streptomycin and spectinomycin. Likewise, the In-t1-carried *aadB* gene broadens the aminoglycoside resistance to tobramycin and gentamicin.

## Conclusions

Our retrospective investigation provides novel evidence for the involvement of integrons in the development of MDR through sequential acquisition of new resistance determinants within IncFI plasmids. This family of replicons could play a role in the future development of resistance in *S.* Typhimurium and, more generally, in Enterobacteriaceae. A dramatic outcome of this evolutionary pathway may be predicted from the intrinsic structural and functional properties of mobile DNA elements found on these R-plasmids. Integrons provide a unique mechanism for the recruitment of additional resistance genes. Moreover, spreading resistance genes among different replicons is favored by the location of integrons within transposable elements. Finally, horizontal transmission is expected because of the conjugative or mobilizable properties of IncFI carriers, which enables them to traverse species and genus boundaries. Future success in controlling resistance depends, at least in part, on a complete understanding of the mechanisms involved in dissemination. A systematic search

for integrons and transposable elements could provide a useful genetic basis for MDR in human and animal *S.* Typhimurium isolates.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## References


1. Glynn MK, Bopp C, Dewitt W, Dabney P, Mokhtar M, Angulo FJ. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. N Engl J Med 1998;338:1333-8.
2. Prager R, Liesegang A, Rabsch W, Gericke B, Thiel W, Voigt W, et al. Clonal relationship of *Salmonella enterica* serovar Typhimurium phage type DT104 in Germany and Austria. Zentralb Bakteriol 1999;289:399-414.
3. Anderson ES, Threlfall EJ, Carr JM, McConnell MM, Smith HR. Clonal distribution of resistance plasmid-carrying *Salmonella typhimurium*, mainly in the Middle East. J Hyg (Lond) 1977;79:425-48.
4. Domart A, Robineau M, Stroh A, Dubertret LM, Modai J. Septicemia due to *Salmonella wien*. Diagnostic, therapeutic and epidemiologic problems. Ann Med Interne (Paris) 1974;125:915-8.
5. Casalino MA, Comanducci A, Nicoletti M, Maimone F. Stability of plasmid content in *Salmonella wien* in late phases of the epidemic history. Antimicrob Agents Chemother 1984;25:499-501.
6. Frost JA, Rowe B, Ward LR, Threlfall EJ. Characterization of resistance plasmids and carried phages in an epidemic clone of multi-resistant *Salmonella typhimurium* in India. J Hyg (Lond) 1982;88:193-204.
7. Tosini F, Visca P, Luzzi I, Dionisi AM, Pezzella C, Petrucca A, Carattoli A. Class 1 integron-borne multiple-antibiotic resistance carried by IncFI and IncL/M plasmids in *Salmonella enterica* serotype Typhimurium. Antimicrob Agents Chemother 1998;42:3053-8.
8. Hall RM, Collis CM. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol Microbiol 1995;15:593-600.
9. Liebert CA, Hall RM, Summers AO. Transposon Tn*21*, a flagship of the floating genome. Microbiol Mol Biol Rev 1999;63:507-22.
10. Colonna B, Bernardini M, Micheli G, Maimone F, Nicoletti M, Casalino M. The *Salmonella wien* virulence plasmid pZM3 carries Tn*1935*, a multiresistance transposon containing a composite IS*1936*-kanamycin resistance element. Plasmid 1988;20:221-31.
11. Maimone F, Colonna B, Bazzicalupo P, Oliva B, Nicoletti M, Casalino MA. Plasmids and transposable elements in *Salmonella wien*. J Bacteriol 1979;139:369-75.
12. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disc susceptibility tests. M2A2. Villanova (PA): The Committee; 1992.
13. Kado CI, Liu S. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 1981;145:1365-73.

# Decreased Susceptibility to Ciprofloxacin in *Salmonella enterica* serotype Typhi, United Kingdom

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In 1999, 23% of *Salmonella enterica* serotype Typhi isolates from patients in the United Kingdom exhibited decreased susceptibility to ciprofloxacin (MIC 0.25-1.0 mg/L); more than half were also resistant to chloramphenicol, ampicillin, and trimethoprim. Increasing numbers of treatment failures have been noted. Most infections have been in patients with a recent history of travel to India and Pakistan.

*Salmonella enterica* serotype Typhi is endemic in developing countries in Africa, South and Central America, and the Indian subcontinent, with an estimated incidence of 33 million cases each year (1). By contrast, in developed countries such as the United Kingdom or the USA, incidence is much lower, and most cases are in travelers returning from endemic areas. For example, 150 to 300 cases occur each year in the U.K., at least 70% in patients with a history of recent foreign travel.

For patients with typhoid fever, administration of an effective antibiotic should begin as soon as clinical diagnosis is made, without recourse to results of antimicrobial sensitivity tests. From 1948 to the mid-1970s, chloramphenicol was the first-line drug of choice, and in developed countries its use resulted in a reduction in mortality rates from 10% to <2%. After extensive outbreaks of typhoid fever occurred in Mexico and India in the early and mid-1970s, in which epidemic strains were resistant to chloramphenicol (2,3), the efficacy of this antimicrobial agent was in doubt.

Alternative drugs for typhoid fever are ampicillin and trimethoprim. However, following outbreaks in the Indian subcontinent, the Arabian Gulf, the Philippines, and South Africa in the late 1980s and early 1990s, in which causative strains were resistant to ampicillin and trimethoprim in addition to chloramphenicol, the efficacy of these antimicrobial agents has also been impaired (4).

The Laboratory of Enteric Pathogens of the Public Health Laboratory Service of England and Wales is the reference center in the U.K. for strains of *S.* Typhi. Strains are identified by Vi-phage typing; all strains are tested with an agar dilution method for resistance to a panel of antimicrobial drugs. The final plate concentrations for selected antimicrobial drugs were chloramphenicol 8 mg/L, ampicillin 8 mg/L, trimethoprim 2 mg/L, nalidixic acid 16 mg/L, ciprofloxacin 0.125 mg/L, ceftriaxone 1 mg/L, and cefotaxime 1 mg/L.

For isolates resistant to ciprofloxacin at 0.125 mg/L, full MICs are determined either by incorporating doubled concentrations of the antimicrobial agent into the agar substrate or by E-test. All isolates resistant to ciprofloxacin at 0.125 mg/L were also resistant to nalidixic acid at 16 mg/L. In contrast, isolates sensitive to nalidixic acid at 16 mg/L had MICs to ciprofloxacin of <0.025 mg/L. All strains with resistance to chloramphenicol, ampicillin, trimethoprim, or nalidixic acid/ciprofloxacin were tested for the ability to transfer these resistances to a drug-sensitive strain of *Escherichia coli* K12. Resultant resistance plasmids were characterized by incompatibility grouping and agarose gel electrophoresis after extraction of plasmid DNA from donor strains of *S.* Typhi and recipient strains of *E. coli* K12.

From 1978 to 1985, resistance to chloramphenicol was identified in 11 (0.47%) of 2,356 strains studied (5); therefore, chloramphenicol remained the first-line drug for typhoid fever before results of laboratory sensitivity tests became available. From 1986 to 1989, chloramphenicol resistance increased threefold: 12 (1.5%) of 790 isolates were resistant. However, this increase was not considered sufficient to change recommendations about therapy. In 1990, there was a dramatic change, with 20% of 248 isolates resistant to chloramphenicol; most were also resistant to ampicillin and trimethoprim (6). In 1991, because of this increased chloramphenicol resistance, ciprofloxacin was recommended as an alternative for patients with a history of recent travel to epidemic areas (7).

From 1990 to 1999, 151 to 291 (mean 210) patients per year in the U.K. had typhoid fever. The incidence of multidrug resistance (MDR) to chloramphenicol, ampicillin, and trimethoprim increased from 21% in 1991 to 36% in 1994, declined to 13% in 1997, and then increased to 26% in 1999 (Table 1). More than 90% of patients infected with MDR strains had recently returned from the Indian subcontinent, particularly Pakistan and India. The reasons for the decline in MDR strains in 1997 followed by the return to 1996 levels in 1998 and 1999 are not known but may be related to changes in climatic conditions in the Indian subcontinent in the mid-1990s, followed by reestablishment of MDR strains in different parts of India in the late 1990s. Epidemiologic investigations to test these hypotheses are in progress.

In the early 1990s, the most common Vi-phage type in MDR strains was phage type M1, and almost all patients

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Table 1. Incidence of multidrug resistance and decreased susceptibility to ciprofloxacin in *Salmonella enterica* serotype Typhi, U.K., 1990–1999

| Year | Strains[a] | MDR[b] no. (%) | Decreased susceptibility to $CP_L$ no. (%) | $CP_L$ strains also resistant to: C | A | Tm | Ct | Cf |
|---|---|---|---|---|---|---|---|---|
| 1990 | 248 | 50 (20) | 0 (0) | 0 | 0 | 0 | 0 | 0 |
| 1991 | 226 | 48 (21) | 2 (0.9) | 1 | 1 | 1 | 0 | 0 |
| 1992 | 204 | 49 (24) | 1 (0.5) | 0 | 0 | 0 | 0 | 0 |
| 1993 | 194 | 49 (25) | 1 (0.5) | 1 | 1 | 1 | 0 | 0 |
| 1994 | 259 | 94 (36) | 5 (2) | 5 | 5 | 5 | 0 | 0 |
| 1995 | 291 | 100 (34) | 8 (3) | 5 | 5 | 5 | 0 | 0 |
| 1996 | 210 | 52 (25) | 11 (7) | 7 | 7 | 7 | 0 | 0 |
| 1997 | 174 | 22 (13) | 9 (5) | 6 | 6 | 6 | 0 | 0 |
| 1998 | 151 | 34 (23) | 32 (21) | 19 | 19 | 19 | 0 | 0 |
| 1999 | 179 | 47 (26) | 42 (23) | 25 | 25 | 25 | 0 | 0 |

[a]Strains referred to Laboratory of Enteric Pathogens.
[b]MDR = multidrug resistant (to chloramphenicol, ampicillin, and trimethoprim). Resistance symbols: C, chloramphenicol; A, ampicillin; Tm, trimethoprim; $CP_L$, ciprofloxacin (MIC 0.25-1.0 mg/L); Ct, ceftriaxone; Cf, cefotaxime. Percentages of total isolates in parentheses.

infected with strains of this phage type had acquired the infections in Pakistan. The last isolations of MDR phage type M1 in the U.K. were in 1994 (4). Since 1993, the most common MDR phage type has been E1. Most patients infected with MDR strains of phage type E1 had acquired the infections in India or Pakistan. However, infections were also recorded in patients returning from Bangladesh, Sri Lanka, and Afghanistan. Regardless of phage type, in all MDR strains resistance to chloramphenicol, ampicillin, and trimethoprim has been encoded by plasmids of approximately 100 megadaltons belonging to the $H_1$ incompatibility group.

In 1991, a strain of *S.* Typhi with plasmid-encoded resistance to chloramphenicol, ampicillin, and trimethoprim and with chromosomally encoded resistance to nalidixic acid (MIC 512 mg/L) was isolated from a 1-year-old child who had recently returned from India. The strain also showed a marked decrease in sensitivity to ciprofloxacin (MIC 0.6 mg/L). The patient did not respond to treatment with ciprofloxacin despite serum levels of 1.5 mg/L. In 1995, 8 (3%) of 291 isolates showed decreased sensitivity to ciprofloxacin (MICs 0.38–0.75 mg/L by E-test); 5 were also resistant to chloramphenicol, ampicillin, and trimethoprim. In 1998, 32 (21%) of 151 strains exhibited decreased susceptibility to ciprofloxacin. One patient, a 65-year-old woman who returned from India infected with a strain of phage type E1 (MIC to ciprofloxacin of 1.0 mg/L) did not respond to twice a day treatment with ciprofloxacin, 400 mg intravenously. After 5 days, treatment was changed to amoxicillin and ceftriaxone. Within 3 days, the patient's condition improved, and after a further 5 days she was apyrexial (8).

*S.* Typhi with decreased susceptibility to ciprofloxacin increased to 23% in the U.K. in 1999 (Table 1). All strains with decreased sensitivity to ciprofloxacin were also resistant to nalidixic acid (MIC 512 mg/L). The predominant phage types have been E1 (81% of cases) and E9 (4%). However, strains of phage types C2, E7, M1, untypeable Vi (UVS), and Vi-negative have also been identified. Most patients had recently returned from India or Pakistan. However, in 1998 and 1999, strains with decreased susceptibility to ciprofloxacin were also isolated from travelers returning from Sri Lanka, Nepal, Bangladesh, and Thailand (Table 2). Furthermore, in both years >50% of isolates with decreased susceptibility to ciprofloxacin were also MDR (Table 1). In 1999, at least 10 patients infected with strains with decreased susceptibility to ciprofloxacin did not respond to treatment with fluoroquinolone antimicrobials. In such cases, ceftriaxone was the most frequently used alternative. In contrast to resistance to chloramphenicol, ampicillin, and trimethoprim, resistance to ciprofloxacin has been chromosomally encoded in all isolates with decreased sensitivity to this antimicrobial agent.

Since 1993, strains of *S.* Typhi with decreased susceptibility to ciprofloxacin have been isolated with increasing frequency in Vietnam (9). In 1997, >6,000 cases occurred in an extensive epidemic in Tajikistan of nalidixic acid-resistant *S.* Typhi with decreased susceptibility to ciprofloxacin (10). The epidemic strain was untypeable with the Vi typing phages but had a pulsed-field profile indistinguishable from that of isolates of MDR Vi-phage type E1 from patients infected in India (11). In both Vietnam and Tajikistan, treatment failures with fluoroquinolone antibiotics have been noted.

The accepted British Society for Antimicrobial Chemotherapy and National Committee for Clinical Laboratory Standards' zone size equivalents for resistance to ciprofloxacin in disc diffusion tests are 2 mg/L and 4 mg/L, respectively, for Enterobacteriaceae. However, testing for resistance at these levels could result in decreased susceptibilities not being detected. As all strains with decreased susceptibility to ciprofloxacin have also been resistant to nalidixic acid, we suggest that the latter antimicrobial agent be included in the panel of drugs used for sensitivity testing. If resistance to nalidixic acid is detected, full MICs to ciprofloxacin should be performed in the event of treatment failure.

Table 2. Phage types in *Salmonella* Typhi isolates with decreased susceptibility to ciprofloxacin ($C_{pL}$), United Kingdom, 1991–1999

| Year | $C_{pL}$ | Phage types E1 | M1 | E9 | Others | Country of origin (no.) |
|---|---|---|---|---|---|---|
| 1991 | 2 | 2 | 0 | 0 | 0 | India (1), Nepal (1) |
| 1992 | 1 | 0 | 0 | 0 | 1 (B2) | India (1) |
| 1993 | 1 | 0 | 0 | 0 | 1 (E14) | Bangladesh (1) |
| 1994 | 5 | 5 | 0 | 0 | 0 | India (3), Nepal (1), Bangladesh (1) |
| 1995 | 8 | 6 | 1 | 0 | 1 (D1) | India (4), Pakistan (3), NS[a] (1) |
| 1996 | 11 | 11 | 0 | 0 | 0 | India (9), Pakistan (2) |
| 1997 | 9 | 8 | 0 | 0 | 1(A) | India (5), Pakistan (2), Nepal (1), NS (1) |
| 1998 | 32 | 23 | 0 | 6 | 3 (G2,2;O,1) | Pakistan (14), India (9), Sri Lanka (1), Bangladesh (1), NS (7) |
| 1999 | 42 | 27 | 1 | 3 | 11 (UVS, 7; E7, 1; C2, 1; Vi-negative, 1) | India (31), Pakistan (6), Bangladesh (1), Thailand (1), NS (3) |

[a]NS = details of travel itinerary not provided; UVS = untypeable with the Vi typing phages.

Our findings suggest that strains of *S.* Typhi with decreased sensitivity to ciprofloxacin are now endemic in several countries in the Indian subcontinent and that such strains are increasing in travelers returning to the U.K. Despite the low level of resistance, treatment failures are being increasingly noted. In such cases, possible alternatives such as ceftriaxone or cefotaxime could be considered. In this respect, it is reassuring that all strains of *S.* Typhi so far tested were sensitive to these antimicrobial drugs.

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## References

1. Ivanoff B. Typhoid fever: global situation and WHO recommendations. Southeast Asian J Trop Med Public Health 1995; 26(Suppl 2):1-6.
2. Anderson ES, Smith HR. Chloramphenicol resistance in the typhoid bacillus. BMJ 1972;3:329-31.
3. Paniker CKJ, Vilma KN. Transferable chloramphenicol resistance in *Salmonella typhi*. Nature 1972;239:109-10.
4. Rowe B, Ward LR, Threlfall EJ. Multidrug-resistant *Salmonella typhi*: a worldwide epidemic. Clin Infect Dis 1997;24(Suppl 1): S106-9.
5. Rowe B, Threlfall EJ, Ward LR. Does chloramphenicol remain the drug of choice for typhoid? Epidemiol Infect 1987;98:379-83.
6. Rowe B, Ward LR, Threlfall EJ. Spread of multiresistant *Salmonella typhi*. Lancet 1990;336:1065-6.
7. Rowe B, Ward LR, Threlfall EJ. Treatment of multiresistant typhoid fever. Lancet 1991;337:1422.
8. Threlfall EJ, Ward LR, Skinner JA, Smith HR, Lacey S. Ciprofloxacin-resistant *Salmonella typhi* and treatment failure. Lancet 1999;353:1590-1.
9. Parry C, Wain J, Chinh NT, Vinh Ha, Farrar JJ. Quinolone-resistant *Salmonella typhi* in Vietnam. Lancet 1998;351:1289.
10. Murdoch DA, Banatvala NA, Bone A, Shoismatulloev BI, Ward LR, Threlfall EJ, et al. Epidemic ciprofloxacin-resistant *Salmonella typhi* in Tajikistan. Lancet 1998;351:339.
11. Hampton MD, Ward LR, Rowe B, Threlfall EJ. Molecular fingerprinting of multidrug-resistant *Salmonella enterica* serotype Typhi. Emerg Infect Dis 1998;4:317-20.

# The First Reported Case of California Encephalitis in More Than 50 Years

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A recent case of California encephalitis, a rare mosquito-borne viral disease, represents only the fourth ever reported and the first since the initial three cases in 1945. This case was diagnosed retrospectively on the basis of a rise in antibody titer between acute- and convalescent-phase serum samples.

The arbovirus California encephalitis virus was first isolated in 1943 from mosquitoes collected in Kern County, California (1). Two years later, three human cases of encephalitis were attributed to this new virus (2); all three cases were in residents of Kern County in the Central Valley of California. The best-documented case occurred in a 2-month-old Hispanic boy who had encephalitis and resultant developmental delay. There was strong laboratory evidence confirming infection from the presence of neutralizing antibodies to California encephalitis, but not to St. Louis encephalitis virus or western equine encephalomyelitis virus. Serum from a 7-year-old boy hospitalized with encephalitis also had neutralizing antibodies to California encephalitis. Serologic tests were inconclusive in a third possible case in a 22-year-old agricultural worker with mild encephalitis; neutralizing antibodies against both California encephalitis and St. Louis encephalitis were detected.

Since the original virus was isolated, other viruses have been isolated that are closely related to California encephalitis. This group of related viruses is now classified as the California serogroup, one of 16 serogroups within the genus *Bunyavirus*, family Bunyaviridae. Several other human pathogens (e.g., Jamestown Canyon virus, La Crosse virus, and Tahyna virus) also belong to the California serogroup. Little human disease was associated with these viruses until 1960, but now California serogroup virus infections are the most commonly reported cause of arboviral encephalitis in the United States. From 1996 to 1998, approximately three times as many reported human cases of arboviral encephalitis were caused by California serogroup viruses as were reported for western equine encephalomyelitis virus, St. Louis encephalitis, and eastern equine encephalomyelitis viruses combined (3). However, since the three original cases from California, no further cases of human disease caused by the prototype California encephalitis had been reported (4). Campbell et al. summarized results of surveys for human antibodies to California serogroup viruses in California (5).

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## Case Report

In June 1996, a 65-year-old man who lived in Marin County, California, became ill with blurred vision and dizziness. Eight days after the onset of symptoms, he visited his primary physician. A physical examination was remarkable only for nystagmus. Laboratory studies included leukocytes $8.2 \times 10^3$, hematocrit 45.8%, and a chemistry panel that was normal except for phosphorus 4.5 mg/dL (normal range 2.7-4.4 mg/dL), cholesterol 265 mg/dL (normal range 125-200 mg/dL), and high-density lipoprotein cholesterol 67 mg/dL (normal range 35-60 mg/dL). A magnetic resonance image and arteriogram were normal. One month after the initial visit, the patient no longer complained of blurred vision or vertigo, and nystagmus had disappeared. Two years after the episode, he had no neurologic sequelae.

The patient lived in a suburban area of Marin County, approximately 1 km from a large brackish marsh bordering San Francisco Bay. He had traveled outside the United States the previous February, when he had visited Egypt and several Caribbean islands. He had not traveled outside California between this time and the onset of his illness in June, 4 months later.

An acute-phase serum specimen was sent to the Viral and Rickettsial Disease Laboratory, California Department of Health Services. Indirect immunofluorescence antibody tests were negative for St. Louis encephalitis and Western equine encephalomyelitis virus, as were serum samples taken 8 and 16 days after onset of illness. However, when this series of samples was tested by neutralization test 2 years later at the Arbovirus Research Unit of the University of California Center for Vector-Borne Disease Research as part of a retrospective study of arboviruses, the 8-day sample showed an antibody titer of 1:80 and the 16-day sample an antibody titer $\leq$ 1:320 to California encephalitis (Table).

California encephalitis-related arboviruses included in the tests were snowshoe hare, La Crosse, Jamestown Canyon, Morro Bay, and Tahyna. Tahyna, an important California serogroup virus widely distributed in Europe and Asia, was included because of the patient's travel history. Northway, Main Drain, and Lokern viruses, members of the Bunyamwera serogroup occurring in California, were also included, as were western equine encephalomyelitis virus and St. Louis encephalitis.

Table. Neutralizing antibody titers to 10 arboviruses in paired serum samples of a patient with neurologic symptoms, California, 1996

| Virus (strain) | Acute-phase serum[a] | Convalescent-phase serum[b] |
|---|---|---|
| California encephalitis (BFS-283) | 1:80 | 1:320 |
| Snowshoe hare (original) | 1:80 | 1:160 |
| La Crosse (prototype) | 1:40 | 1:40 |
| Jamestown Canyon (BFS 4474) | 1:20 | 1:20 |
| Morro Bay (DAV 457) | 1:80 | 1:40 |
| Tahyna (Bardos 92) | 1:40 | 1:20 |
| Northway (BFN 2654) | 1:20 | <1:20 |
| Main Drain (BFS 5015) | <1:20 | <1:20 |
| Lokern (BFS 5183) | <1:20 | <1:20 |
| Western equine encephalomyelitis (BFS 1703) | <1:20 | <1:20 |
| St. Louis encephalitis (BFS 1750) | <1:20 | <1:20 |

[a]Sample taken 8 days after onset of symptoms.
[b]Sample taken 16 days after onset of symptoms.

The sera were tested by plaque reduction-serum dilution neutralization with African green monkey kidney (Vero) cell cultures, according to the protocol of Campbell et al. (6). An increase in titer between the acute- and convalescent-phase samples was found only for California encephalitis and snowshoe hare virus. The titers for the convalescent-phase serum were 1:320 for California encephalitis and 1:180 for the closely related snowshoe hare virus (Table), which has never been reported in California. The patient had not traveled recently to alpine areas of the state, where snowshoe hare virus might be expected to occur.

## Conclusions

Because the patient's travel occurred several months before his illness, exposure to mosquitoes near his home is the most likely route of infection. The most common mosquito species in salt marshes in Marin County are *Aedes washinoi* and *Ae. squamiger* (7). Bloodsucking adult females of both these species are usually present in late winter to early spring. *Ae. dorsalis* also occurs in this area, but later in the year. *Culiseta inornata* occurs frequently near salt marshes. California encephalitis has not been recovered from any of these species in California; most isolates have come from *Ae. melanimon* in the Central Valley (8).

Campbell et al. (5) found that most seropositive samples from humans, mostly from high elevations (>1,000 m) in California, were apparently infected with Jamestown Canyon virus. However, these investigators also reported that approximately 35% of samples from horses in low elevations (<1,000 m) in California showed evidence of prior infection with California encephalitis (9), but they did not test samples from the San Francisco Bay area. Fulhorst et al. (10) reported that 57% of the horses sampled in Marin County showed evidence of prior infection with Jamestown Canyon virus, but none with California encephalitis.

Awareness of arboviruses in the United States has been heightened as a result of the recent outbreak of human illnesses caused by West Nile virus. That outbreak was originally thought to be due to St. Louis encephalitis (11). The case we describe is a further reminder that clinicians should consider several causative agents when a patient has a central nervous system infection, especially if mosquito exposure has occurred.

Further studies are needed to assess the risk for human infection by California encephalitis in coastal California and the role of various mosquito species in transmission.

## Acknowledgment

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Dr. Eldridge is Emeritus Professor of Entomology at the University of California, Davis, and continues to serve as director of the UC Mosquito Research Program. His area of specialization is the ecology of mosquitoes and mosquito-borne viral diseases of humans.

## References

1. Hammon WM, Reeves WC, Sather G. California encephalitis virus, a newly described agent. II. Isolations and attempts to identify and characterize the agent. J Immunol 1952;69:493-510.
2. Hammon WM, Reeves WC. California encephalitis virus, a newly described agent. I. Evidence of infection in man and other animals. California Medicine 1952;77:303-9.
3. Centers for Disease Control and Prevention. Summary of notifiable diseases, United States, 1998. MMWR Morb Mortal Wkly Rep 1998;47:22-5.
4. McJunkin JE, Khan RR, Tsai T. California-La Crosse encephalitis. Infect Dis Clin North Am 1998;47:83-93.
5. Campbell GL, Reeves WC, Hardy JL, Eldridge BF. Seroepidemiology of California and Bunyamwera serogroup viruses in humans in California. Am J Epidemiol 1992;136:308-19.
6. Campbell GL, Eldridge BF, Hardy JL, Reeves WC, Jessup DA, Presser SB. Prevalence of neutralizing antibodies against California and Bunyamwera serogroup viruses in deer from mountainous areas of California. Am J Trop Med Hyg 1989;40:428-37.
7. Lanzaro GC, Eldridge BF. A classical and population genetic description of two new sibling species of *Aedes* (*Ochlerotatus*) *increpitus* Dyar. Mosquito Systematics 1992;24:85-101.
8. Reeves WC, editor. Epidemiology and control of mosquito-borne arboviruses in California, 1943-1987. Sacramento: Calif Mosq Vector Control Assoc; 1990. p. 508.
9. Campbell GL, Reeves WC, Hardy JL, Eldridge BF. Distribution of neutralizing antibodies to California and Bunyamwera serogroup viruses in horses and rodents in California. Am J Trop Med Hyg 1990;42:282-90.
10. Fulhorst CF. The epidemiology and ecology of mosquito-borne viruses in coastal areas of California [dissertation]. Berkeley (CA): University of California, Berkeley; 1994.
11. Centers for Disease Control and Prevention. Outbreak of West Nile-like viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:845-9.

# Reiter Syndrome Following Protracted Symptoms of *Cyclospora* Infection

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Two large outbreaks of diarrheal illness associated with *Cyclospora cayetanensis*, a coccidian parasite, provided an opportunity to evaluate clinical syndromes associated with this enteric pathogen. Reiter syndrome, a triad of ocular inflammation, inflammatory oligoarthritis, and sterile urethritis, has been associated with enteric infections. We describe the first case of Reiter syndrome following protracted symptoms of *Cyclospora* infection.

*Cyclospora*, a protozoan pathogen that causes a syndrome of diarrhea and fatigue, was responsible for two large-scale outbreaks in North America in 1996 and 1997 (1,2). These outbreaks, along with studies in *Cyclospora*-endemic areas (Nepal and Peru) and treatment of travelers returning from these areas, have increased our understanding of the clinical illness associated with this pathogen. Chronic diarrhea is not uncommon, especially in patients whose condition is untreated or partially treated. Recently, Guillain-Barré syndrome has been reported as an extraintestinal complication of *Cyclospora* infection (3). We describe Reiter syndrome diagnosed in a patient after prolonged gastrointestinal illness associated with *Cyclospora* infection.

## The Study

A 31-year-old man had onset of gastrointestinal illness on May 11, 1997, 7 days after attending a dinner at a country club. His symptoms (extreme fatigue, dizziness, fever of 101°F, intermittent vomiting and diarrhea) were similar to those of the 10 other guests at this party and caused him to miss work for 2 weeks. He visited his internist on May 22, 1997, and was hospitalized with predominant symptoms of fatigue and dehydration; he also reported constipation. Fluids and gentamicin were administered intravenously. Stool cultures and microscopy were negative; however, *Cyclospora* was not searched for specifically. After the patient was discharged from the hospital, diarrhea resumed, and a stool specimen was examined by our laboratory by a concentration technique and modified acid-fast staining. This examination identified *Cyclospora* oocysts. Standard therapy, trimethoprim/sulfamethoxazole, could not be administered because of the patient's allergy to sulfa, and as no effective alternative antibiotic therapy for *Cyclospora* infections exists, antibiotic treatment was not offered. Symptoms of epigastric pain and discomfort, bloating, and alternating diarrhea and constipation predominated, along with intermittent anorexia. An 8-pound weight loss (5% of body weight) was noted. By mid-June 1997, the patient's symptoms had abated slightly but were not completely resolved. He was enrolled at our institution in an open-label trial of albendazole, 400 mg 2 times a day for 14 days, and reported no change in his symptoms during this time. *Cyclospora* was present in stool after treatment with albendazole. Soft stool, intermittent diarrhea, and abdominal cramping persisted, along with proctalgia. Results of blood tests, including a complete blood count, biochemical profile, and liver function tests, were normal.

The patient's medical history included mild asthma with flare-ups approximately monthly, for which he had used theophylline. A diagnosis of prostatitis was made on clinical grounds in December 1996, 6 months before the onset of diarrheal illness and 10 months before the diagnosis of Reiter syndrome. These symptoms resolved with antibiotic therapy and did not recur.

When the patient first came to our institution on July 29, 1997, he was not in acute distress. His abdomen was soft and tender only to deep palpation in the left and right lower quadrants. There were no masses or enlarged organs. Stool specimens were negative for occult blood. As part of an ongoing study of small-bowel histopathologic changes associated with *Cyclospora* infections, the patient agreed to endoscopic evaluation. Upper gastrointestinal endoscopy was performed on August 15, 1997, with examination to the descending duodenum. This examination revealed inflammation of the distal esophagus and mild erythema of the gastric cardia and antrum, as well as erythema of the duodenal bulb and descending duodenum. Flexible fiber-optic sigmoidoscopy to the mid-descending colon was unremarkable. No *Cyclospora* oocysts were identified on either duodenal or stool aspirates. Biopsies of the duodenum revealed partial villous atrophy and moderate crypt hyperplasia with increased intraepithelial lymphocytes and rare intraepithelial neutrophils. Biopsies of the gastric antrum and cardia were normal. A rapid urease test performed on the gastric biopsy was negative for *Helicobacter pylori*. Electron microscopy evaluation of the small bowel biopsies revealed acute and chronic inflammation evidenced by focally intense epithelial injury, including vacuolization, lipid accumulation, and abundant interstitial and epithelial reactive elements. Sigmoid and rectal biopsies revealed no histopathologic changes.

Gastrointestinal symptoms persisted, including abdominal bloating and cramping and intermittent soft stool, along with fatigue. In September 1997, the patient noted pain and

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soreness in knees, ankles, right first toe, and plantar heels. During early October 1997, he noted a constellation of symptoms, including left eye pain, dysuria, arthralgias, and a painful ulcer of the right buccal mucosa. An ophthalmologist confirmed that the left eye pain resulted from iritis and episcleritis. A rheumatologist suspected the diagnosis of Reiter syndrome on the basis of the constellation of findings and negative serologic tests for Lyme disease and *Chlamydia*. A urinalysis was negative for leukocytes and bacteria, although no attempts were made to recover *Chlamydia* from the urine. Stool microscopy was negative for parasites, and a stool culture was negative for bacterial pathogens. The patient was noted to be negative for HLA-B27. Oral corticosteroids were begun for the iritis symptoms, and he was treated with doxycycline (100 mg twice a day) for its antiinflammatory effects. The arthritis symptoms improved promptly and dramatically. Four days after beginning doxycycline therapy, the patient complained of progressively severe odynophagia, and a clinical diagnosis of pill-induced esophagitis or ulcer was made. The symptoms resolved with a combination of sucralfate suspension and omeprazole, and the patient has remained clinically well.

## Conclusions

Reiter syndrome, characterized by the triad of ocular inflammation (conjunctivitis, iritis, episcleritis), inflammatory oligoarthritis, and sterile urethritis, usually occurs several weeks after a triggering infection (4). Infections associated with Reiter syndrome include genitourinary *Chlamydia* or enteric infections with *Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*, *Clostridium difficile*, and *Cryptosporidium*, although the incidence of Reiter syndrome with each of these infections is suspected to be low. This is the first reported case of Reiter syndrome following *Cyclospora* infection. Although Reiter syndrome in this patient could have been coincidental, we propose *Cyclospora* as another infectious trigger for Reiter syndrome.

This patient's history is unique in several respects. Because of sulfa allergy, he was inadequately treated for the *Cyclospora* infection. An experimental trial of albendazole, which is used to treat other parasitic infections, did not shorten his illness. Chronic gastrointestinal symptoms persisted for 12 weeks before endoscopy, when variable crypt hyperplasia and villous atrophy were noted—characteristic findings in small bowel biopsies of patients with *Cyclospora* infection (5). No *Cyclospora* oocysts were noted on stool examination at the time of endoscopy. The pathogenesis of Reiter syndrome may involve molecular mimicry between microbial fragments in synovial fluid and the HLA-B27 molecule; however, it is unknown whether microbial fragments of *Cyclospora* could be found in synovial fluid, and in this case the patient was HLA-B27 negative (6). Inflammatory lesions in the small intestine may allow antigen priming of receptive T lymphocytes and trigger an inflammatory response in affected organs. Depending on the length of this process, it can be hypothesized that prompt, effective treatment of *Cyclospora* infections may minimize the risk for postinfectious complications such as Reiter syndrome.

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## References

1. Herwaldt BL, Ackers ML, and the Cyclospora Working Group. An outbreak in 1996 of Cyclosporiasis associated with imported raspberries. N Engl J Med 1997;336:1548-56.
2. Herwaldt BL, Beach MJ, and the Cyclospora Working Group. The return of *Cyclospora* in 1997: Another outbreak of cyclosporiasis in North America associated with imported raspberries. Ann Intern Med 1999;130:210-20.
3. Richardson RF, Remler BF, Katirji B, Murad MH. Guillain-Barré syndrome after *Cyclospora* infection. Muscle Nerve 1998;21:669-71.
4. Konttinen YT, Nordstrom DC, Bergroth V, Leirisalo-Repo M, Santavirta S. Occurrence of different ensuing triggering infections preceding reactive arthritis: a follow-up study. BMJ 1988;296:1644-5.
5. Connor BA, Shlim DR, Scholes JB, Rayburn JL, Reidy J, Rajah R. Pathologic changes in the small bowel in nine patients with diarrhea associated with a coccidia-like body. Ann Intern Med 1993;119:377-82.
6. Hermann E, Yu DT, Meyer zum Buschenfelde KH, Fleischer B. HLA-B27 restricted CD8 T-cells derived from synovial fluids of patients with reactive arthritis and ankylosing spondylitis. Lancet 1993;342:646-50.

# Presence of Class I Integrons in Multidrug-Resistant, Low-Prevalence *Salmonella* Serotypes, Italy

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In 1997 to 1999, we detected class I integrons in multidrug-resistant isolates of *Salmonella enterica* serovars Anatum, Blockley, Brandenburg, Bredeney, Derby, Heidelberg, Livingstone, Newport, Ohio, Panama, Paratyphi B, Saintpaul, Sandiego, and Stanley.

Bacterial resistance to antimicrobial agents is a serious problem worldwide. Of particular concern is the increasing frequency of multidrug resistance within *Salmonella* strains isolated from zoonotic foodborne infections (1,2). This aspect has been extensively investigated in *Salmonella enterica* serovar Typhimurium in relation to the worldwide spread of multidrug-resistant (MDR) strains of definitive phage type (DT) 104, with chromosomally integrated genes coding for resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (3,4).

Recently, a basic role in dissemination and evolution of antimicrobial resistance in MDR *S.* Typhimurium DT104 (MDR-DT104) and many other organisms has been attributed to integrons, gene expression elements that potentially account for rapid and efficient transmission of drug resistance because of their mobility and ability to collect resistance gene cassettes (5,6). These elements have been described in a wide range of pathogenic organisms (7), including *S.* Typhimurium and *S.* Enteritidis (8,9); reports of these integrons in other *Salmonella* serotypes are anecdotal (10).

Although *S.* Enteritidis and *S.* Typhimurium account for approximately 45% and 25%, respectively, of the strains of *Salmonella* identified at the Centre for Enteric Pathogens of southern Italy, other serotypes, such as Brandenburg, Derby, Livingstone, and Thompson, are frequently identified from various sources, exhibiting sometimes unusually wide patterns of antibiotic resistance. We investigated the presence of class I integrons in MDR strains of *Salmonella* serotypes other than *S.* Typhimurium and *S.* Enteritidis, identified in 1997 to 1999, to obtain information on the presence of these elements in low-prevalence serotypes and to determine their association with multidrug-resistance phenotypes.

## The Study

Seventy-four strains of *Salmonella* (of serotypes other than *S.* Enteritidis and *S.* Typhimurium) resistant to three or more antibacterial drugs were identified from January 1997 to December 1999. Isolates were from human and nonhuman sources. Sixty-two isolates were available for further investigation. Identification was performed by the API 20E system (Biomerieux, Marcy l'Etoile, France) and serotyping (11) by commercially obtained antisera (Sanofi Diagnostics Pasteur, Marnes-La Coquette, France).

Susceptibility to ampicillin, amoxicillin-clavulanic acid, cefotaxime, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, nitrofurantoin, sulfonamides, streptomycin, tetracycline, and trimethoprim was tested by disk-diffusion assay, according to National Committee for Clinical Laboratory Standard Guidelines (12).

The rifampin-resistant strain of *Escherichia coli* K12J5 $Rif_r$ was used as the recipient in conjugation experiments (13). Transconjugants were selected on Luria-Bertani agar containing 250 µg/mL of rifampin plus 50 µg/mL of ampicillin or sulfonamides or 30 µg/mL of chloramphenicol, streptomycin, tetracycline, or trimethoprim.

Plasmid DNA was extracted by the procedure of Birnboim and Doly (14), electrophoresed on 0.7% agarose, and stained with ethidium bromide simultaneously with reference size plasmids (39R861, MIP 233, R27, and R477).

Screening of isolates for presence of class I integrons was performed by a high-stringency protocol with oligonucleotide primers specific for the sequence of the published 5'-CS and 3'-CS regions adjacent to the site-specific recombinational insertion sequence (15). Primer sequences were: 5'-CS, GGCATCCAAG-CAGCAAG and 3'-CS, AAGCAGACTTGACCTGA (15).

Further polymerase chain reaction (PCR) analysis was performed on the 26 isolates harboring class I integrons to better characterize the antibiotic resistance genes associated with the integron structure. This was done by using primers located at the beginning extremities of the inserted resistance genes in combination with that specific for the 5'-CS conserved segment. The following sequences were tested: sulfonamide resistance gene *sul*I; beta-lactam resistance genes *oxa*2, *pse*1, and *tem*; aminoglycoside resistance genes *aac*(3)-Ia, *aac*(3)-IIa, *aac*(6')-Ib, *ant*(3")-Ia, *aad*A2 [also named *ant*(3")-Ib]; and trimethoprim resistance gene *dhfr*I (15). The presence of the *pasppflo*-like (*flor*) and *tet*G genes, conferring resistance to chloramphenicol, florfenicol, and tetracycline in MDR-DT104, was also investigated by using PCR primers specific for these sequences (16).

From 1997 to 1999, 18 *Salmonella* serotypes were identified, including isolates resistant to three or more antibacterial drugs: Anatum, Blockley, Brandenburg, Bredeney, Derby, Hadar, Heidelberg, Livingstone, Muenchen,

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Newport, Ohio, Panama, Paratyphi B, Saintpaul, Sandiego, Stanley, Thompson, and Virchow. Seventy-four multidrug-resistant isolates were identified, which accounted for 10.0% of the strains belonging to the serotypes under study. The proportion of isolates with a pattern of resistance to three or more drugs is summarized (Table 1); 26 isolates belonging to 14 serotypes contained class I integrons (Table 2). Screening for the presence of plasmid DNA detected no plasmids in 14 strains and plasmids, between 35 and 140 megadaltons in size, in the remaining 12. Three isolates of serotype Brandenburg clustered as an epidemic, according to epidemiologic data and shared identical plasmid DNA and integron profiles. Transfer of plasmids was associated with transmission to *E. coli* of the complete or partial resistance pattern (Table 2). In all but one case, PCR analysis with the 5'CS and 3'CS primers confirmed the presence of integrons in the recipient cells.

Heterogeneous integron-associated resistance genes were present in the isolates under study, despite the extensive similarities of the antibiotic resistance phenotypes (Table 3). Strains belonging to serotypes Ohio, Panama, and Saintpaul carried the *ant*(3")-Ia and *pse*I gene cassettes previously described in two different chromosome-located integrons in MDR-DT104 but inserted in a single integron transferable by conjugation. The integron-associated aminoglycoside resistance genes *aac*(3)-IIa and *aac*(6')-Ib were not detected in the strains tested.

Both *tet*G and *flor* resistance determinants, known to characterize MDR-DT104 strains (16), were found in one strain of Paratyphi B isolated from tropical fish imported from Singapore.

Table 1. Proportion of low-prevalence *Salmonella* serotypes resistant to three or more antibacterial drugs

| Serotype | No (% of isolates) |
|---|---|
| Sandiego | 3 (33.3) |
| Blockley | 22 (31.9) |
| Heidelberg | 6 (21.4) |
| Thompson | 11 (20.4) |
| Stanley | 2 (16.7) |
| Saintpaul | 2 (12.5) |
| Muenchen | 1 (11.1) |
| Brandenburg | 6 (10.7) |
| Anatum | 5 (10.0) |
| Hadar | 1 (7.7) |
| Ohio | 2 (7.1) |
| Bredeney | 2 (5.7) |
| Paratyphi B | 1 (5.0) |
| Newport | 1 (4.5) |
| Panama | 1 (4.3) |
| Virchow | 3 (3.3) |
| Livingstone | 2 (2.9) |

## Conclusions

The emergence of multidrug resistance in *Salmonella* serotypes is causing growing concern because of the high potential of human involvement through food and animal contact. We have detected integrons in MDR-resistant isolates of *Salmonella* identified in southern Italy in the last 3 years. Our findings confirmed not only that integrons are not confined to *S.* Typhimurium DT104 but also that they can be found in many less- prevalent serotypes with extensive reservoirs, encompassing animal species (swine, poultry, domestic pets) and environmental sites (rivers, sewage effluents). A further concern is the presumed location of integrons on the chromosome, detected in isolates of nine different serotypes. This resistance gene location has proved to be very efficient in acquiring and establishing resistance traits and in supporting spread of *S.* Typhimurium DT104 through the food chain in western countries (17).

Table 2. Phenotypic and molecular characteristics of multidrug-resistant and class I integron carrying strains of *Salmonella*

| Serotype | Year and source of isolation | Resistance pattern | Plasmid pattern (mDa) | Integron sizes (kb) | Resistance pattern of recipient *Escherichia coli* | Integron sizes (kb) of *E. coli* |
|---|---|---|---|---|---|---|
| Derby | 1997 Human | Ap Cm Sm Su Tc Tp Gm | **120**[b] | 2.0 | Ap Cm Sm Su Tc Tp | 2.0 |
| Newport | 1997 Human | Ap Cm Sm Su Tc Tp Gm | **120** | 2.0 | Ap Cm Sm Su Tc Tp | 2.0 |
| Paratyphi B | 1997 Tropical fish | Ap Cm Sm Su Tc | | 1.2, 1.0 | | |
| Saintpaul | 1997 Poultry | Ap Cm Sm Su Tc Tp | **120** | 1.8 | Ap Cm Sm Su Tc Tp | 1.8 |
| Sandiego | 1997 Poultry | Ap Cm Sm Su Tc Tp Kf | | 1.4 | | |
| Anatum | 1998 Food (not specified) | Ap Sm Su Tc Tp F | | 1.8 | | |
| Blockley | 1998 River water | Cm Sm Su Tc Tp F Na | | 1.8, 1.0 | | |
| Brandenburg[a] | 1998 Human | Cm Sm Su Tc Tp | **120** | 1.0 | Cm Sm Su Tc Tp | 1.0 |
| Livingstone | 1998 River water | Ap Cm Sm Su Tc Tp | **120** | 1.8 | Ap Cm Sm Su Tc Tp | 1.8 |
| Ohio | 1998 River water | Ap Cm Sm Su Tc Tp | **120** | 1.8 | Ap Cm Sm Su Tc Tp | 1.8 |
| Ohio | 1998 Swine | Ap Sm Su Tc Tp | **120** | 1.6 | Ap Sm Su Tc Tp | 1.6 |
| Panama | 1998 Swine | Ap Cm Sm Su Tc Tp | **120** | 1.8 | Ap Cm Sm Su Tc Tp | 1.8 |
| Saintpaul | 1998 Human | Ap Cm Sm Su Tc Tp Kf | **140** | 1.8 | Ap Cm Sm Su Tc Tp | 1.8 |
| Anatum | 1999 Sewage | Ap Sm Su Tc Tp | | 1.8 | | |
| Anatum | 1999 River water | Ap Su Tc Tp F | | 1.8 | | |
| Anatum | 1999 River water | Su Tc Tp Na | | < 0.1 | | |
| Blockley | 1999 Human | Cm Sm Su Tc F Na | | < 0.1 | | |
| Brandenburg | 1999 Tropical fish | Cm Su Tc Tp | | 0.8, 0.2, <0.1 | | |
| Brandenburg | 1999 Tropical fish | Cm Sm Su Tc Tp | | 1.8 | | |
| Brandenburg | 1999 Poultry | Cm Sm Su Tc Tp Gm F Na | **120** | 1.8 | Cm Sm Su Tc Tp | 1.8 |
| Bredeney | 1999 Sewage | Sm Su Tc | | 1.8, 1.0 | | |
| Derby | 1999 Sewage | Ap Sm Su Tc | **60,** 35 | 1.0 | Ap | |
| Heidelberg | 1999 Human | Ap Sm Su Tc | | 1.8, 1.0, 0.2 | | |
| Stanley | 1999 Tropical fish | Cm Sm Su Tc Tp | | 1.8, 1.0 | | |

[a]Outbreak strain.
[b]Numbers in bold indicate the approximate molecular size of self-transferable resistance plasmids.

Table 3. Resistance genetic sequences identified in class I integron-carrying multidrug-resistant strains of *Salmonella*

| | Integron | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Serotype | sulI | pse1 | tem | oxa2 | aadA2 | ant(3")-Ia | aac(3)-IA | dhfrI | tetG | pasppflo-like (flor) |
| Derby | + | | + | | | | | | | |
| Newport | + | | + | | | | | | | |
| Paratyphi B | + | | | + | | | | | + | + |
| Saintpaul | + | + | | | | + | | + | | |
| Sandiego | + | | | | | + | | + | | |
| Anatum | + | + | | | | | | | | |
| Blockley | + | | | | | | | + | | |
| Brandenburg[a] | + | | | | + | | | + | | |
| Livingstone | + | + | | | | | | | | |
| Ohio | + | + | | | | | | + | | |
| Ohio | + | + | | | | + | | + | | |
| Panama | + | + | | | | + | | + | | |
| Saintpaul | + | + | | | | + | | + | | |
| Anatum | + | | + | | | | | + | | |
| Anatum | + | | + | | | | | | | |
| Anatum | + | | | | | | | | | |
| Blockley | + | | | | | | | | | |
| Brandenburg | + | | | | | | | | | |
| Brandenburg | + | | | | | | + | | | |
| Brandenburg | + | | | | | + | | + | | |
| Bredeney | + | | | | | | | | | |
| Derby | + | | + | | + | | | | | |
| Heidelberg | + | + | | | | | | | | |
| Stanley | + | | | | | | + | | | |

[a]Outbreak strain.

We also recognized in different serotypes a pattern of resistance similar to the five-drug pattern typical of DT104, a phenomenon reported by Glynn et al. (10). The heterogeneous distribution and organization of resistance genes within several low-prevalence serotypes of *Salmonella* suggest the possible emergence of MDR-DT104-like patterns in serotypes other than *S.* Typhimurium that share a similar selective pressure because of intensive use of antimicrobial agents in farming. Moreover, *tet*G and *flor* resistance sequences in one *S.* Paratyphi B isolate from Singapore tropical fish suggest that the use of antimicrobial agents in aquaculture in Asia is contributing to the emergence and spread of multidrug resistance within fish pathogens and, subsequently, MDR-DT 104 strains (18).

The association between emergence of MDR *Salmonella* strains and excessive use of antibiotics in animal husbandry (as growth promoters and for disease prevention and therapy) is receiving increasing attention in developed countries. The presence of integrons in zoonotic serotypes such as Blockley, Brandenburg, Derby, or Saintpaul, which in southern Italy are epidemiologically linked to farming practices, underscores the public health problem of antibiotic resistance diffusion.

Surveillance and monitoring of antimicrobial-drug resistance, including screening for class I integrons as likely indicators of evolution of drug resistance mechanisms and acquisition of new resistance traits, are necessary steps in planning effective strategies for containing this phenomenon within foodborne infectious organisms.

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## References

1. Threlfall EJ, Rowe B, Ward LR. A comparison of multiple drug resistance in salmonellas from humans and food animals in England and Wales, 1981 and 1990. Epidemiol Infect 1993;111:189-97.
2. Tassios PT, Chadjichristodoulou C, Lambiri M, Kansouzidou-Kanakoudii A, Sarandopoulou Z, Kourea-Kremastinou J, et al. Molecular typing of multidrug-resistant *Salmonella* blockley outbreak isolates from Greece. Emerg Infect Dis 2000;6:604.
3. Glynn MK, Bopp C, Dewitt W, Dabney P, Mokhtar M, Angulo FJ. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. N Engl J Med 1998;338:1333-8.
4. Mølbak K, Baggesen DL, Aarestrup FM, Ebbesen JM, Engberg J, Frydendahl K, et al. An outbreak of multidrug-resistant, quinolone-resistant *Salmonella enterica* serotype Typhimurium DT104. N Engl J Med 1999;341:1420-5.
5. Recchia GD, Hall RM. Gene cassettes: a new class of mobile elements. Microbiology 1995;141:3015-27.
6. Tosini F, Visca P, Luzzi I, Dionisi AM, Pezzella C, Petrucca A, et al. Class I integron-borne multiple-antibiotic resistance carried by IncF1 and IncL/M plasmids in *Salmonella enterica* serotype Typhimurium. Antimicrob Agents Chemother 1998;42:3053-8.
7. Jones ME, Peters E, Weersink AM, Fluit A, Verhoef J. Widespread occurrence of integrons causing multiple antibiotic resistance in bacteria [letter]. Lancet 1997;349:1742-3.
8. Rankin SC, Coyne MJ. Multiple antibiotic resistance in *Salmonella enterica* serotype enteritidis [letter]. Lancet 1998;351:1740.
9. Brown AW, Rankin SC, Platt DJ. Detection and characterization of integron in *Salmonella enterica* serotype Enteritidis. FEMS Microbiol Lett 2000;191:145-9.
10. Glynn MK, Ribot EM, Barrett TJ. Multidrug-resistant *Salmonella enterica* serotype Typhimurium infections [letter]. N Engl J Med 1998;339:922.
11. Kauffman F. Serological diagnosis of *Salmonella* species. Copenhagen (Denmark): Munksgaard; 1972.
12. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4. Villanova (PA): The Committee; 1997.

13. Datta N. Transmissible drug-resistance in an epidemic strain of *Salmonella typhimurium*. J Hyg 1997;78:297-300.
14. Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 1979;7:1513-23.
15. Lévesque C, Piché L, Larose C, Roy PH. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob Agents Chemother 1995;39:185-91.
16. Ng LK, Mulvey MR, Martin I, Peters GA, Johnson W. Genetic characterization of antimicrobial resistance in Canadian isolates of *Salmonella* serovar Typhimurium DT104. Antimicrob Agents Chemother 1999;43:3018-21.
17. Casin I, Breuil J, Brisabois A, Moury F, Grimont F, Collatz E. Multidrug-resistant human and animal *Salmonella* typhimurium isolates in France belong predominantly to a DT104 clone with the chromosome- and integron-encoded beta-lactamase PSE-1. J Infect Dis 1999;179:1173-82.
18. Angulo FJ, Griffin PM. Changes in antimcrobial resistance in *Salmonella enterica* serovar Typhimurium [letter]. Emerg Infect Dis 2000;6:436-7.

# Risk for Human Tick-Borne Encephalitis, Borrelioses, and Double Infection in the Pre-Ural Region of Russia

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We assessed the risk for human tick-borne encephalitis (TBE), ixodid tick-borne borrelioses, and double infection from 1994 to 1998 in Perm, which has among the highest rates of reported cases in Russia. We studied 3,473 unfed adult *Ixodes persulcatus* ticks collected from vegetation in natural foci and 62,816 ticks removed from humans. TBE virus and *Borrelia* may coexist in ticks.

Tick-borne encephalitis (TBE) and infections of the Lyme borreliosis group, or ixodid tick-borne borrelioses (ITBB) (1), are widespread in Russia. In 1996, 1997, and 1998, cases of these diseases totaled approximately 16,650, 11,350, and 14,700, respectively. Most cases were acquired from the bite of an adult *Ixodes persulcatus* tick. In some regions west of the Volga River, the *I. ricinus* tick may also transmit these infections. The geographic distribution and epidemiology of TBE and ITBB in Eurasia are almost identical (2).

These data suggest that double infection by TBE virus and *Borrelia* may result from transmission of both pathogens from double-infected ticks to humans (2). Such ticks are present in natural foci, and the occurrence of TBE virus and *Borrelia* is independent in ticks rather than mutually exclusive. The prevalence of *Borrelia* in unfed *I. persulcatus* ticks with and without TBE virus is virtually identical, and the same is true for TBE virus prevalence in ticks with and without *Borrelia* infection. Concentrations of virus and *Borrelia* in double-infected ticks are not correlated. In the natural mixed foci of TBE and ITBB, interannual changes in the prevalence of virus and spirochetes in ticks are virtually parallel. The coexistence of these microorganisms in their principal vectors, which promotes simultaneous infection in humans bitten by ticks, is apparently an important precondition for the relative autonomy of conjugate parasitic systems formed by TBE virus and *Borrelia* (3). However, prevalence of both pathogens in ticks collected from vegetation has not previously been compared with their prevalence in ticks removed from humans.

Cases of double infection were identified for the first time during 1986 to 1988 in Austria and northwestern European Russia (4,5). They have been described in the species range of the *I. ricinus* tick (6) but occur more frequently in areas where *I. persulcatus* ticks are abundant (7,8). We describe long-term studies assessing the risk for human double infection compared with that for separate TBE and ITBB infection in a region with a consistently high level of both diseases (1).

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## The Study

The study was performed from 1994 to 1998 in the city of Perm, located in the Pre-Ural region, which has among the highest rates of reported cases in Russia. In this area, the *I. persulcatus* tick is the only vector transmitting these infections to humans (1,2,9,10). Only two *Borrelia* species (*B. garinii* and *B. afzelii*) circulate in this area, but the frequency of *B. garinii* in ticks, rodents, and patients is substantially higher (11). Each year, unfed adult ticks were collected from vegetation in several areas where people are exposed to ticks. In most cases (>98%), people are bitten by adult *I. persulcatus* ticks, and only rarely by nymphs (12). Adult ticks were removed from patients who sought medical treatment at a city laboratory designated for the diagnosis and prophylaxis of TBE and ITBB. We studied 66,289 adult *I. persulcatus* ticks, including 3,473 collected from vegetation and 62,816 removed from humans. Some ticks remained attached 1 to 6 days before being removed, but in most cases (~90%) people came to the laboratory <1 day after the tick attached (12), virtually before it began to feed.

Only living ticks were studied for *Borrelia* infection by dark-field microscopic analysis of tick gut contents in standard vital preparations at a magnification of X600. Two hundred fifty microscopic fields per preparation were studied, in 1,797 unfed ticks collected from vegetation and 7,442 ticks removed from patients. For virologic analysis, 1,676 unfed ticks were individually homogenized in 1 mL of medium 199 with Earle's salts (Sigma M3769, St. Louis, MO) containing 25% inactivated normal calf serum. Virus was isolated from the supernatant in cultures of an established pig kidney cell line and identified by the direct fluorescent antibody method and in hemagglutination inhibition and complement fixation tests with specific antibodies from ascitic fluid (13). In addition, all ticks removed from humans were studied on the same day to determine TBE virus by indirect immunofluorescent assay

(14); 7,442 of these ticks were concurrently analyzed for infection with *Borrelia*.

Both TBE and ITBB are reportable diseases for official statistics throughout Russia. Diagnosis is established on the basis of clinical signs and serologic evidence. We used these data for the total population of Perm (slightly over 1 million) on the number of TBE and ITBB cases and requests for medical aid to remove attached ticks.

Calculated percentages were analyzed by using a confidence interval based on double sampling error ($\pm 2m_p$). Significance of differences between mean values ($p<0.05$) was assessed by the Student $t$ test. The correlation coefficient r was determined by Kendall rank correlation analysis. The degree of independence of phenomena under study was estimated by 2x2 contingency analysis for calculating chi square at $p<0.05$ and df = 1.

*Borrelia* prevalence in *I. persulcatus* ticks removed from humans varied from 25% to 35% in different seasons (Table 1). A 5-year series of data on the proportion of *Borrelia*-infected ticks from vegetation in natural foci compared with those removed from people showed good correlation (r = 0.71), indicating that changes in prevalence occurred in parallel.

Table 1. Prevalence of infection by *Borrelia burgdorferi* sensu lato (%) in unfed adult *Ixodes persulcatus* ticks collected from vegetation and removed from humans

| | Ticks collected from vegetation | | Ticks removed from humans | | |
|---|---|---|---|---|---|
| Year | No. | Prevalence of infection($p \pm 2m_p$) | No. | Prevalence of infection ($p \pm 2m_p$) | $t$ |
| 1994 | 220 | 31.3 ± 6.2 | 878 | 27.3 ± 3.0 | 1.2 |
| 1995 | 232 | 34.9 ± 6.3 | 833 | 25.2 ± 3.0 | 2.7 |
| 1996 | 500 | 46.8 ± 4.5 | 2,388 | 35.5 ± 2.0 | 4.5 |
| 1997 | 451 | 25.0 ± 5.7 | 1,535 | 27.9 ± 2.3 | 0.9 |
| 1998 | 394 | 27.4 ± 4.5 | 1,808 | 28.9 ± 2.1 | 0.6 |
| Total | 1,797 | 35.1 ± 2.2 | 7,442 | 30.0 ± 1.1 | 4.1 |

$\pm 2m_p$ = confidence interval based on double sampling error.

Pairwise comparison for each year (1994, 1997, and 1998) of *Borrelia* prevalence in ticks removed from patients and collected from vegetation demonstrated that they were almost similar ($t<2$), whereas in 1995 and 1996, as well as over the whole study period, the proportions of specimens with *Borrelia* among ticks that attached to people were slightly lower than in nature.

As in the case of *Borrelia*, the lowest incidence of specimens with TBE antigen among ticks removed from patients was recorded in 1995, and the highest was observed in 1996 (Table 2). As the virologic analysis of ticks collected from vegetation and removed from patients was performed by different methods, direct pairwise comparison, as in the case of *Borrelia* infection (Table 1), would not be valid. However, in any year, the proportion of infected ticks from humans did not exceed that in ticks collected in nature (Table 2).

The degree of independence of TBE virus and *Borrelia* prevalence in concurrently studied ticks removed from humans was estimated by 2x2 contingency analysis. The resulting chi-square values, ranging from 0.22 to 1.01, provided evidence for lack of any dependence between the prevalence of TBE virus and *Borrelia* in ticks.

Table 2. Prevalence of infection by tick-borne encephalitis virus (%) in unfed adult *Ixodes persulcatus* ticks collected from vegetation and removed from humans

| | Ticks collected from vegetation | | Ticks removed from humans | |
|---|---|---|---|---|
| Year | No. | Prevalence of infection($p \pm 2m_p$) | No. | Prevalence of infection($p \pm 2m_p$) |
| 1994 | 664 | 21.5 ± 3.2 | 9,815 | 9.5 ± 0.6 |
| 1995 | 265 | 10.9 ± 3.8 | 8,523 | 5.5 ± 0.5 |
| 1996 | 167 | 35.3 ± 7.4 | 17,905 | 11.0 ± 0.5 |
| 1997 | 336 | 38.7 ± 5.3 | 12,443 | 8.8 ± 0.5 |
| 1998 | 244 | 15.2 ± 4.6 | 14,129 | 8.4 ± 0.5 |
| Total | 1,676 | 23.7 ± 2.1 | 62,816 | 9.0 ± 0.2 |

$\pm 2m_p$ = confidence interval based on double sampling error; Coefficient of correlation was r = 0.7.

Among ticks removed from patients, proportions with double infection from 1994 to 1998 were as follows: 2.2 ±1.0%, 1.2 ±0.7%, 3.3 ±0.7%, 2.3 ±0.8%, and 2.2 ±0.7%, respectively. In general, annual changes in TBE, ITBB, and double infection illness occur in parallel (Figure). Coefficients of correlation (r) between prevalence of these infections are as follows: TBE and ITBB, r = 0.73; ITBB and double infection, r = 0.53; TBE and double infection, r = 0.94; and TBE + ITBB and double infection, r = 0.84.

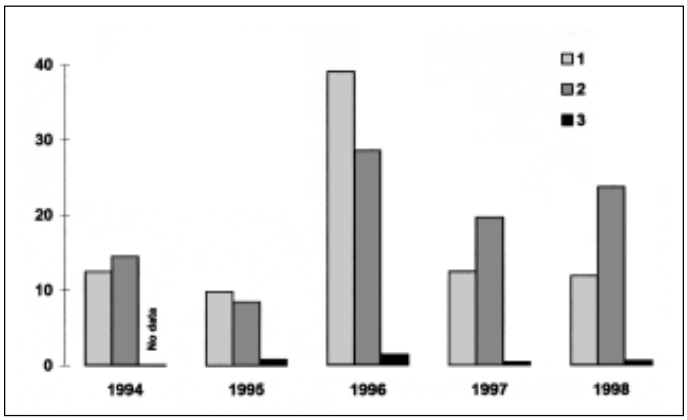


Figure. Rates of disease in Perm, Russia (per 100,000 residents), of 1) tick-borne encephalitis (TBE), 2) Ixodid tick-borne borreliosis (ITBB), and 3) double infection.

Based on the number of reported requests for medical treatment after tick bites and the pathogen prevalences in ticks, the probable frequency of contact with infected ticks was estimated (Table 3). These estimates, characterizing only the study group, substantially exceed the actual prevalence of illness for the population of Perm (Figure).

## Conclusions

Our data provide evidence for a distinct correlation between prevalence of TBE virus and *Borrelia* in *I. persulcatus* ticks collected from vegetation in natural foci and removed from humans. However, we found no evidence that ticks infected by TBE virus attach to people more often than uninfected ticks (15). *Borrelia* prevalence in ticks collected from vegetation and from patients is virtually identical. A trend toward increasing prevalence of spirochetes in unfed ticks in some years may be explained by technical difficulties in visualizing *Borrelia* under a microscope, which increase with engorgement (16).

Table 3. Frequency of possible contacts of people with ticks infected by pathogens of tick-borne encephalitis, ixodid tick-borne borrelioses, and both (double infection)

| Year | No. seeking medical treatment after tick bite | No. who had probable contact with ticks infected with: TBE[a] | ITBB | Both (double infection) |
|---|---|---|---|---|
| 1994 | 1,260 | 120 | 345 | 27 |
| 1995 | 970 | 55 | 240 | 11 |
| 1996 | 2,070 | 230 | 735 | 68 |
| 1997 | 1,340 | 110 | 370 | 30 |
| 1998 | 1,280 | 115 | 370 | 28 |

[a]TBE = tick-borne encephalitis; ITBB = ixodid tick-borne borreliosis.

The results of our microbiologic and virologic testing of individual ticks removed from patients, as well as those of unfed *I. persulcatus* ticks from a natural focus (3), showed that the prevalences of their infection by TBE virus and *Borrelia* are independent. Our data do not support the concept that, in double-infected ticks, *Borrelia* circulation restricts TBE virus circulation; at the same time, the presence of virus in ticks may even promote *Borrelia* transmission (15,17,18). In fact, in double-infected *I. persulcatus* ticks, *Borrelia* and TBE virus do not interfere with one another.

This is why the prevalences of TBE, ITBB, and double infection illness correlate well, and human cases of double infection are more frequent in the regions where the incidence of both TBE and borrelioses is especially high. Hence, the hypothesis that TBE is replaced by borreliosis in conjugate foci (18) appears to be erroneous.

Knowing the frequency of human contact with ticks and the prevalence of both TBE virus and *Borrelia* in ticks, the probable annual frequency of combined exposure to these pathogens may be calculated for residents of a certain area. Our data show, however, that such calculations substantially exceed the current level of illness and the actual risk for acquiring not only double infection, but also each infection separately. This discrepancy results because not all bites from infected ticks transmit a dose of pathogen sufficient to cause clinical illness. In TBE, for example, most bites result in asymptomatic infection and immune response, and the mean ratio of clinically manifested to asymptomatic cases is 1:60 (19). However, this ratio varies widely by regions and year. Because most people remove ticks promptly (within <1 day [12]), a physiologic phenomenon called reactivation, which is required for some microorganisms before infectivity is attained, does not have time to develop. The risk for clinical illness of TBE and borreliosis is largely determined by highly infected ticks (20), which always account for only a few of all infected ticks in a population (21). In addition, among *I. persulcatus* ticks infected by *B. garinii* and *B. afzelii*, no more than half contain spirochetes in the salivary glands after a blood meal and are capable of transmitting them to bitten people (16). At the same time, only a fraction of bitten people seek medical help in removing an attached tick, and the actual number of people bitten by ticks is many times greater than reported (1). Therefore, prevalences of risk for human TBE, ITBB, and double infection may be no more than estimates. Nevertheless, the epidemiologic and clinical aspects of widespread tick-borne coinfections merit close attention from researchers and clinicians.

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## References

1. Korenberg EI. Ixodid tick-borne borrelioses (ITBBs), infections of the Lyme borreliosis group, in Russia: country report. In: Report of WHO Workshop on Lyme Borreliosis Diagnosis and Surveillance. Warsaw: Sanitati; 1995. p. 128-36.
2. Korenberg EI. Comparative ecology and epidemiology of Lyme disease and tick-borne encephalitis in the former Soviet Union. Parasitol Today 1994;4:157-60.
3. Korenberg EI, Kovalevskii YuV, Karavanov AS, Moskvitina GG. Mixed infection by tick-borne encephalitis virus and *Borrelia* in ticks. Med Vet Entomol 1999;13:204-8.
4. Kristoferitsch W, Stanek G, Kunz Ch. Doppelinfection mit Fruhsommermeningoenzephalitis- (FSME-) und *Borrelia burgdorferi*. Dtsch Med Wochenscrift 1986;111:861-4.
5. Mebel' BD, Beitrishvili GA, Zhivich MB, Osetrov BA, Kuznetsova RI, Korenberg EI, et al. Clinical progression of Lyme disease in the acute period. Med Parazitol Parazit Bolezni 1988;3:30-3 (in Russian).
6. Cimperman J, Maraspin V, Lotricfurlan S, Ruzicsabljic E, Avsic-Zupanc T, Picken RN, et al. Concomitant infection with tick-borne encephalitis virus and *Borrelia burgdorferi* sensu lato in patients with acute meningitis or meningoencephalitis. Infection 1998;26:160-4.
7. Korenberg EI, Kuznetsova RI, Kovalevskii YuV, Vasilenko ZE, Mebel' BD. Basic epidemiological features of Lyme disease in the northwestern Soviet Union. Med Parazitol Parazit Bolezni 1991;3:14-7 (in Russian).
8. Laikovskaya EE, Lesnyak OM, Volkova LI, Turova EL, Sokolova ZI, Khodyrev VN. Mixed infection by Lyme borreliosis and tick-borne encephalitis agents. In: Korenberg EI, editor. Problems of tick-borne borrelioses. Moscow; 1993. p. 93-8 (in Russian).
9. Ustinova OYu, Volegova GM, Devyatkov MYu, Gusmanova AG. Clinical-epidemiological features of tick-borne encephalitis in the Perm region. Zh Microbiol Epidemiol Immunobiol 1997;3:33-6 (in Russian).
10. Vorobyeva NN. Clinical picture, treatment and prevention of ixodid tick-borne borrelioses. Perm; 1998. p. 1-132 (in Russian).
11. Postic D, Korenberg E, Gorelova N, Kovalevskii Yu, Bellenger E, Baranton G. *Borrelia burgdorferi* sensu lato in Russia and neighbouring countries: high incidence of mixed isolates. Res Microbiol 1997;148:691-702.
12. Korenberg EI, Moskvitina GG, Vorobyeva NN. Prevention of human borreliosis after infected tick's bite. In: Cevenini R, Sambri V, La Placa M, editors. Advances in Lyme borreliosis research. Proceedings of the VI International Conference on Lyme Borreliosis. Bologna: Esculapio; 1994. p. 209-11.
13. Korenberg EI, Horakova M, Kovalevskii YuV, Hubalek Z, Karavanov AS. Probability models of the rate of infection with tick-borne encephalitis virus in *Ixodes persulcatus* ticks. Folia Parasitol 1992;39:85-92.
14. Matveev LE, Karavanov AS, Rubin SG, Semashko IV, Tsekhanovskaya NA, Presman EK. Comparative estimation of two immunofermantal test systems for determining antibodies to TBE virus. Vopr Virusol 1989;4:488-491 (in Russian).
15. Alekseev AN. Symbiotic relationships in the complex vector-pathogen system. Dokl Akad Nauk SSSR 1994;338:259-61 (in Russian).
16. Korenberg EI, Moskvitina GG. Interrelationships between different Borrelia genospecies and their principal vectors. J Vector Ecol 1996;21:178-85.

17. Alekseev AN, Burenkova LA, Vasil'eva IS, Dubinina EV, Chunikhin SP. The functioning of the foci of mixed tick-borne infections on the Russian territory. Med Parazitol Parazit Bolezni 1996;4:9-16 (in Russian).
18. Alekseev AN, Dubinina EV. Epidemiological aspects of mixed (encephalitis and borreliosis) tick-borne infections. Zh Infekts Patol 1996;3:5-9 (in Russian).
19. L'vov DK, Pokrovsky VI. Encephalites. In: Pokrovsky VI, editor. A manual of zoonoses. Leningrad: Meditsina; 1983. p. 7-10 (in Russian).
20. Korenberg EI, Kovalevskii YuV. Main features of tick-borne encephalitis eco-epidemiology in Russia. Zentralbl Bacteriol 1999;289:525-39.
21. Korenberg EI, Kovalevskii YuV. Variation in parameters affecting risk for human disease due to TBE virus. Folia Parasitol 1995;42:307-12.

# Outbreak of Influenza in Highly Vaccinated Crew of U.S. Navy Ship

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An outbreak of influenza A (H3N2) occurred aboard a U.S. Navy ship in February 1996, despite 95% of the crew's having been appropriately vaccinated. Virus isolated from ill crew members was antigenically distinct from the vaccination strain. With an attack rate of 42%, this outbreak demonstrates the potential for rapid spread of influenza in a confined population and the impact subsequent illness may have upon the workplace.

Influenza is a highly contagious virus transmitted by the respiratory route by inhalation of aerosols as well as by direct contact with animate or inanimate objects (1). Because it can spread rapidly in persons in semiclosed or crowded environments, influenza epidemics have posed major risks to military populations (2). The impact of influenza on the U.S. Navy and Marine Corps has been greatly reduced since an annual immunization program began in 1954 (3,4). This mandatory program uses the influenza vaccine reformulated annually by the Vaccine and Related Biological Products Advisory Committee, Food and Drug Administration (5). The 1995/1996 influenza vaccination program contained A/Texas/36/91(H1N1), A/Johannesburg/33/94(H3N2), and B/Harbin/7/94 (6).

Data from the Centers for Disease Control and Prevention (CDC) indicate that the earliest specimen collection date for a new variant, A/Wuhan/359/95(H3N2), was July 1995. The variant was later identified in China (during August, September, and October 1995), Hong Kong (October 1995), and Guam (November 1995). During the winter months of 1996, A/Wuhan/359/95-like viruses were isolated in Asia, Europe, and North America (7). In February 1996, this virus caused an outbreak onboard a U.S. Navy ship whose crew had received the 1995-96 influenza vaccine. We describe the details of this outbreak.

The USS Arkansas is a nuclear-powered, guided missile cruiser with a complement of >500 men. It was in its home port of Bremerton, Washington, in January 1996. On February 1, this cruiser and her sister ship, the USS California (crew >550 men), departed for a 3-week training exercise in the waters off southern California. Up to departure date, the primary contact the two crews had was a shared dining facility. On February 5, the USS Arkansas contacted the Navy Environmental and Preventive Medicine Unit in San Diego, California (NEPMU-5), to report the onset of an acute febrile respiratory disease in many crew members. Subsequently, high rates of an incapacitating illness forced the ship to dock in San Diego, where it remained for 2 days.

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## The Study

On February 6, >60 crew members reported to the USS Arkansas medical department with respiratory symptoms; the 16 most symptomatic patients underwent a complete medical examination and additional laboratory testing. Pharyngeal swabs were collected from 50 ill crew members and submitted for viral culture. After influenza A was diagnosed, amantadine was flown to the ship and offered, 100 mg twice a day, to unvaccinated persons and those in the first days of illness. Follow-up surveys and interviews were conducted in early March after the ship returned from its training exercises. The crew's medical, berth, work site, and immunization records were reviewed.

### Case Finding

Several case-finding methods were used in this outbreak. Initially, cases were identified by the ship's medical officer and corpsmen. Their case definition included crew members seeking medical evaluation with symptoms of fever, headache, sore throat, and/or cough along with nonspecific symptoms of fatigue and malaise. NEPMU-5, notified of the outbreak, sent an epidemiology team to meet the ship. This team used a symptom-based questionnaire, administered to the entire crew at the outbreak peak (February 6), to seek additional cases.

On February 23, the ship returned to Bremerton. A follow-up investigation then took place, which included review of sick-call logs and medical department reports, interviews with select crew members, and a follow-up questionnaire given to all crew members onboard during the outbreak. This questionnaire sought to identify the impact of the outbreak on the ship's function and the effects of intervention with amantadine. Those persons who reported a influenza-like illness on the follow-up questionnaire and had not yet completed the initial questionnaire were asked to do so.

A person was considered a case if he had a documented illness consistent with influenza or if he had indicated on the

initial questionnaire that he had had an influenza-like illness and associated symptoms in the first 3 weeks of February. A possible case was a person who had an influenza-like illness but either did not complete the symptom questionnaire or completed it before becoming ill.

Fifty pharyngeal swabs collected in viral transport media were submitted for culture to the virology lab of Green Hospital at Scripps Clinic in La Jolla, California. Influenza A virus was identified by immunofluorescent staining of infected cell cultures with commercially available monoclonal antibodies. Successfully cultured viruses were forwarded to San Diego County's public health department laboratory for identification of influenza A subtype. The laboratory forwarded five of these isolates to CDC for antigenic characterization by hemagglutination-inhibition reactions with ferret antisera directed against a reference battery of influenza viral antigens, including currently circulating strains received from the global World Health Organization (WHO) influenza network.

## Conclusions

A total of 548 Navy crew members and 3 civilians were onboard February 1-23; 440 crew members (80%) completed the first questionnaire and 509 (93%) completed the follow-up questionnaire. All respondents were male, age 18 to 48 years (mean 26 ± 6 years). Some 523 crew members (95%) had received the 1995-96 influenza vaccine in December 1995. Administration had been uniform, and the cold chain was maintained. The vaccine lot number and administration time were the same as those used for the crew of the companion ship, the USS California.

A total of 232 USS Arkansas crew members were identified with an influenza-like illness during the outbreak (attack rate = 42%); 158 cases were identified by the medical department; 74 patients did not seek medical attention but met case criteria. An additional 63 crew members (11%) reported having some influenza-like symptoms (probable cases) but did not meet the definite case criteria. The outbreak peaked on February 6; the last case was identified on February 20 (Figure 1). Review of medical logs and interviews with the medical department and crew of the USS California revealed that no one aboard that ship had sought medical attention for influenza-like illness during the training exercise.

Ill members of the Arkansas crew missed 106 working days (Figure 2). One patient with severe chest pain as a result of infection was medically evacuated by helicopter. An additional 8 work days were lost by probable cases (N = 63).

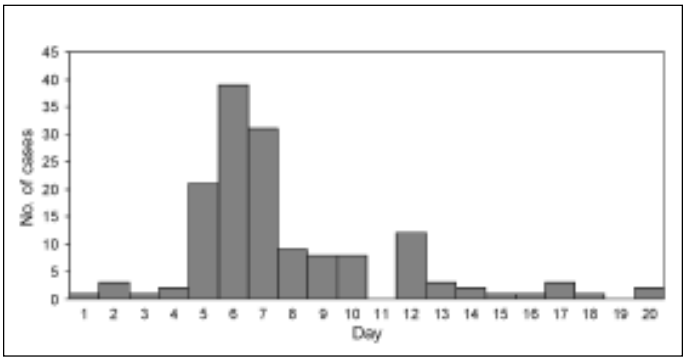


Figure 1. Influenza outbreak aboard USS Arkansas, February 1996.

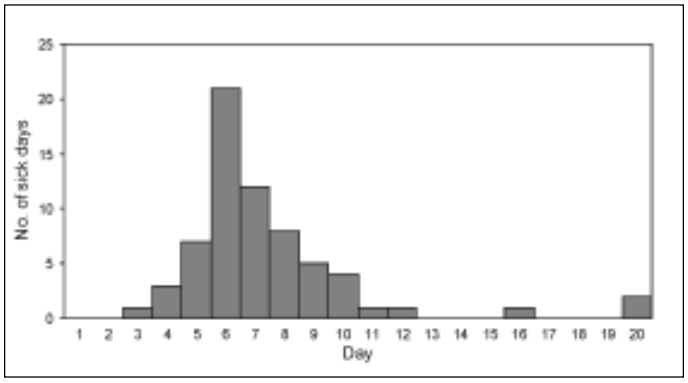


Figure 2. Number of lost workdays during February 1–20, 1996. A lost workday = day a patient is placed at bed rest with influenza.

After influenza A was identified in the crew, amantadine, 100 mg twice a day for 5 days, was offered to all unvaccinated persons (until the outbreak subsided) and to those in the first 2 days of illness. A total of 59 persons chose to take the antiviral drug, 49 for therapy and 10 for prophylaxis; 11 of 59 had side effects (insomnia, 4; headache, 4; nausea, 2; dizziness, 1; and bad dreams, 1). One person discontinued therapy because of headaches. A total of 28 unvaccinated crew members were onboard; 11 (61%) of the 18 unvaccinated persons who did not take amantadine met case criteria for influenza, but none of 10 unvaccinated persons who took amantadine prophylaxis had influenza-like symptoms (p-value <0.03, Fisher's exact test, 1-tailed).

Interviews and record reviews yielded a possible explanation for an outbreak's occurring on the Arkansas but not on the California, a ship with the same home port and vaccination schedule and a crew in close proximity until the day of departure. A sailor returned by plane to Bremerton from vacation in North Carolina on January 27 and became ill on January 28. He reported to the USS Arkansas on the day of departure (February 1). On January 28, a second sailor visited the first sailor in his home. The second sailor became ill on January 31 and was immediately placed on the sick list upon reporting to the Arkansas on February 1. On February 3, members of the crew who worked and lived in the same parts of the ship as the second sailor became ill. The outbreak peaked 3 days later. This correlated with a 3-day incubation period and efficient transmission. Four of six members of the investigating team (all Navy personnel vaccinated with the 1995-96 influenza vaccine) had an influenza-like illness that appeared to respond to amantadine therapy.

Influenza A was isolated from 30 of 50 throat culture specimens. They were confirmed as influenza A (H3N2) by the San Diego County Public Health Laboratory. All five isolates submitted to CDC were antigenically characterized as A/Wuhan/359/95-like.

This outbreak demonstrates the potential for rapid spread of influenza A throughout a confined population despite appropriate vaccination. The efficiency of human-to-human transmission is emphasized by the fact that there was no discernible difference in attack rates between various areas of the ship by the end of the outbreak. Although over 95% of the Arkansas crew were appropriately immunized with the 1995-96 influenza vaccine, at least 42% became ill

with influenza; when definite and probable cases were included, the attack rate was 54%, for an estimated 46% efficacy of the 1995-96 influenza vaccine against the Wuhan strain.

The outbreak reiterates that optimal prevention of influenza by vaccination depends on the antigenic fit between the vaccine strain and the infecting virus, which, in turn, is dependent on the early identification of drift variants. The identification of the drift variant, A/Wuhan/359/95(H3N2), as the causative pathogen underscores the need for increasing the number as well as the capabilities of surveillance laboratories worldwide to rapidly isolate and identify strains of currently circulating influenza viruses. In recent years, the Department of Defense has augmented CDC and WHO surveillance efforts through establishing a collaborative network of >30 domestic and international influenza surveillance sites (8,9). Influenza isolates from this surveillance have proven valuable in decisions about vaccine content.

### Acknowledgments

We thank the medical department of the USS Arkansas for assistance with data collection; the crews of the USS Arkansas and USS California for their cooperation during the investigation; Helen Regnery and the Influenza Branch, Strain Surveillance Section, CDC, for antigenic characterization; and Capt. Patrick E. Olson, for critical review of the manuscript.

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### References

1. Cox NJ, Fukuda K. Influenza. Infect Dis Clin North Am 1998;2:27-38.
2. White DC. A shipboard epidemic of Asian influenza. US Armed Forces Medical Journal 1957;8:1717-25.
3. Ninety-first annual report of the Surgeon General, US Navy: Statistics of diseases and injuries in the United States Navy, calendar year 1955. Washington: Department of the Navy, Bureau of Medicine and Surgery; 1956.
4. Seal JR. Reactions to influenza vaccine. US Armed Forces Medical Journal 1955;6:1559-63.
5. Immunizations and chemoprophylaxis. Bureau of Medicine and Surgery Instruction 6230.15. Washington: Department of the Navy, Bureau of Medicine and Surgery;1995.
6. Marwick C. Antigenically different subtype in new flu vaccine. JAMA 1996;275:825.
7. Centers for Disease Control and Prevention. Update: influenza activity—worldwide, 1996. MMWR Morb Mortal Wkly Rep 1996;45:816-9.
8. Gambel J, Shlim D, Canas LC, Cox NJ, Regnery HL, Scott RM, et al. Partnerships for detecting emerging infectious diseases: Nepal and global influenza surveillance. Emerg Infect Dis 1998; 4:128-30.
9. Gray GC, Callahan JD, Hawksworth AW, Fisher CA, Gaydos JC. Respiratory diseases among U.S. military personnel: countering emerging threats. Emerg Infect Dis 1999; 5:379-85.

# HIV-1 Group O Infection in Cameroon, 1986–1998

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We report a survey of HIV-1 group O infection in Cameroon during 1986 to 1998. The prevalence of HIV-1/O decreased from 0.6% to 0.4%, while HIV-1/M increased from 19.2% to 31.5% from 1994 to 1998. We concluded that HIV-1/O infection is stable in Cameroon and may be declining slightly.

HIV-1 group O (HIV-1/O) was first identified in Cameroon in 1994 (1), raising concern about the emergence of a new HIV-1 variant, with implications for public health and blood safety. Anti-HIV-1/O antibodies are weakly detected by some HIV-1 screening tests (2), and the natural resistance of most HIV-1/O strains to nonnucleoside inhibitors of reverse transcriptase limits their use in preventing vertical transmission (3).

## The Study

All HIV-1-positive samples stored at the Centre Pasteur from 1986 to 1991, from January 1994 to December 1995, and from July 1997 to June 1998 were systematically tested to differentiate HIV-1/M and HIV-1/O infections by using a group-specific, synthetic peptide-based enzyme immunoassay with high reliability (4). The Centre Pasteur is the public health national reference laboratory and a reference center for HIV confirmation in Cameroon.

We compared data from two main regions (encompassing more than 47% of the country's population) on opposite sides of Cameroon. We chose these regions because they differ in environment (rain forest in the central region and grasslands in the north) and sociocultural characteristics (mostly Christians in the central region and Muslims in the north). Data from other regions were insufficient, especially for the total number of samples tested, to be included in our analysis.

We tested all samples (18,921) collected in 1994-95 and 1997-98 in the north and central regions and referred by physicians to our laboratory for HIV screening or sent by collaborating laboratories for confirmation of HIV-positive results. The 4,722 samples confirmed as HIV-1 positive were further differentiated into groups M and O (Table 1).

ELAVIA Mixt (Sanofi Diagnostics Pasteur, Marnes La Coquette, France) was used as the screening enzyme-linked immunosorbent assay (ELISA). Positive samples were further tested with Enzygnost HIV1/2 (Behring, Marburg, Germany), and subsequently, Western blot (LAV blot, Sanofi Diagnostics Pasteur) was systematically performed. After January 1992, GeneElavia Mixt from Sanofi Diagnostics Pasteur was used as the screening ELISA, followed by the above-mentioned assays until July 1993, when Wellcozyme HIV-1 recombinant (Murex, Dartford, UK) began to be used as the confirmatory second reagent. Samples giving discordant results were further tested by Enzygnost HIV1/2 and Western blot.

Table 1. Trends in HIV-1/O and /M positivity in two major regions of Cameroon, 1994–1998

| Periods | Total tested | Positive (%) | HIV-1/M (%) | HIV-1/O (%) |
|---|---|---|---|---|
| Central region | | | | |
| 1994-95 | 6,008 | 908 (15.1) | 859 (14.3) | 49 (0.8) |
| 1997-98 | 5,470 | 1,823 (33.3) | 1,800 (32.9) | 23 (0.4) |
| Total | 11,478 | 2,731 (23.8) | 2,659 (23.2) | 72 (0.6) |
| Northern region | | | | |
| 1994-95 | 4,818 | 1,231 (25.6) | 1,215 (25.2) | 16 (0.3) |
| 1997-98 | 2,625 | 760 (29.0) | 750 (28.6) | 10 (0.4) |
| Total | 7,443 | 1,991 (26.7) | 1,965 (26.4) | 26 (0.3) |

Data from stored samples indicate a substantial drop in the relative frequency of HIV-1/O (Table 2). The apparent relative frequencies of HIV-1/M and HIV-1/O varied significantly from 1986 to 1998, with an increase in HIV-1/M and decrease in HIV-1/O infections (chi square for trend = 297.52; $p<10^{-8}$). To determine whether the observed decrease of HIV-1/O is absolute or relative, we averaged the number of positive samples by the total number of sera tested.

Pooled results from the two regions show that the prevalence of HIV-1/M was significantly higher in 1997-98 than in 1994-95 (2,550 [31.5%] of 8,095] versus 2,074 [19.2%] of 10,826). The odds ratio (OR) (1.94; 95% confidence intervals [CI] 1.81-2.08) is highly significant ($p<10^{-7}$). The prevalence of

Table 2. Differentiation into group M and O of HIV-1 positive samples, 1986–1998

| Periods | No. of samples | HIV-1/M (%) | HIV-1/O (%) |
|---|---|---|---|
| 1986-1988 | 301 | 239 (79.4) | 62 (20.6) |
| 1989-1991 | 625 | 571 (91.4) | 54 (8.6) |
| 1994-1995 | 2,458 | 2,376 (96.7) | 82 (3.3) |
| 1997-1998 | 4,160 | 4,100 (98.6) | 60 (1.4) |
| Total | 7,544 | 7,286 (96.6) | 258 (3.4) |

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HIV-1/O was lower in the second period, but not significantly: 33 (0.4%) of 8,095 versus 65 (3%) of 2,139 (OR 0.68; 95% CI [0.44-1.05]; p=0.07). Data were then analyzed separately by region. In the central region, HIV-1/O infections declined significantly, from 49 (0.8%) of 6,008 to 23 (0.4%) of 5,470 (OR 0.51; p=0.008) (Table 1).

HIV-1/O infections did not change significantly in the north from 1994-95 to 1997-98: 16 (0.3%) of 4,818 to 10 (0.4%) of 2,625 (OR 1.15; p=0.7) (Table 1). In both regions, a significant increase of HIV-1/M was observed from 1994 to 1998. The prevalence of HIV-1/M infections almost doubled in the central region during that period (OR 2.94; $p<10^{-7}$).

## Conclusions

Our data show a decline of HIV-1/O in HIV-1-positive samples from 1986 to 1998. Refined analysis for a defined time (1994 to 1998) in two major Cameroonian regions shows stable prevalence of HIV-1/O infections in the north and a decrease in the central region, contrasting with a concomitant dramatic increase in HIV-1/M in both regions. These trends suggest that HIV-1/O may have decreased fitness compared with group M HIV. Experimental studies are in progress to corroborate these epidemiologic observations.

We checked for the possibility of sampling biases, especially for the period 1986 to 1988, when the apparent relative frequency of HIV-1/O was the highest. We found no differences in recruitment. Moreover, the same diagnostic algorithm was used from 1986 to December 1991.

Our 12-year survey demonstrates that HIV-1/O infection was not emerging at the time it was first described (1). Moreover, as the first well-documented case of HIV infection in the world was in a patient infected in 1966 with an HIV-1/O virus (5) and HIV-1/O viruses seem as pathogenic as HIV-1/M (Saimot AG et al., unpubl. data) understanding why this variant remains stable or is even declining merits further study.

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Dr. Ayouba conducts research in the virology laboratory of the Centre Pasteur du Cameroun. His work focuses on the biodiversity of HIV and the mechanisms of mother-to-child transmission of HIV.

### References

1. Gurtler LG, Hauser PH, Eberle J, von Brunn A, Knapp S, Zekeng L, et al. A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. J Virol 1994;68:1581-5.
2. Loussert-Ajaka I, Ly TD, Chaix ML, Ingrand D, Saragosti S, Courouce AM, et al. HIV-1/HIV-2 seronegativity in HIV-1 subtype O infected patients. Lancet 1994;343:1393-4.
3. Descamps D, Collin G, Letourneur F, Apetrei C, Damond F, Loussert-Ajaka I, et al. Susceptibility of HIV-1 group O isolates to antiretroviral agents: in vitro phenotypic and genotypic analyses. J Virol 1997;71:8893-8.
4. Mauclere P, Damond F, Apetrei C, Loussert-Ajaka I, Souquiere S, Buzelay L, et al. Synthetic peptide ELISAs for detection of and discrimination between group M and group O HIV type 1 infection. AIDS Res Hum Retroviruses 1997;13:987-93.
5. Jonassen TO, Stene-Johansen K, Berg ES, Hungnes O, Lindboe CF, Froland SS, et al. Sequence analysis of HIV-1 group O from Norwegian patients infected in the 1960s. Virology 1997;231:43-7.

# VEB-1-Like Extended-Spectrum β-Lactamases in *Pseudomonas aeruginosa*, Kuwait

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Two clinical *Pseudomonas aeruginosa* isolates from patients in intensive care units in Kuwait were resistant to expanded-spectrum cephalosporins and showed a synergistic effect between ceftazidime and clavulanic acid. This is the first report of extended-spectrum enzymes from nosocomial isolates from the Middle East.

*Pseudomonas aeruginosa* has an inducible, naturally occurring cephalosporinase that confers low-level resistance to aminopenicillins and narrow-spectrum cephalosporins such as cephalothin and cefoxitin (1). Resistance to extended-spectrum cephalosporins may arise from overexpression of this cephalosporinase, acquired beta-lactamases, or both (1). The acquired beta-lactamases may be either clavulanic-acid inhibited (mostly Ambler class A enzymes) or clavulanic-acid resistant (class B and class D enzymes) (2). The class A extended-spectrum beta-lactamases (ESBLs) may derive from narrow-spectrum beta-lactamases of TEM and SHV types, as extensively reported for *Enterobacteriaceae* and rarely for *P. aeruginosa* (2). Other class A enzymes reported in *P. aeruginosa* include PER-1, which we first identified as chromosomally located and which is widespread in *P. aeruginosa* isolates in Turkey (11% of the hospital isolates) (3,4). Lately, another class A ESBL integron-located gene, $bla_{\text{VEB-1}}$, has been identified from *P. aeruginosa* and enterobacterial isolates from Southeast Asia (5-7).

We report on two novel VEB-1-like beta-lactamases from *P. aeruginosa* clinical isolates from Kuwait. This is the first report of extended-spectrum enzymes from nosocomial isolates from this part of the world.

## The Study

*P. aeruginosa* KU-1 was isolated in January 1999 at Ibn Sina Hospital in Kuwait from endotracheal secretions of a 1-day-old infant with respiratory tract infection, hospitalized in the intensive care unit (ICU) unit because of severe enterocolitis. He was first treated with cefotaxime, amikacin, and metronidazole. These antibiotics were discontinued, and he was given imipenem. He improved and was discharged 7 days later. As it was a single isolate of a multidrug-resistant strain, isolation precautions were carried out only in the neonatal ICU. The infant's mother could not remember whether she had received antibiotic therapy during her pregnancy (which was uneventful). She had not traveled outside Kuwait.

*P. aeruginosa* KU-2 was also isolated in June 1999 from the urine of a 73-year-old man admitted to the ICU of another Kuwaiti hospital, Mubarak Al-Kabeer, with ischemic chest pain and bronchectiasis. On day 2 of his hospitalization, fever developed, but blood and urine cultures were negative. He was treated with ceftazidime for 12 days beginning on day 2. During his hospital stay, hematuria developed, followed by urine retention. On day 28, pus from a urinary catheter infection after transurethral prostatitic surgery grew *P. aeruginosa* KU-2 that was susceptible to norfloxacin. *P. aeruginosa* KU-2 was the only *P. aeruginosa* strain isolated from this patient's clinical specimens. He was treated with norfloxacin. Repeated urine cultures did not yield any organism, and he was discharged 15 days later. He had no history of travel outside Kuwait.

Strains from both patients were identified by using an API-20 NE system (Biomerieux, Marcy-l'Etoile, France). Preliminary antibiotic susceptibility testing by disc diffusion (5) revealed a slight synergy between ceftazidime- and clavulanic acid-containing discs for two clinical isolates, *P. aeruginosa* KU-1 and KU-2. Susceptibility testing of beta-lactams for *P. aeruginosa* KU-1 and KU-2 was then performed by a Mueller-Hinton agar dilution method (8). Both strains showed decreased susceptibility to all beta-lactams except imipenem and piperacillin/tazobactam (Table). MICs of

Table. MICs of beta-lactams for *Pseudomonas aeruginosa* KU-1 and KU-2 clinical isolates, *P. aeruginosa* PU21(pROT-1), and PU21 reference strain

| | MICs (mg/L) | | | |
|---|---|---|---|---|
| Antibiotic[a] | *P. aeruginosa* KU-1 | *P. aeruginosa* KU-2 | *P. aeruginosa* PU21 (pROT-1) | *P. aeruginosa* PU21 |
| Amoxicillin | >512 | >512 | >512 | 64 |
| Ticarcillin | >512 | 512 | 512 | 16 |
| Ticarcillin+CLA | 64 | 16 | 16 | 16 |
| Piperacillin | 64 | 16 | 16 | 2 |
| Piperacillin+TZB | 32 | 8 | 8 | 1 |
| Ceftazidime | 512 | 512 | 512 | 0.25 |
| Ceftazidime+CLA | 16 | 8 | 8 | 0.25 |
| Ceftazidime+TZB | 8 | 4 | 8 | 0.50 |
| Cefotaxime | 512 | 128 | 128 | 4 |
| Cefotaxime+CLA | 64 | 16 | 16 | 2 |
| Imipenem | 2 | 2 | 2 | 0.12 |
| Aztreonam | >512 | >512 | 512 | 0.5 |
| Aztreonam+CLA | 64 | 8 | 8 | 0.5 |

[a]CLA: clavulanic acid at a fixed concentration of 2 mg/L; TZB: tazobactam at a fixed concentration of 4 mg/L.

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ceftazidime were decreased by addition of clavulanic acid and tazobactam, indicating the presence of a clavulanic-acid inhibited ESBL (Table). According to antibiotic susceptibility testing by Mueller-Hinton agar disc diffusion, *P. aeruginosa* KU-1 and KU-2 were also resistant to aminoglycosides (amikacin, gentamicin, netilmicin, tobramycin), chloramphenicol, tetracyclines, sulfonamides, and fluoroquinolones (except for KU-2).

Beta-lactamase extracts from cultures of *P. aeruginosa* KU-1 and KU-2 were obtained (8). Isoelectric focusing analysis (8) revealed beta-lactamases with isoelectric points of 7.4 and 8-8.4, the latter likely corresponding to *P. aeruginosa* AmpC cephalosporinases. Whole-cell DNAs of *P. aeruginosa* KU-1 and KU-2 were then obtained (3). Preliminary polymerase chain reaction (PCR) experiments were performed with DNAs of *P. aeruginosa* KU-1 and KU-2 as templates and primers specific for the following class A beta-lactamases: TEM, SHV, CARB (PSE-1), GES-1, PER-1, and VEB-1 (3,5,9-11). Only PCR using internal primers for $bla_{\text{VEB-1}}$ gave a positive result with an identical 642-bp fragment. External $bla_{\text{VEB-1}}$ specific primers gave 1,070-bp PCR fragments with DNAs of both *P. aeruginosa* strains as templates that were sequenced on both strands (6). The deduced amino acid sequences, obtained over the internet (8), identified VEB-1-like sequences that shared 99% amino acid identity with VEB-1 (Figure). Compared with VEB-1, the amino acid changes in VEB-1a and VEB-1b from *P. aeruginosa* KU-1 and KU-2, respectively, occurred in the putative leader peptide sequence (Figure). Thus, the hydrolytic activity of VEB-1a and VEB-1b should be identical to that reported for VEB-1 beta-lactamase (5).

The genetic background of $bla_{\text{VEB-1a}}$ and $bla_{\text{VEB-1b}}$ was further characterized. Plasmid extraction, conjugation, and electroporation experiments were performed (8). A plasmid (pROT-1) of ca. 70 kb carrying $\text{bla}_{\text{VEB-1a}}$ gene was identified according to hybridization results by using an internal PCR-obtained probe for $bla_{\text{VEB-1}}$ (5). Plasmid pROT-1 was self-conjugative from *P. aeruginosa* KU-1 to in vitro obtained rifampin-resistant *P. aeruginosa* PU21 reference strain after selection of transconjugants onto Mueller-Hinton agar plates each containing 150 µg/mL rifampin and 200 µg/mL ticarcillin. As assessed by antibiotic susceptibility testing by disc diffusion, plasmid pROT-1 conferred additional resistance to gentamicin, netilmicin, sulfonamides, and tobramycin. MICs of beta-lactams for *P. aeruginosa* PU21 (pROT-1) mirrored those obtained for *P. aeruginosa* KU-1 (Table). While the plasmid location of $bla_{\text{VEB-1}}$ gene is known only in Enterobacteriaceae (5-7), its report in *P. aeruginosa* may signal the evolution of its spread. The $bla_{\text{VEB-1b}}$ gene was not plasmid located, but a PCR-obtained 642-bp internal probe for $bla_{\text{VEB-1}}$ hybridized at chromosomal position of whole-cell DNA of *P. aeruginosa* KU-2.

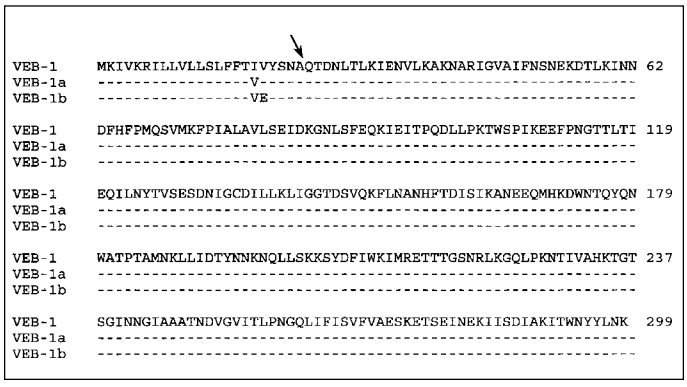


Figure. Comparison of the amino acid sequence of VEB-1, VEB-1a, and VEB-1b beta-lactamases from *Pseudomonas aeruginosa* JES from Thailand and KU-1 and KU-2 from Kuwait. Dashed lines indicate identical amino acids. Arrow indicates position of the putative cleavage site of the peptide leader. Numbering is according to Ambler designation (12).

The $bla_{\text{VEB-1}}$ gene is located on different structures of class 1 integrons (5-7). Integrons comprise two conserved regions (5'-CS and 3'-CS) flanking an internal variable region usually containing several gene cassettes (13). Integrons are in fact expression vectors for antibiotic resistance genes that are included as gene cassettes and are neighbored (13). By using primers located either in the 5'-CS sequence and the 5' end of $bla_{\text{VEB-1}}$ or in the 3' end of $bla_{\text{VEB-1}}$ and the 3'-CS sequence (5,14), PCR amplification experiments were performed with whole-cell DNAs of *P. aeruginosa* KU-1 and KU-2 as templates. In one case (strain KU-1), a PCR fragment was obtained by using $bla_{\text{VEB-1}}$ and 5'-CS primers, indicating that $bla_{\text{VEB-1a}}$ was located downstream of a class 1 integrase gene. In this case, the 4-kb PCR fragment differed from those of known $bla_{\text{VEB-1}}$ containing integrons identified in *Escherichia coli* and *P. aeruginosa* isolates (5-7). Amplimers of 1 kb were obtained for both strains using $bla_{\text{VEB-1}}$ and 3'-CS primers, showing that the 3'-CS end was present in both cases and that the $bla_{\text{VEB-1}}$-like sequences were located next to the 3'-CS end within class 1 integrons. The *attC (59-be)* recombination sites (15) located downstream of gene cassettes were identical for $bla_{\text{VEB-1a}}$ and $bla_{\text{VEB-1b}}$ to those described for $bla_{\text{VEB-1}}$ in *P. aeruginosa* and enterobacterial isolates identified so far from Southeast Asia (5-7). Therefore, an identical $\text{bla}_{\text{VEB-1}}$-like gene cassette may be located on different class 1 integrons. Using 5'-CS and 3'-CS primers, two additional PCR fragments were obtained for each *P. aeruginosa* strain, showing that both strains contained another $bla_{\text{VEB}}$-negative class 1 integron. For *P. aeruginosa* KU-1, a 950-bp PCR fragment for an *aadA1a* gene coding for an aminoglycoside modifying enzyme was found to be plasmid- and integron-located in *Salmonella enterica* serotype Typhimurium (16). For *P. aeruginosa* KU-2, a 500-bp PCR fragment encoding a putative 95 amino acid protein of unknown function was PCR amplified. It shared 71% amino acid identity with an amino acid sequence from a gene that was Tn*1696* transposon-located and In4 integron-located in *P. aeruginosa* (17).

Finally, *P. aeruginosa* KU-1 and KU-2 isolates containing VEB-1-like beta-lactamases were compared with VEB-1 positive *P. aerugionosa* strain JES from Thailand by using random amplified polymorphic DNA technique (10,18). The isolates were not clonally related (data not shown). Although the patients had not traveled outside Kuwait, introduction of *P. aeruginosa* into Kuwaiti hospitals by travelers or patients from Southeast Asia cannot be ruled out.

## Conclusions

The presence of clavulanic-acid inhibited ESBLs in *P. aeruginosa* isolates may account for part of the 50% resistance to ceftazidime of *P. aeruginosa* strains isolates from ICUs in Kuwait (19). ESBLs in *P. aeruginosa* in Kuwait

and other Middle Eastern hospitals may be underestimated because routine detection with a double disc synergy test may be difficult. Identification of ESBLs is of interest since they confer resistance to all extended-spectrum cephalosporins and aztreonam, whatever their MICs. This has been confirmed by experimental data using a model of pneumonia in rats with the Ambler class A ESBL, PER-1 (20).

This work underscores that very similar ESBLs may be identified in different parts of the world. It is the first report of ESBL genes characterized from *P. aeruginosa* isolates from the Middle East.

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## References

1. Chen HY, Yuan M, Livermore DM. Mechanisms of resistance to ß-lactam antibiotics amongst *Pseudomonas aeruginosa* isolates collected in the UK in 1993. J Med Microbiol 1995;43:300-9.
2. Nordmann P, Guibert M. Extended-spectrum ß-lactamases in *Pseudomonas aeruginosa*. J Antimicrob Chemother 1998;42:128-31.
3. Nordmann P, Naas T. Sequence analysis of PER-1 extended-spectrum ß-lactamase from *Pseudomonas aeruginosa* and comparison with class A ß-lactamases. Antimicrob Agents Chemother 1994;38:104-14.
4. Vahaboglu H, Ozturk R, Aygun G, Coskunkan F, Yaman A, Kaygusuz A, et al. Widespread detection of PER-type extended-spectrum ß-lactamases among nosocomial *Acinetobacter* and *Pseudomonas aeruginosa* isolates in Turkey: a nationwide multicenter study. Antimicrob Agents Chemother 1997;41:2265-9.
5. Poirel L, Naas T, Guibert M, Chaibi EB, Labia R, Nordmann P. Molecular and biochemical characterization of VEB-1, a novel class A extended-spectrum ß-lactamase encoded by an *Escherichia coli* integron gene. Antimicrob Agents Chemother 1999;43:573-81.
6. Naas T, Poirel L, Karim A, Nordmann P. Molecular characterization of In50, a class 1 integron encoding the gene for the extended-spectrum ß-lactamase VEB-1 in *Pseudomonas aeruginosa*. FEMS Microbiol Lett 1999;176:411-9.
7. Tribuddharat C, Fennewald M. Integron-mediated rifampin resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1999;43:960-2.
8. Poirel L, Naas T, Nicolas D, Collet L, Bellais S, Cavallo JD, et al. Characterization of VIM-2, a carbapenem-hydrolyzing metallo-ß-lactamase and its plasmid- and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. Antimicrob Agents Chemother 2000;44:891-7.
9. Mercier J, Lévesque RC. Cloning of SHV-2, OHIO-1 and OXA-6 ß-lactamase and cloning and sequencing of SHV-1 ß-lactamase. Antimicrob Agents Chemother 1990;34:1577-83.
10. Poirel L, Guibert M, Bellais S, Naas T, Nordmann P. Integron-and carbenicillinase-mediated reduced susceptibility to amoxicillin-clavulanic-acid in isolates of multidrug-resistant *Salmonella enterica* serotype typhimurium DT104 from French patients. Antimicrob Agents Chemother 1999;43:1098-2004.
11. Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P. Biochemical-sequence analyses of GES-1, a novel class A extended-spectrum ß-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. Antimicrob Agents Chemother 2000;44:622-32.
12. Ambler RP. The structure of ß-lactamases. Philos Trans R Soc Lond B Biol Sci 1980;289:321-31.
13. Fluit AC, Schmitz FJ. Class 1 integrons, gene cassettes, mobility, and epidemiology. Eur J Clin Microbiol Infect Dis 1999;18:761-70.
14. Lévesque C, Piché L, Larose C, Roy PH. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob Agents Chemother 1995;39:185-91.
15. Stokes HW, O'Gorman DB, Recchia GD, Parsekhian M, Hall RM. Structure and function of 59-base element recombination sites associated with mobile gene cassettes. Mol Microbiol 1997;26:731-45.
16. Tosini F, Visca P, Luzzi I, Dionisi AM, Pezella C, Petrucca A, et al. Class 1 integron-borne multiple-antibiotic resistance carried by IncFI and IncL/M plasmids in *Salmonella enterica* serotype typhimurium. Antimicrob Agents Chemother 1998;42:3053-8.
17. Hall RM, Brown HJ, Brookes DE, Stokes HW. Integrons found in different locations have identical 5' ends but variable 3' ends. J Bacteriol 1994;176:6286-94.
18. Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 1990;18:6531-5.
19. Jamal WY, El Din K, Rotimi VO, Chugh TD. An analysis of hospital-acquired bacteraemia in intensive care unit patients in a university hospital in Kuwait. J Hosp Infect 1999;43:49-56.
20. Mimoz O, Elhelali N, Léotard S, Jacolot A, Laurent F, Samii K, et al. Treatment of experimental pneumonia in rats caused by a PER-1 extended-spectrum ß-lactamase-producing strain of *Pseudomonas aeruginosa*. J Antimicrob Chemother 1999;44:91-7.

# *Borrelia lonestari* DNA in Adult *Amblyomma americanum* Ticks, Alabama

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Polymerase chain reaction analysis of 204 *Amblyomma americanum* and 28 *A. maculatum* ticks collected in August 1999 near the homes of patients with southern tick-associated rash illness and in control areas in Choctaw County, Alabama, showed *Borrelia lonestari* flagellin gene sequence from two adult *A. americanum*. The presence of *B. lonestari* in *A. americanum* ticks from Alabama suggests that this suspected pathogen may be widespread in the southeastern United States.

Lyme disease is the most commonly reported vector-borne disease in the United States. In the northeastern, midwestern, and western coastal states, the pathogen *Borrelia burgdorferi* sensu lato is well established and is maintained by *Ixodes scapularis* and *I. pacificus* in a variety of rodent reservoirs.

In the southeastern states, where *I. scapularis* is widespread but is less commonly found infected with *B. burgdorferi* or attaching to humans (1,2), isolations from humans are uncommon (3). However, a clinical condition similar to Lyme disease, termed southern tick-associated rash illness (STARI), has been described in humans in the southeastern region of the United States associated with the bite of *Amblyomma americanum* ticks (1,4,5). Moreover, a new spirochete, *B. lonestari*, was described from *A. americanum* on the basis of polymerase chain reaction (PCR) amplification of the flagellin and 16s rRNA genes (6,7). Virtually identical sequences have been found in ticks from geographic regions as disparate as New Jersey and Texas (6), suggesting this organism is widely distributed. Likewise, *Borrelia* spirochetes have been detected in *A. americanum* and *I. scapularis* in Alabama (8,9).

As part of an epidemiologic investigation of a reported cluster of STARI cases in Choctaw County, Alabama, we collected both *A. americanum* and *A. maculatum* ticks adjacent to the houses of suspected patients and in control areas, and the *B. lonestari* flagellin gene sequence was amplified from DNA extracted from *A. americanum*.

## The Study

Ticks were collected with drag cloths in areas around the homes of persons with suspected cases, as well as in control areas. Clinical cases were defined as illness characterized by acute onset of an annular, expanding erythema migrans-like rash at least 5 cm in diameter, when no alterative explanation for the rash can be found; and there is a history of tick bite at the rash site or potential exposure to ticks within 14 days before rash onset. Ticks were identified to species by using standard taxonomic keys.

DNA was isolated from *Amblyomma* sp. by using an extraction procedure reported previously (10). Briefly, individual ticks were frozen in liquid nitrogen, macerated between metal plates, and then homogenized by adding 1 mL of DNA STAT-60 (Tel-Test, Friendswood, TX). The tick homogenate was then incubated with chloroform for phase separation of DNA, which was precipitated with 100% isopropanol. To ensure PCR-quality DNA, all tick extracts were tested for the presence of tick mitochondrial DNA (11). *Amblyomma* DNA was then subjected to a nested PCR procedure for *B. lonestari* by using primers FlaLL/FlaRL, then FlaLS/FlaRS (6). Flagellin-positive samples were further analyzed by using OspA primers as a control for possible flagellin false-positive samples. Subsequent DNA sequencing of positive samples for *Borrelia* sequence identification was done with a Taq Dyedeoxy terminator cycle kit (Applied Biosystems, Foster City, CA) and run on an Applied Biosystems 377 automated sequencer. The derived sequences were aligned with MegAlign (DNASTAR, Inc., Madison, WI) by using the clustal algorithm. Aligned sequences were transferred to PAUP (Sinauer Associates Inc., Sutherland, MA) for phylogentic analysis. Accession numbers D88295 (*B. afzelii*), X75201 (*B. anserina*), D82857 (*B. bissettii*), Y15097 (*B. burgdorferi*), D63372 (*B. garinii*), AF228034 (*B. hermsii*), D43777 (*B. miyamotoi*), D82863 (*B. parkeri*), U26705 (*B. lonestari,* New Jersey isolate), and U26704 (*B. lonestari,* Texas isolate) were used in this comparative genetic analysis.

Two hundred four *A. americanum* (21 adults and 183 nymphs) were collected: 13 adults and 44 nymphs from the properties of controls, and 8 adults and 139 nymphs near homes of persons meeting the STARI case definition. Twenty-nine *A. maculatum* adults were collected from control and STARI case areas. All but five ticks yielded PCR-quality DNA, as determined by PCR amplification of tick mitochondrial DNA (11).

Two (11%) of 19 of adult *A. americanum* ticks analyzed were positive for *B. lonestari* flagellin gene DNA (Table).

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Table. Ticks collected in Choctaw County, Alabama

| Ticks analyzed | *Amblyomma americanum* Adult male | Adult female | Nymphs | *A. maculatum* Adult male | Adult female |
|---|---|---|---|---|---|
| Controls[a] | 11[b] | 2 | 44 | 6 | 12 |
| Cases[c] | 1 | 5[b] | 139 | 7 | 1 |
| Total | 12 | 7 | 183 | 13 | 13 |

[a]Denotes ticks collected near the homes of persons not diagnosed with southern tick-associated rash illness (STARI).
[b]Positive results as demonstrated by amplification of the flagellin gene of *Borrelia lonestari.* A single positive tick was found among 11 adult male *A. americanum* males collected near the homes of controls and 1 of 5 adult females collected near the homes of STARI controls was positive.
[c]Denotes ticks collected near the homes of persons diagnosed with STARI.

Positive results for *B. lonestari* were confirmed by amplification of the 16s rRNA gene as described by Barbour et al. (6). All 183 nymphs and all 26 adult *A. maculatum* were PCR negative for *B. lonestari* flagellin gene DNA. Moreover, all tick DNA samples were PCR negative when analyzed for the *B. burgdorferi* OspA gene. Sequence analyses for both positive samples showed >99% homology with the published *B. lonestari* sequences from New Jersey and Texas. Alabama isolates numbers 1 and 2 were 100% homologous to *B. lonestari* NJ and differed by 1 bp when compared with published sequences of the Texas isolate of *B. lonestari*. Phylogenetic analysis, using maximum likelihood and bootstrap analysis with 500 replications of derived sequences (Figure), illustrated that both isolates clustered with reported sequences of the *B. lonestari* NJ and TX strains and demonstrated considerable divergence from *B. burgdorferi* sensu stricto, the only genospecies shown to cause disease in the United States.

The nucleotide sequences of the *B. lonestari* flagellin gene have been submitted to GenBank and assigned accession numbers AF298653 (Alabama isolate 1) and AF298654 (Alabama isolate 2).

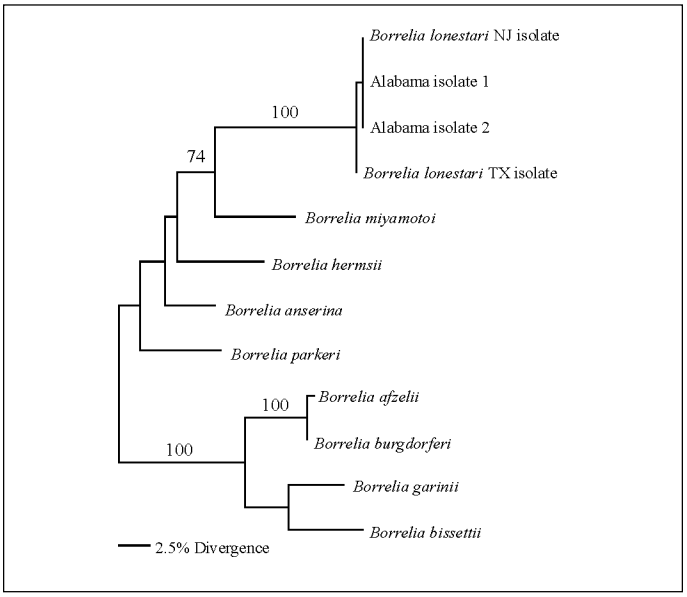


Figure. Maximum parsimony phylogenetic tree generated by using PAUP. Numbers indicate parsimony bootstrap scores for the branch. Only bootstrap scores >70 are included in the phylogenetic tree.

## Conclusions

Spirochetes have been reported in *A. americanum* ticks from New York, New Jersey, Virginia, North Carolina, Alabama, Missouri, and Texas. In contrast to *B. burgdorferi* spirochetes, attempts to propagate these spirochetes from *A. americanum* in Barbour-Stoenner-Kelly (BSK-H) culture have been unsuccessful. Likewise, attempts to coculture *A. americanum* samples with an *I. scapularis* cell line (IDE2) failed to propogate *B. lonestari* (B. Johnson, pers. comm.). Thus, we used genetic analysis to determine the presence of this organism.

Erythema migrans associated with the bites of *A. americanum* has been reported from the southeastern United States, including Missouri (4) and North Carolina (5). Serum samples from these patients did not recognize *B. burgdorferi* antigens (4,5), and spirochetes from these cases have not been successfully cultured in BSK-H medium. Hence, these cases have been diagnosed as STARI. Recently, novel DNA sequences, amplified by PCR with primer sets recognizing the flagellin and 16s rRNA genes, have identified a new spirochete in *A. americanum* and *B. lonestari* (6). Nearly identical sequences have been amplified from *A. americanum* collected from Texas and New Jersey (2) and Maryland (1). *B. lonestari* is the suspected pathogen responsible for the STARI-related erythema migrans associated with bites by *A. americanum* (1). Moreover, *B. lonestari* was isolated from a patient in Westchester County, New York, who had traveled to Maryland and North Carolina and had an attached *A. americanum* at the site of an erythema migrans rash. This isolate differed only slightly from *B. lonestari* isolates reported in New Jersey and Texas (A. James et al., unpub. data).

Although the numbers of ticks we analyzed were small, our results suggest that adult *A. americanum* may transmit *B. lonestari* to persons in this area. Finding *B. lonestari* sequences in *A. americanum* from Alabama suggests that this spirochete is widely distributed in the United States. These first sequences from the southeastern United States are noteworthy because this region is a focus for numerous reports of erythema migrans associated with the bites of *A. americanum.* Further investigation is needed to formally isolate and propagate *B. lonestari*, as well as to determine its host reservoir.

### Acknowledgments

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### References

1. Felz MW, Durden LA. Attachment sites of four tick species (Acari:Ixodidae) parasitizing humans in Georgia and South Carolina. J Med Entomol 1999;36:361-4.
2. Merten HA, Durden LA. A state-by-state survey of ticks recorded from humans in the United States. J Vector Ecol 2000;25:102-13.

3. Oliver JH Jr, Clark KL, Chandler FW, Tao L, James AM, Banks CW, et al. Isolation, cultivation and characterization of *Borrelia burgdorferi* from rodents and ticks in the Charleston area of South Carolina. J Clin Microbiol 2000;38:120-4.
4. Campbell GL, Paul WS, Schriefer ME, Craven RB, Robbins KE, Dennis DT. Epidemiologic and diagnostic studies of patients with suspected early Lyme disease, Missouri, 1990-1993. J Infect Dis 1995;172:470-80.
5. Kirkland KB, Klimbo TB, Meriwether RA, Schriefer M, Levin M, Levine J, et al. Erythema migrans-like rash illness at a camp in North Carolina: a new tick-borne disease? Arch Intern Med 1997;157:2635-41.
6. Barbour AG, Maupin GO, Teltow GJ, Carter CJ, Piesman J. Identification of an uncultivatable *Borrelia* species in the hard tick *Amblyomma americanum*: possible agent of a Lyme disease–like illness. J Infect Dis 1996;173:403-9.
7. Armstrong PM, Rich SM, Smith RD, Hart DL, Spielman A, Telford SR III. A new *Borrelia* infecting lone star ticks. Lancet 1996; 347:67-8.
8. Luckhart SG, Mullen GR, Wright JC. Etiologic agent of Lyme disease, *Borrelia burgdorferi*, detected in ticks (Acari:Ixodidae) collected at a focus in Alabama. J Med Entomol 1991;28:652-7.
9. Luckhart SG, Mullen GR, Durden LA, Wright JC. *Borrelia* sp. in ticks recovered from white-tailed deer in Alabama. J Wildl Dis 1992;28:449-52.
10. Zeidner NS, Burkot TR, Massung R, Nicholson WL, Dolan MC, Rutherford JS, et al. Transmission of the agent of human granulocytic ehrlichiosis by *Ixodes spinipalpis* ticks: evidence of an enzootic cycle of dual infection with *Borrelia burgdorferi* in northern Colorado. J Infect Dis 2000;182:616-9.
11. Black WC, Piesman J. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proc Natl Acad Sci U S A 1994;91:10034-8.

# Physicians' Database Searches as a Tool for Early Detection of Epidemics

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We analyzed retrospectively the use of Physician Desk Reference Database searches to identify epidemics of tularemia, nephropathy, Pogosta disease, and Lyme disease and compared the searches with mandatory laboratory reports to the National Infectious Diseases Register in Finland during 1995. Continuous recording of such searches may be a tool for early detection of epidemics.

Epidemics are conventionally recognized through the observation of spatial or temporal clustering of patients with similar illnesses or through laboratory findings indicating unusually high incidence of a specific disease. Surveillance systems based on passive physician reports frequently have low sensitivity and may not be timely. Recognizing an epidemic by detecting clusters of microbiologically verified cases may also involve delays, depending on such factors as the severity and familiarity of the clinical illness, time from the appearance of the first clinical cases of an epidemic to the first appropriate samples taken and transported, and time required for laboratory testing. Samples from geographically scattered cases from the same epidemic may be sent to different laboratories, reducing the sensitivity of detection. A recent study in infectious disease surveillance used as an index the number of cases per week per sentinel medical institution in the area covered by a health center in Japan (1).

Physicians may consult electronic databases for selecting appropriate diagnostic measures or treatment for specific infectious diseases. A computerized set of primary-care guidelines, the Physicians' Desk Reference and Database (PDRD), has been available in Finland since 1989 (2). This database contains structured information on diseases and conditions that are common or important to recognize in primary care. Physicians often seek information about infectious diseases from the PDRD guidelines (3).

We hypothesized that the frequency of physician searches in a popular database could be useful as a complementary tool in early recognition of infectious disease epidemics. In a pilot study, we analyzed retrospectively the feasibility of using surveillance of database searches in the PDRD to identify epidemics of four specific infectious diseases, as recorded in a recently revised National Infectious Diseases Register (NIDR).

## The Study

The PDRD computerized guidelines on CD-ROM are updated three times a year. We collected the frequency of infection guideline-specific searches by producing a log file of all searches. This data-collecting version was piloted in spring 1994 and mailed to all registered PDRD subscribers in 1995 (3). The data for searches were collected from the computer hard disks of individual users during each updating of the program and were mailed to the PDRD maintenance team.

The NIDR in Finland, which was thoroughly revised in 1994, consists of mandatory laboratory reporting of diagnostic findings for more than 70 pathogens or pathogen groups and mandatory physician reporting of 32 microbiologically confirmed infectious diseases (4). Microbiologic laboratories reported approximately 41,000 cases in 1995. From NIDR, we recorded the sampling dates of cases or the date of the report if the sampling date was not available.

We chose four infectious diseases for comparison between PDRD searches and NIDR laboratory reports: tularemia, ICD-10 A21, caused by *Francisella tularensis*; epidemic nephropathy, ICD-10 A98.5, caused by a Puumala virus (a hantavirus); Pogosta disease, ICD-10 A92.8, caused by the Sindbis virus (an arbovirus); and Lyme disease, ICD-10 A69.2, caused by *Borrelia burgdorferi*. In Finland, epidemic nephropathy (758 to 1,305 laboratory-reported infections per year during 1995-1999) and Lyme disease (346 to 538 laboratory reports of *B. burgdorferi* infections per year) are endemic, with pronounced seasonal variation. Laboratory-confirmed cases of tularemia, mostly of the glandular type, and Pogosta disease, an acute syndrome of fever with rash and self-limiting arthritis, are usually rare, but epidemics occur at intervals of several years.

We compared distributions of PDRD searches and NIDR laboratory reports by disease and month for 1995. Because the number of observations was small, we did not test statistical significance between distributions but based our observations on time-frequency graphs produced by calculating each month's proportion of the total number of cases.

PDRD had 477 subscribers in 1995: 48% of the users returned 306 log files on 15,267 searches; 23,083 specific guidelines were read. The five most popular subject areas were dermatology (9% of searches), infectious diseases (8%), cardiology (6%), gastroenterology (6%), and pediatrics (5%). The Lyme disease guideline was the third most frequently

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read, with 144 readings; Pogosta disease was fifth, with 91; epidemic nephropathy eighth, with 87; and tularemia nineteenth, with 68 readings.

Three hundred forty-six laboratory-confirmed *B. burgdorferi* cases were reported to NIDR, distributed throughout the year (incidence 0.68 per 100,000; 95% confidence interval [CI] 0.60-0.75), but peaking in August. A large epidemic of Pogosta disease took place in late summer and autumn 1995. Cases were distributed throughout the country, with 1,310 laboratory-confirmed (incidence 2.56 per 100,000; 95% CI 2.42-2.70). The 888 laboratory-confirmed cases of Puumala virus infection (1.74; 95% CI 1.62-1.85) peaked in late 1995 and occurred throughout Finland. A major epidemic of tularemia, involving 467 laboratory-reported cases (0.91; 95% CI 0.83-1.00) and distributed over central and southern Finland, began in July 1995 and continued until late autumn.

The distributions of PDRD readings and laboratory-reported cases of the four infectious diseases fell into two patterns (Figure). For tularemia (Figure, panel D) and Pogosta disease (Figure, panel C), the PDRD searches and the cases in NIDR rose from a low baseline level, peaked sharply, and then declined in parallel. For epidemic nephropathy (Figure, panel B), the curves followed the same pattern only partly, with PDRD searches peaking twice, the major peak occurring earlier than in NIDR, in the latter half of 1995. For Lyme disease (Figure, panel A), PDRD search data had a peak well before that of NIDR reports during June to September.

## Conclusions

The temporal correlation observed between the distribution of database searches and laboratory reports for Pogosta disease and tularemia supports the concept that continuous monitoring of database searches for specific infections could be a novel tool for surveillance and detection of epidemics. We are not aware of previous reports of this application for electronic desk reference database searches. For this investigation, we used retrospectively collected logs of CD-ROM–based searches from computers of physicians who used the widely distributed and popular electronic guideline database. The statistics on searches were created automatically into log files without active input by the physicians, and the users had free access to the data they were providing. Although only a few users expressed negative attitudes towards data collection, fewer than half of subscribers returned the data.

Monitoring database searches has the potential to provide timely recognition of an epidemic, as physicians are more likely to use searches to seek guidance for the diagnostics and management of the first patients of an unusual cluster, even before an order for laboratory work is given. Another potential benefit of monitoring database searches at a central facility is that it consolidates consultations from different geographic areas, making it possible to detect subtle changes in widely distributed cases.

Access through the Internet to databases such as the PDRD provides an opportunity to monitor the searches for specific topics. In 1999, the PDRD had 3,500 subscribers throughout Finland, a sevenfold increase from 1995, the year

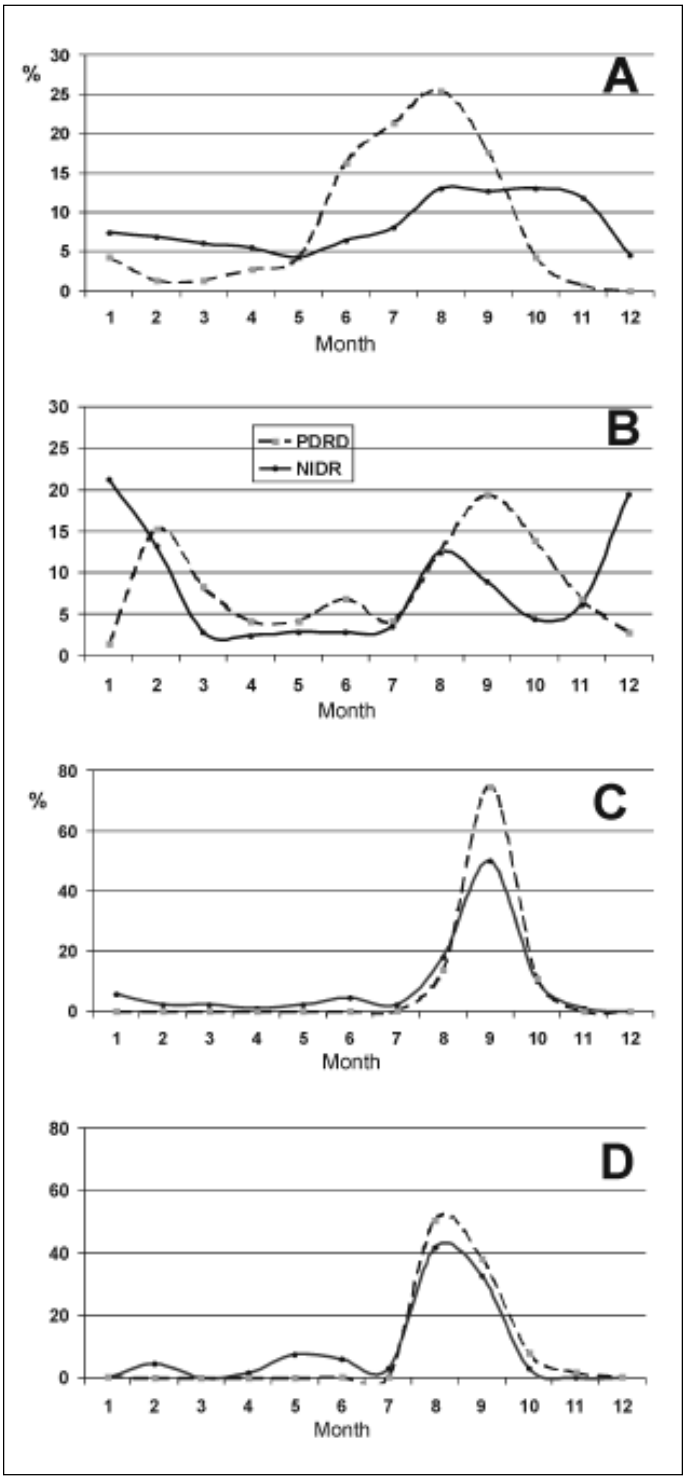


Figure. Laboratory reports to the National Infectious Diseases Register compared with searches on the Physicians' Desk Reference and Database, Finland, 1995. Panel A, *Borrelia burgdorferi* and Lyme disease; panel B, Puumala virus and epidemic nephropathy; panel C, Sindbis virus and Pogosta disease; panel D, *Francisella tularensis* and tularemia.

of this study. An Internet version of the PDRD guidelines (now renamed Evidence-Based Medicine Guidelines) (5), which collects a log file of all searches to the database, was introduced in October 2000. In the future, log files will automatically be sent from CD-ROM users, with their permission. As no patient data are transmitted, no concerns about confidentiality can arise.

Reference databases are likely to be used by clinicians more frequently for syndromes or suspected infections if the physician encounters the specific problem infrequently, e.g., infectious diseases with low transmission levels between epidemics. This observation is supported by the close correlation between the distributions in PDRD searches and NIDR reports of tularemia, epidemics of which have not occurred annually, and Pogosta disease, for which the interepidemic interval has been long (previous epidemics in 1981 and 1988). Search-frequency-based surveillance can never achieve the specificity of laboratory reporting. However, it can provide an effective early warning for infectious diseases in which the clinical syndromes are specific enough to prompt the clinician to search specific guidelines.

For Lyme disease, the increase and peak distribution of the database searches substantially preceded the increase and peak in the laboratory reports of *B. burgdorferi* to the NIDR. The diagnosis of early Lyme disease, with its characteristic skin rash (6), is entirely clinical; serologic diagnosis can be made no earlier than 3 weeks after a tick bite. This delay could explain why the database searches peaked well before the NIDR reports. The earlier peak in database searches could also reflect a search for preventive measures during summer outdoor activities or for tick bite management before symptoms appear.

Dr. Jormanainen works as a consulting physician at the Health Care Division of the General Staff of Finnish Defense Forces, where his research interests include infectious disease epidemiology, surveillance, and public health issues.

## References

1. Hashimoto S, Murakami Y, Taniguchi K, Nagai M. Detection of epidemics in their early stage through infectious disease surveillance. Int J Epidemiol 2000;29:905-10.
2. Jousimaa J, Kunnamo I. PDRD-a computer-based primary care decision support system. Med Inform 1993;18:103-12.
3. Jousimaa J, Kunnamo I, Mäkelä M. Physicians' patterns of using a computerized collection of guidelines for primary health care. Int J Technol Assess Health Care 1998;14:484-93.
4. National Public Health Institute. Infectious diseases in Finland in 1995. Publications of the National Public Health Institute, KTL B8/1996. Helsinki: 1997.
5. Finnish Medical Society Duodecim. Available at: URL: *Http://www.ebm-guidelines.com*. [Note: The English version of the guidelines will be available for registered Internet users in 2001.]
6. Benenson AS, editor. Control of communicable diseases manual. 16th ed. Washington, DC: American Public Health Association; 1995.

# Filamentous Phage Associated with Recent Pandemic Strains of *Vibrio parahaemolyticus*

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A group of pandemic strains of *Vibrio parahaemolyticus* has recently appeared in Asia and North America. We demonstrate that a filamentous phage is specifically associated with the pandemic *V. parahaemolyticus* strains. An open reading frame unique to the phage is a useful genetic marker to identify these strains.

*Vibrio parahaemolyticus*, a halophilic gram-negative rod, causes seafood-borne gastroenteritis in humans. Infections caused by this organism have been associated with diverse serovars: 13 O serotypes and 75 K serotypes have been identified. Recent studies, however, have revealed the emergence and pandemic spread of a single serovar, O3:K6 (1-6). Strains belonging to the O3:K6 serovar abruptly appeared in India in 1996 and have since been isolated in Southeast Asian countries, from travelers at quarantine stations in Japan, and from foodborne outbreaks in the United States (1-5). This serovar accounts for more than half of the *V. parahaemolyticus* isolates from diarrheal patients in Japan (6). Such widespread occurrence of a single serovar of *V. parahaemolyticus* had not previously been reported. Since 1998, *V. parahaemolyticus* strains belonging to other two serovars, O4:K68 and O1:K untypeable (KUT), have also been isolated with increasing frequency from diarrheal patients (2,6,7). The genetic background of the O4:K68 and O1:KUT isolates is almost indistinguishable from that of the recent O3:K6 strains, suggesting a common origin (2,7).

In a previous study, we reported on a filamentous phage that is specifically associated with the recent O3:K6 serovar strains of *V. parahaemolyticus* (8). This phage, f237, has several genes in common with and a similar genomic structure to another filamentous phage, CTX (9), which is known to carry the genes for cholera enterotoxin (*ctxAB*), the most important virulence factor of *V. cholerae*. Instead of *ctxAB*, f237 possesses a unique open reading frame, ORF8, which has no homology with other sequences in DNA databases (8). In this study, we examined the distribution of f237 in recent clinical isolates of *V. parahaemolyticus*.

## The Study

We studied 96 strains of *V. parahaemolyticus* isolated from diarrheal patients during January to May 1999 at the Kansai International Airport quarantine station, Osaka, Japan. Detection of f237 was by colony hybridization using a digoxigenin-labeled DNA probe targeted for ORF8 (8) and prepared as described (8). In brief, after amplification of a partial DNA sequence of ORF8 (746 bp in size) by polymerase chain reaction (PCR) with the genomic DNA of *V. parahaemolyticus* KXV237 strain (RIMD2210633) (8) as a template, the amplified DNA was labeled with digoxigenin using a PCR DIG labeling kit (Boehringer GmbH, Mannheim, Germany). PCR primers for ORF8 were 8S (5'-GTTCGCATA-CAGTTGAGG-3') and 8A (5'-AAGTACAGCAGGAGTGAG-3'). The conditions for PCR were as follows: After heating at 94°C for 3 minutes, a cycle of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 1 minute was repeated 30 times, followed by end extension step at 72°C for 5 minutes. PCR was done on a Perkin-Elmer (Foster City, CA) thermal cycler type 9700. Pulsed-field gel electrophoresis (PFGE) and Southern hybridization were performed as described (10). For PFGE, the *V. parahaemolyticus* genomic DNA in agarose blocks was digested with restriction enzymes *Not*I or *Sfi*I. A contour-clamped homogeneous electric field method was used for PFGE on a CHEF Mapper System (Bio-Rad Laboratories, Richmond, CA). The conditions for PFGE were 1% agarose gel in 0.5x Tris-borate-EDTA buffer at 6 V $cm^{-1}$, with pulse times increasing linearly from 1 to 60 seconds within 24 hours.

Of the 96 strains examined, 53 tested positive for ORF8, suggesting the presence of phage f237 (Table). In addition to O3:K6, the ORF8-positive strains were found in strains with serotypes O4:K68 and O1:KUT. None of the strains with other serovars showed evidence of ORF8. When the genomic DNA of the strains was digested with *Not*I or *Sfi*I endonuclease, PFGE showed very similar restriction patterns for the bacterial genomes of the ORF8-positive strains, irrespective

Table. Distribution of ORF8 in clinical isolates of *Vibrio parahaemolyticus*

| Serovar | ORF8[a] | PFGE genotype[b] | No. of strains |
|---|---|---|---|
| O3:K6 | + | A | 34 |
| | - | A | 1 |
| O4:K68 | + | A | 11 |
| | - | - | 0 |
| O1:KUT | + | A | 8 |
| | - | A | 1 |
| | - | B | 8 |
| Others | + | - | 0 |
| | - | B | 33 |
| Total | | | 96 |

[a]Possession of ORF8: (+) denotes presence of ORF8; (-) denotes absence of ORF8.
[b]Pulsed-field gel electrophoresis (PFGE) pattern of the genomic DNA of strains; A, genotype closely related to recent O3:K6 strains (7); B, genotype distinct from that of recent O3:K6 strains.

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of their serovars (data not shown). Southern hybridization with a probe for ORF8 after PFGE demonstrated that, in all the ORF8-positive strains, the largest *Not*I fragment (size approximately 1,080 kb) reacted with the probe, suggesting that f237 integrated into the bacterial chromosome.

## Conclusions

Our study shows that the filamentous phage f237 is associated not only with O3:K6 serovar but also with other recently emerging serovars of *V. parahaemolyticus*. In the 55 strains showing PFGE genotypes closely related to those of the recent O3:K6 strains, >96% was positive for the ORF8 (Table). Such high prevalence of the phage f237 in the *V. parahaemolyticus* strains showing pandemic spread suggests that the phage might confer a so-far-unknown phenotype to the bacterium. The phenotype might, in turn, protect the organism against selective pressure in a certain environment before it infects humans. Whether phage f237 has played a part in the recently increasing pandemic potency of strains of *V. parahaemolyticus* is a subject for further study.

The virulence of *V. parahaemolyticus* extends beyond acute gastroenteritis: it can also cause wound infections and septicemia (5). Physicians everywhere need to be alert to the possibility of infection from these recently emergent strains. To distinguish the new and increasingly common *V. parahaemolyticus* strains from classic ones, ORF8 is a useful genetic marker.

### Acknowledgments

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### References

1. Okuda J, Ishibashi M, Hayakawa E, Nishino T, Takeda Y, Mukhopadhyay AK, et al. Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. J Clin Microbiol 1997;35:3150-5.
2. Matsumoto C, Okuda J, Ishibashi M, Iwanaga M, Garg P, Rammamurthy T, et al. Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *toxRS* sequence analyses. J Clin Microbiol 2000;38:578–85.
3. Centers for Disease Control and Prevention. Outbreak of *Vibrio parahaemolyticus* infections associated with eating raw oysters—Pacific Northwest, 1997. MMWR Morb Mortal Wkly Rep 1998;47:457-62.
4. Centers for Disease Control and Prevention. Outbreak of *Vibrio parahaemolyticus* infection associated with eating raw oysters and clams harvested from Long Island Sound—Connecticut, New Jersey, and New York, 1998. MMWR Morb Mortal Wkly Rep 1999;48:48-51.
5. Daniels NA, MacKinnon L, Bishop R, Altekruse S, Ray B, Hammond RM, et al. *Vibrio parahaemolyticus* infections in the United States, 1973–1998. J Infect Dis 2000;181:1661–6.
6. World Health Organization. *Vibrio parahaemolyticus*, Japan, 1996-1998. Wkly Epidemiol Rec 1999;74:361-3.
7. Chowdhury NR, Chakraborty S, Eampokalap B, Chaicumpa W, Chongsa-Nguan M, Moolasart P, et al. Clonal dissemination of *Vibrio parahaemolyticus* displaying similar DNA fingerprint but belonging to two different serovars (O3:K6 and O4:K68) in Thailand and India. Epidemiol Infect 2000;125:17-25.
8. Nasu H, Iida T, Sugahara T, Yamaichi Y, Park K-S, Yokoyama K, et al. A filamentous phage associated with recent pandemic *Vibrio parahaemolyticus* O3:K6 strains. J Clin Microbiol 2000;38:2156–61.
9. Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 1996;272:1910–4.
10. Yamaichi Y, Iida T, Park K-S, Yamamoto K, Honda T. Physical and genetic map of the genome of *Vibrio parahaemolyticus*: presence of two chromosomes in *Vibrio* species. Mol Microbiol 1999;31:1513-21.

## *Candida dubliniensis* Candidemia in Australia

**Letter to the Editor:** We read with interest the recent publications of Meis et al. (1) and Brandt et al. (2) describing the first reported cases of *Candida dubliniensis* fungemia from Europe and North America, respectively. To contribute to the growing recognition of the pathogenic role of this organism, we report the first case of *Candida dubliniensis* fungemia from Australia.

A 68-year-old Caucasian woman with a long history of alcohol abuse visited the emergency department with a 5-week history of progressive weakness. On physical examination she was afebrile, cachectic, and confused. Poor oral hygiene with gingivitis and tooth decay was noted, but no oral candidiasis was seen. Stigmata of chronic liver disease, including palmar erythema and spider nevi, were present. Neurologic examination showed generalized muscle weakness, mild cerebellar ataxia, and peripheral neuropathy.

Abnormal laboratory tests included hemoglobin 8.7 g/dL with macrocytosis, neutrophils 0.2 x $10^9$/L, platelets 98 x $10^9$/L, alkaline phosphatase 229 U/L, gamma glutamyltransferase 128 U/L, calcium 1.62 mmol/L, and albumin 2.4 g/dL. Coagulopathy was identified, with a prothrombin time of 23 seconds and an automated partial thromboplastin time of 44 seconds. The HIV 1 and 2 antibody test was negative.

The patient was admitted to the hospital, where she was rehydrated through a peripheral venous cannula, which was removed on day 5. Ticarcillin-clavulanic acid and gentamicin were administered for 4 days until her neutrophil count increased to >1.0 x $10^9$/L. The cause of the transient neutropenia was not identified. On day 7, the patient was increasingly unwell, with confusion, postural hypotension, and a temperature of 38°C. Yeasts were isolated from a blood culture taken on day 9. Oral and vaginal cultures collected on day 10 did not grow yeast. Treatment with intravenous fluconazole (400 mg/day) was begun. The patient's fever resolved within 48 hours, and her clinical condition improved gradually. Fluconazole was ended on day 37. There was no evidence of metastatic candidemia.

Positive cultures were detected at 31 hours by the BacTAlert (Organon Teknika Corp., Durham, NC). blood culture system, and yeast were present on Gram stain. Subculture on ChromAgar (ChromAgar Candida, Paris, France) grew apple-green colonies, which were germ tube positive. The Analytical Profile Index 20C profile at 48 and 72 hours was 6 1 5 2 0 1 4 (*C. dubliniensis* - 99.9% certainty) and the API 32C profile was 7 1 4 2 1 4 0 0 1 5, which matches the reported profile for *C. dubliniensis* (3). The isolate grew well at 35°C but did not grow at 42°C. Identification was confirmed by Professor T. Patterson, University of Texas (San Antonio) by Ca3 probe, contour clamped homogeneous electric field (CHEF) karyotype, and CHEF pulsed-field gel electrophoresis. The fluconazole MIC was <0.125 mg/mL by the National Committee for Clinical Laboratory Standards broth microdilution method (4).

The initial description by Meis et al. (1) of *C. dubliniensis* fungemia included three patients with chemotherapy-induced immunosuppression and hematologic malignancy. The subsequent publication of Brandt et al. (2) broadens the spectrum of susceptible patients, with two of four patients having end-stage liver disease and one with HIV infection, although the CD4+ count was in the normal range. Our patient also had advanced liver disease but had few other recognizable risk factors for candidemia, with transient neutropenia and a short course of broad-spectrum antimicrobial therapy the only apparent predisposing factors.

Identification of *C. dubliniensis* as the causative pathogen in cases of candida fungemia is important, as concerns have been expressed that resistance may develop rapidly in oral isolates of *C. dubliniensis* in HIV-infected patients treated with fluconazole (5). However, all eight bloodstream isolates reported to date have been susceptible to fluconazole, and treatment with this agent was initiated in every patient. Our patient had a rapid and complete clinical response to fluconazole therapy. Fluconazole appears to be appropriate empiric therapy in patients with no prior azole exposure. However, susceptibility testing should be performed on each isolate to confirm azole sensitivity.

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### References

1. Meis JFGM, Ruhnke M, DePauw BE, Odds FC, Siegert W, Verweij PE. *Candida dubliniensis* candidemia in patients with chemotherapy-induced neutropenia and bone marrow transplantation. Emerg Infect Dis 1999;5:150-3.
2. Brandt ME, Harrison LH, Pass M, Sofair AN, Huie S, Li R, et al. *Candida dubliniensis* fungemia: the first four cases in North America. Emerg Infect Dis 2000;6:46-9.
3. Sullivan DJ, Westerneng TJ, Haynes KA, Bennett DE, Coleman DC. *Candida dublinensis* sp. nov.: phenotypic and molecular characterisation of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology 1995;141:1507-152.
4. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A, vol. 17, no. 9. Wayne (PA): The Committee; 1997.
5. Moran GP, Sullivan DJ, Henman MC, McCreary CE, Harrington BJ, Shanley DB, et al. Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. Antimicrob Agents Chemother 1997;41:616-23.

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## Characterization of a Human Granulocytic Ehrlichiosis-like Agent from *Ixodes scapularis*, Ontario, Canada

**To the Editor:** Human granulocytic ehrlichiosis (HGE), a tick-associated febrile illness first described in Minnesota and Wisconsin in 1994 (1), has recently been reported in a number of European countries (2,3). Molecular and serologic characterization has shown that the HGE agent is closely related or identical to *Ehrlichia equi* and *E. phagocytophila* (4,5). In the United States, human cases of HGE overlap the range of the blacklegged tick, *Ixodes scapularis*, and the detection of HGE agent DNA in this species

provides direct evidence that this arthropod is a competent vector (4). We report the first identification and characterization of an HGE-like agent in a blacklegged tick collected in a tick-endemic area of Canada (6).

Sixty male and 60 female *I. scapularis* were collected from five white-tailed deer shot on Long Point Peninsula, Ontario, during November 1999. Live ticks were cut longitudinally into halves, and half of each specimen was placed in lysis buffer from a QIAamp DNA Mini Kit (Qiagen Inc., Canada); DNA was extracted per manufacturer's instructions. Five microliters of extracted tick DNA was then added to a polymerase chain reaction (PCR) mixture containing primers Ehr 521 and Ehr 790 (7), and the resulting amplification products were run on agarose gels. Extracted DNA from one male tick generated the expected 293-bp HGE agent amplicon. Preliminary DNA sequencing analysis of the putative granulocytic *Ehrlichia* PCR product indicated a high sequence identity with HGE agent 16S rDNA. To further genotype this HGE-like agent, an 894-bp portion of 16S rDNA was amplified by using primers ge3a, ge9f, ge10r (8), and primer mdge9r (5' ATGTCAAGGAGTGGTAAGGT) in a nested PCR reaction.

Genetic characterization of the Long Point HGE-like agent (designated here as L3H) was carried out by sequencing an 849-bp portion of the rDNA gene and comparing it with other HGE-like agents in GenBank. Within the rDNA portion sequenced, L3H shares 99.6% identity with the HGE agent and *E. equi*/*E. phagocytophila*. In the 849-bp portion of the rDNA gene amplified and sequenced, the L3H strain differed from the HGE agent by three nucleotides. Comparison of L3H with HGE-like agents from the United States, Europe, and China suggests a high degree of sequence identity at the rDNA level; however, a number of nucleotide positions did show variation. (GenBank accession number for L3H is AF311343.)

This study documents for the first time (by rDNA sequence comparisons) that *I. scapularis* from a tick-endemic site in Canada can harbor an ehrlichia of the *E. equi* genogroup and is closely related to the HGE agent. The taxonomic significance of HGE-like agents that vary somewhat in their rDNA sequence is still unclear. HGE-like agents from diverse geographic locations and various hosts can exhibit nucleotide differences at a number of positions and still be >99% similar at the level of rDNA sequence identity. Recently it has been shown that sequencing of a more variable genomic region, such as the *ank* gene of granulocytic ehrlichia, can group these agents into different North American and European genetic clades or genogroups (9). Whether all HGE-like "variants" that differ somewhat in their rDNA or *ank* gene sequences can cause human or animal disease remains to be determined.

The identification of an HGE-like agent further highlights the concern that *I. scapularis* may transmit a number of pathogens to humans or other animals in Canada. Public health officials and veterinarians should be aware of this finding and consider HGE in the differential diagnosis of patients or clients with relevant clinical presentations. Further studies documenting the prevalence of the HGE-like agent(s) in ticks from Canada and characterization of any agents identified are warranted to better define potential human and animal health risks.

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### References

1. Bakken JS, Dumler JS, Chen SM, Eckman MR, Van Etta LL, Walker DH. Human granulocytic ehrlichiosis in the upper midwest United States. A new species emerging? JAMA 1994;272:212-8.
2. Petrovec M, Furlan SL, Zupanc TA, Strle F, Brouqui P, Roux V, et al. Human disease in Europe caused by a granulocytic *Ehrlichia* species. J Clin Microbiol 1997;35:1556-9.
3. Laferl H, Hogrefe W, Kock T, Pichler H. A further case of acute human granulocytic ehrlichiosis in Slovenia. Eur J Clin Microbiol Infect Dis 1999;18:385-92.
4. Walker DS, Dumler JS. Emergence of the ehrlichioses as human health problems. Emerg Infect Dis 1996;2:18-28.
5. Chen SM, Dumler JS, Bakken JS, Walker DS. Identification of a granulocytotropic *Ehrlichia* species as the etiological agent of human disease. J Clin Microbiol 1994;32:589-95.
6. Watson TG, Anderson RC. *Ixodes scapularis* Say on white-tailed deer (*Odocoileus viginianus*) from Long Point, Ontario. J Wildl Dis 1976;12:66-77.
7. Kolbert C. Detection of the agent of human granulocytic ehrlichiosis by PCR. In: Persing D, editor. PCR protocols for emerging infectious diseases. Washington: American Society for Microbiology Press; 1996. p. 106-11.
8. Massung RF, Slater K, Owens JH, Nicholson WL, Mather TN, Solberg VB, et al. Nested PCR assay for detection of granulocytic ehrlichia. J Clin Microbiol 1998;36:1090-5.
9. Massung RF, Owens JH, Ross D, Reed KD, Petrovec M, Bjoersdorff A, et al. Sequence analysis of the *ank* gene of granulocytic ehrlichiae. J Clin Microbiol 2000;38:2917-22.

## High Prevalence of Sin Nombre Virus in Rodent Populations, Central Utah: A Consequence of Human Disturbance?

**To the Editor:** Sin Nombre virus (SNV) (Bunyaviridae) is a newly discovered hantavirus responsible for hantavirus pulmonary syndrome (HPS) in humans. The deer mouse, *Peromyscus maniculatus,* is its primary reservoir (1,2). To address a gap in our understanding of the temporal dynamics of SNV, we conducted a longitudinal study in the Great Basin.

Our study site was the West Tintic Mountains, Jericho, Utah, 39°57' N, 112°22' W. We trapped on May 29 to 31, July 10 to 12, and October 7 to 9, 1999. Previous research (3) indicated woodrats (*Neotoma lepida*) were reservoirs for SNV; therefore, we concentrated our trapping efforts at woodrat middens. Middens (2 m diam) are structures of thousands of sticks built by woodrats and serve as nesting sites for a variety of small rodents, including deer mice (4,5). Each night of the 3-night trapping session, we set ~3 live traps baited with oats, peanut butter, and cotton at each of ~40 middens.

Captured rodents were collected each morning and anesthetized with Metafane (methoxyflurane). Animals were identified to species and ear-clipped for future identification. Scarring, body mass, sex, tail length, and reproductive status were recorded (data available by request).

Animals were bled via the retroorbital sinus. We performed an enzyme-linked immunosorbent assay for detection of hantavirus antibody (3).

We trapped six species of rodents; *P. maniculatus* was the most common, followed by *P. truei* and *N. lepida*. Other species captured infrequently were *Dipodomys ordii*, *Largurus curtatus*, and *Chaetodipus* sp.

Over three trapping periods, we captured 212 *P. maniculatus;* 63 were antibody-positive (29.7%). Prevalence of SNV was greater in males than in females (chi-square = 3.8, p = 0.05), and it varied little among sampling periods. Most of the variation was due to changes in prevalence in males, which was 28% to 42%; prevalence among female deer mice was 17% to 20%. *P. truei* also tested positive for SNV. Of 37 *P. truei* tested, 4 were antibody-positive (10.8%).

We found a high and relatively stable level of SNV prevalence in a population of deer mice in Central Utah. Mean antibody prevalence (29.7%) across 3 periods was up to 3 times higher than that of other locations. Prevalence of SNV in this population was comparable with that during the 1993 outbreak of HPS in the Four Corners region.

We propose that the high level of SNV prevalence could be due to disturbance by humans, primarily intensive use of all-terrain vehicles at the study site. Little Sahara Recreation Area, ~4 km from the study site, recorded 180,000 visitors during 1999, mostly all-terrain vehicle users (Bureau of Land Management statistics). Many visitors to Little Sahara camp and recreate on land in our study area. This heavy recreational use has produced numerous dirt roads and campsites. Vehicle movement has denuded the area of vegetation other than large (>1 m tall) shrubs and trees and has removed cryptogamic crust, resulting in compaction of sandy soil into roads, trails, and large open spaces. Open spaces caused by disturbance reduce habitat suitable for species such as *Peromyscus* (6,7) and may cause animal density to increase within a microhabitat. Increased intra- and interspecific interactions would favor the transmission of SNV. Thus, fragmentation of the landscape may alter behavior of deer mice in a manner that enhances transmission of SNV.

Four pieces of evidence corroborate our speculation that habitat disturbance increases prevalence of SNV. First, in experimentally fragmented habitats, the density of deer mice increased dramatically. In one study, density of deer mice in small patches (4 m x 8 m) was consistently 3 times higher during 7 years of the study than that of deer mice in larger patches (10 x 50 m) (6). These small patches are similar in size to patches created by vehicle movement at our study site. Second, deer mice in fragmented habitats travel much longer distances, on average 2 times as far, as deer mice in less fragmented habitats (6). Third, immunocompetence of small rodents may decline as population density increases, making rodents more susceptible to infection than at lower densities (8). These three factors taken together should enhance transmission of SNV by increasing interactions among deer mice with lowered immunocompetence. Finally, prevalence of SNV in deer mice is lower in populations from habitats less impacted but similar to our study site. Across four other sites in the Great Basin, prevalence of SNV was 11% (9). Although we have not quantified the disturbance in these other areas, their general locations suggest they are not as disturbed by humans as the site near Little Sahara Recreational Area.

Further investigation of the effect of human disturbance on SNV prevalence is needed. We have presented several possible mechanisms that may be involved in a causal relationship between these two factors. Given that most HPS cases are contracted in areas where there has been human alteration to the landscape, future investigation of this hypothesis is warranted.

The prevalence and total numbers of infected rodents were much lower in *P. truei* than *P. maniculatus*. Adult *P. truei* are larger than *P. maniculatus* and tend to compete with *P. maniculatus* for food and nesting sites. Interspecific competition could lead to aggressive contact between these two species that could result in interspecific transmission of SNV. *P. truei* were regularly captured at the same middens on the same nights with *P. maniculatus*.

We suggest that the high level of disturbance at this study site could increase the probability of SNV transmission between species through the same mechanisms suggested for the high levels of prevalence within deer mice. Rodents at our study site may be living at higher densities than in other areas. The increased contact between species, especially when SNV prevalence is high in deer mice, could promote transmission to species other than *P. maniculatus*.

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## References

1. Hjelle B, Torrez-Martinez N, Koster FT, Jay M, Ascher MS, Brown T, et al. Epidemiologic linkage of rodent and human hantavirus genomic sequences in case investigations of hantavirus pulmonary syndrome. J Infect Dis 1996;173:781-6.
2. Childs JE, Ksiazek TG, Spiropoulou CF, Krebs JW, Morzunov S, Maupin GO, et al. Serologic and genetic identification of *Peromyscus maniculatus* as the primary rodent reservoir for a new hantavirus in the southwestern United States. J Infect Dis 1994;169:1271-80.
3. Dearing MD, Mangione AM, Karasov WH, Morzunov S, Otteson E, St. Jeor S. Prevalence of hantavirus in four species of *Neotoma* from Arizona and Utah. Journal of Mammalogy 1998;79:1254-9.
4. Egoscue HJ. The desert woodrat: a laboratory colony. Journal of Mammalogy 1957;38:472-81.
5. Stones RC, Hayward CL. Natural history of the desert woodrat, Neotoma lepida. The American Midland Naturalist 1968;80:458-76.
6. Diffendorfer JE, Gaines MS, Holt RD. Habitat fragmentation and movements of three small mammals (*Sigmodon, microtus*, and *Peromyscus*). Ecology 1995;76:827-39.
7. Mader HJ. Animal habitat isolation by roads and agricultural fields. Biological Conservation 1984;29:81-96.
8. Moshkin MP, Dobrotvorsky AK, Mak VV, Panov VV, Dobrotvorskaya EA. Variability of immune response to heterologous erythrocytes during population cycles of red (*Clethrinomys rutilus*) and bank (*C. glareolus*) voles. Oikos 1998;82:131-8.

9. Mills JN, Ksiazek TG, Ellis BA, Rollin PE, Nichol ST, Yates TL, et al. Patterns of association with host and habitat: antibody reactive with Sin Nombre virus in small mammals in the major biotic communities of the southwestern United States. Am J Trop Med Hyg 1997;56:273-84.

## Hantavirus Seroconversion of Wild-Caught *Peromyscus* During Quarantine

**To the Editor:** In 1993 an outbreak of unexplained respiratory deaths in the Four Corners region of the United States led to the discovery of Sin Nombre (SN) hantavirus and the associated human disease, hantavirus cardiopulmonary syndrome (HCPS). Numerous studies have shown that a series of hantaviruses similar to SN virus are maintained in natural reservoirs composed of Sigmodontine rodents, including deer mice (*Peromyscus maniculatus*), white-footed mice (*P. leucopus*), cotton rats (*Sigmodon hispidus*) and western harvest mice (*Reithrodontomys megalotis*) (1). Deer mice, however, are the principal reservoir of SN virus, the primary etiologic agent of HCPS in North America.

Some hantaviruses, thus far not including SN virus, have been transmitted in indoor animal-care facilities through the airborne route (2). The high case-fatality ratio of HCPS (40%), coupled with its airborne transmission by captive rodents, has led to classification of the agents of HCPS as biosafety level 3 (BSL-3) in tissue culture and BSL-4 in reservoir host rodents. Although deer mice mount an antibody response and develop chronic infection, the virus does not harm them. Deer mice are believed to shed SN virus in urine, feces, and saliva. Infection in humans occurs primarily by inhalation of aerosols from dried excreta containing infectious virus, particularly in closed spaces with poor ventilation (3).

Handling mice infected with SN virus in a laboratory requires BSL-4 conditions (4). However, outdoor standards greatly reduce costs and difficulties associated with handling infected rodents safely, since workers wearing respirators and protective clothing may handle infected mice outdoors (5). Thus, we have constructed outdoor quarantine facilities for the temporary housing of potentially infected mice (6). These facilities consist of a series of individual nest boxes enclosed by a partially buried steel plate fence. Mice are placed into individual nest boxes spaced 3 m apart, which prevents transmission of hantavirus among mice during quarantine (J. Botten and B. Hjelle, unpub. data). Each nest box is composed of an artificial burrow enclosed within a small steel container, which serves as a barrier to contain each mouse. These facilities allow safe handling of wild rodents at much lower cost than that associated with BSL-4 laboratories. Very few, if any, patients with HCPS contracted the virus in an open, outdoor environment (3).

Viral infections are characterized by a window period during which the host is infected but diagnostic test (e.g., antibody) results are negative. To detect infections reliably, it is important to conduct antibody tests after the host animal has been given sufficient time to mount a detectable immune response. Mills et al. (5) recommend testing captured rodents for hantavirus antibodies at the beginning and end of a 5-week quarantine period whenever potential reservoir species are used to establish laboratory colonies. Only upon completion of the second test can an animal be considered truly uninfected by a hantavirus.

We describe two cases of seroconversion in *Peromyscus* spp. that were undergoing such quarantine. These results support the use of a quarantine period in combination with hantavirus antibody testing to clear mice for indoor use.

We collected 132 white-footed mice from one southern and two northern areas of Illinois that have not previously been examined for the presence of hantavirus. The average seroprevalence among these populations was 1.5%. Forty-six of these mice were quarantined for 5 weeks (6), and one mouse underwent seroconversion as detected by strip immunoblot assay. The presence of viral RNA in this mouse was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) from lung tissue. In addition, we collected 69 deer mice from an area of New Mexico that had an overall seroprevalence of approximately 20% and placed them in quarantine (6).

One deer mouse delivered four pups while in quarantine and seroconverted 19 days after delivery (6,7). While all four pups were seropositive, viral RNA was detected in the dam by using RT-PCR for lung tissue and immunohistochemistry for heart, lung, and liver tissue (data not shown). Infectiousness of the virus from this mouse was demonstrated by successful passage through uninfected deer mice (7). The fact that the New Mexico pups had not become infected when they were euthanized at 21 days supports other epidemiologic data that suggest that deer mice do not transmit the virus vertically (9-11). These results strongly support the recommendations promulgated by Mills et al. (5) and the Centers for Disease Control and Prevention that wild rodents be used as colony founders only if they remain seronegative for hantavirus after a 5-week quarantine period.

Working in outdoor quarantine facilities is labor-intensive and requires routine maintenance and occasional repair. Building costs depend on the number of nest boxes, but the material cost of a substantial quarantine facility is $10,000 to $20,000. However, safety concerns and the difficulties of maintaining mice alive outdoors without bringing them indoors necessitate their use. A possible exception could be made for very temperate climates, where outdoor cages might be used temporarily.

Our finding that even a recently infected dam, one known to be infectious by horizontal route, did not transmit virus to her pups supports lack of vertical transmission of SN virus as argued previously by workers using less direct methods (9-11).

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### References

1. Schmaljohn C, Hjelle B. Hantaviruses: a global disease problem. Emerg Infect Dis 1997;3:95-104.
2. Tsai T. Hemorrhagic fever with renal syndrome: mode of transmission to humans. Lab Anim Sci 1987;37:428-30.
3. Hjelle B, Glass GE. Outbreak of hantavirus infection in the Four Corners region of the United States in the wake of the 1997-98 El Niño-Southern oscillation. J Infect Dis 2000;181:1569-73.
4. Centers for Disease Control and Prevention. Laboratory management of agents associated with hantavirus pulmonary syndrome: interim biosafety guidelines. MMWR Morb Mortal Wkly Rep 1994;43(RR-7):1-7.
5. Mills JN, Yates TL, Childs JE, Parmenter RR, Ksiazek TG, Rollin PE, et al. Guidelines for working with rodents potentially infected with hantavirus. Journal of Mammalogy 1995;76:716-22.
6. Botten J, Nofchissey R, Kirkendoll-Ahern H, Rodriguez-Moran P, Wortman IA, Goade D, et al. Outdoor facility for quarantine of wild rodents infected with hantavirus. Journal of Mammalogy 2000;81:250-9.
7. Botten J, Mirowsky K, Kusewitt D, Bharadwaj M, Yee J, Ricci R, et al. Experimental infection model for Sin Nombre hantavirus in the deer mouse (*Peromyscus maniculatus*). Proc Natl Acad Sci U S A 2000;97:10578-83.
8. Green W, Feddersen R, Yousef O, Behr M, Smith K, Nestler J, et al. Tissue distribution of hantavirus antigen in naturally infected humans and deer mice. J Infect Dis 1998;177:1696-700.
9. Mills JN, Ksiazek TG, Ellis BA, Rollin PE, Nichol ST, Yates TL, et al. Patterns of association with host and habitat: antibody reactive with Sin Nombre virus in small mammals in the major biotic communities of the southwestern United States. Am J Trop Med Hyg 1997;56:273-84.
10. Bennett SG, Webb JP Jr, Madon MB, Childs JE, Ksiazek TG, Torrez-Martinez, et al. Hantavirus (Bunyaviridae) infections in rodents from Orange and San Diego counties, California. Am J Trop Med Hyg 1999;60:75-84.
11. Borucki MK, Boone JD, Rowe JE, Bohlman MC, Kuhn EA, DeBaca R, et al. Role of maternal antibody in natural infection of *Peromyscus maniculatus* with Sin Nombre virus. J Virol 2000;74:2426-29.

## *Mycobacterium tuberculosis* Isolates of Beijing Genotype in Thailand

**To the Editor:** Anh and colleagues recently reported that in new tuberculosis (TB) cases from Vietnam, *Mycobacterium tuberculosis* isolates of the Beijing genotype are associated with younger age and, in isolates from Ho Chi Minh City, with resistance against isoniazid and streptomycin (1). However, occurrence of Beijing genotype *M. tuberculosis* strains may be different in other Southeast Asian countries such as Thailand.

From May 1999 to June 2000, we obtained 244 *M. tuberculosis* isolates from TB patients at Ramathibodi Hospital, Bangkok, Thailand (the hospital treats approximately 625 TB patients annually). The isolates have been prospectively analyzed by DNA-fingerprinting with the spoligotyping method (2). Drug-resistance testing and recording of patients' data (sex, age, geographic origin) were completed for 204 of 244 patients. The 204 patients originated from all six regions of Thailand, although the central region (comprising the Bangkok area) and the northeast region predominated (59% and 23.5%, respectively). Altogether 111 male and 93 female patients with a median age of 34 years (1 to 89 years) were included. Status of BCG vaccination or HIV infection was not assessed.

The Beijing genotype was found in 90 (44.1%) of the 204 isolates analyzed in detail, without significant differences regarding the respective patients' geographic origin or sex. Thus in Thailand, the frequency of the Beijing type is somewhere between the frequency in Vietnam (53%) (1) and in peninsular Malaysia (estimated at 24%) (3). Using the same age groups as Anh and colleagues, we did not find an association of Beijing genotype with young age ($p = 0.41$; chi-square test for trend). Although Beijing type isolates were more frequent among patients <25 years (18 [56%] of 32) than among those >25 years (69 [43%] of 161), this was not significant ($p = 0.13$). This association remained not significant, if only isolates from the central or the northeast region were analyzed.

Of 204 isolates, 62 (30%) showed resistance to ≥1 of 4 drugs tested (isoniazid, 8.8%; rifampicin, 6.4%; streptomycin, 19.6%; ethambutol, 4.9%). However, overall drug resistance, resistance to single drugs, and multidrug resistance were not associated with the Beijing genotype. The frequency of resistance was similar in distribution but overall lower than reported for the Ho Chi Minh City isolates (isoniazid, 24%; rifampicin, 2%; streptomycin, 31%; ethambutol, 2%) (1).

In both studies, the highest percentage of drug resistance was found for streptomycin. In our sample, this was not associated with particular spoligotypes or with geographic origin of the patient. Furthermore, streptomycin-resistant isolates were not more frequent in older age groups, although there was a nonsignificant trend ($p = 0.12$; $\text{chi-square}_{\text{trend}}$). Streptomycin is still used for standard quadritherapy in Thailand, and occurrence of resistant strains can reflect selection or transmission recently or in the past. This differs from occurrence of streptomycin resistance in countries where streptomycin is no longer used in standard therapy (4).

In the original description of the Beijing family of strains in 1995, Beijing genotype isolates were found in 7 (37%) of 19 Thai isolates (5). In a subsequent IS*6110* restriction fragment length polymorphism analysis, 80 (38%) of 211 isolates from central Thailand collected in 1994 to 1995 belonged to the Beijing family (6). Whether 90 (44%) of 204 among our recent isolates reflect a reliable increase in Beijing type transmission over the last 5 years, is difficult to state. However, the fact that no correlation of Beijing type with (young) age of the patient was observed in the previous analysis (6) supports the notion that increasing incidence of the Beijing strain in Thai cases is not due to recent transmission.

The *M. tuberculosis* population appears to be considerably more heterogeneous in Thailand than in the large urban areas of Vietnam. In our study, the three most common spoligopatterns besides Beijing, S156, S153, and S22 (according to the nomenclature of Soini et al. [7]), together comprised 20% of 244 isolates. However, the second most frequent spoligopattern, the "Vietnam genotype" (S10 according to Soini), reportedly shared by 27% of the Vietnamese isolates, was not found in our sample of

Thai isolates. Only 20 different spoligopatterns were found among 499 isolates in the Vietnam study, compared to 60 among 244 isolates in our study.

Although the Beijing genotype of *M. tuberculosis* has been recognized in settings of emerging drug resistance around the world, the situation in Southeast Asian countries with a high frequency of Beijing type isolates appears to be nonuniform.

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### References

1. Anh DD, Borgdorff MW, Van LN, Lan NTN, van Gorkom T, Kremer K, et al. *Mycobacterium tuberculosis* genotype Beijing emerging in Vietnam. Emerg Infect Dis 2000;6:302-5.
2. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 1997;35:907-14.
3. Dale JW, Nor RM, Ramayah S, Tang TH, Zainuddin ZF. Molecular epidemiology of tuberculosis in Malaysia. J Clin Microbiol 1999;37:1265-8.
4. Niemann S, Richter E, Rusch-Gerdes S, Thielen H, Heykes-Uden H. Outbreak of rifampin and streptomycin-resistant tuberculosis among homeless in Germany. Int J Tuberc Lung Dis 1999;3:1146-7.
5. van Soolingen D, Qian L, de Haas PE, Douglas JT, Traore H, Portaels F, et al. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. J Clin Microbiol 1995;33:3234-8.
6. Palittapongarnpim P, Luangsook P, Tansuphaswadikul S, Chuchottaworn C, Prachaktam R, Sathapatayavongs B. Restriction fragment length polymorphism study of *Mycobacterium tuberculosis* in Thailand using IS*6110* as probe. Int J Tuberc Lung Dis 1997;1:370-6.
7. Soini H, Pan X, Amin A, Graviss EA, Siddiqui A, Musser JM. Characterization of *Mycobacterium tuberculosis* isolates from patients in Houston, Texas, by spoligotyping. J Clin Microbiol 2000;38:669-76.

## Jungle Yellow Fever, Rio de Janeiro

**To the Editor:** Yellow fever control in Brazil through vaccination campaigns began in 1937. However, cases of jungle yellow fever still occur despite the existence of a potent vaccine and immunization campaigns focused on areas endemic for the jungle form of the disease (1). Most of these cases are in men in rural areas.

In Brazil from 1980 to 1998, 376 cases of jungle yellow fever were laboratory confirmed (by virus isolation, with or without immunoglobulin [Ig]M-capture enzyme-linked immunosorbent assay [MAC-ELISA] and immunoperoxidase stain), with 216 deaths (case-fatality rate 57.4%). Most cases were from Maranhão and Goiás States, with 99 and 41 cases, respectively; Goiás, in midwestern Brazil, reported a case-fatality rate of 95%.

During 1998 to 1999, 106 cases of jungle yellow fever were confirmed, with 40 deaths (37.7%). During 1999, 75 cases were confirmed, compared with 34 cases in 1998 and a mean of 20 cases per year from 1980 to 1998 (2). In 2000, 84 cases were confirmed, with 40 deaths (47.6% case-fatality rate). During 2000, the probable site of infection for nearly all cases was in Goiás, with 53 confirmed cases and 23 deaths, suggesting epizootic circulation of the virus (2). These cases were in unvaccinated persons who became ill in their home states after traveling to endemic areas for tourism or work.

In Brazil, almost two thirds of the territory is considered an enzootic area (3). Rio de Janeiro State is not endemic for jungle yellow fever, but in January 2000, the Oswaldo Cruz Institute confirmed a case of yellow fever in a 24-year-old woman who had traveled to a national park in Goiás State on December 28, 1999, with a group of 17 persons. Yellow fever infections were also confirmed in tourists from other states who visited this park in late 1999.

The young woman became ill on January 3 with fever, headache, retroocular pain, prostration, anorexia, and nausea. She returned to Rio de Janeiro on January 5 and visited a private clinic on January 7, when a complete blood count, platelet count, urea, creatinine, liver function tests, and dengue serologic testing were performed. The patient had leukopenia (1,730 leukocytes/$mm^3$), 100,000 platelets/$mm^3$, AST 911 U/L and ALT 680 U/L, creatinine 0.90 mg/dL, urea 10 mg/dL, and normal bilirubin and protein. Anti-dengue IgM serology was negative. A blood sample was collected January 11 for yellow fever diagnosis. Reverse transcription-polymerase chain reaction (RT-PCR) test was performed on RNA extracted from the serum (4), and virus isolation was attempted on C6/36 cells, both with negative results. A MAC-ELISA test was positive for yellow fever, with a serum IgM titer 1/80,000 8 days after onset of symptoms. The patient recovered within a week. After confirmation of this case in the only person who became ill in the travel group, yellow fever IgM serologic testing was performed on the other group members, all of whom tested negative. RT-PCR and virus isolation were not attempted because the sera were taken after the viremia period.

Control measures for the *Aedes aegypti* vector were promptly taken for a radius of 300 m around the patient's home. A vaccination campaign was carried out, during which 735 neighbors were vaccinated. An epidemiologic survey was conducted in the area by using active surveillance for all symptomatic cases of fever during the period of yellow fever transmissibility. Blood samples from patients with fever were assessed for yellow fever virus and antibodies. Surveillance was intensified immediately in Rio de Janeiro State, and our laboratory examined 54 sera from patients who had traveled recently to endemic areas and who had compatible signs and symptoms (in accordance with a nationwide protocol). All these persons tested negative for yellow fever.

From January to July 2000, >16.9 million people were vaccinated against yellow fever (2); however, cases continue to occur. Unvaccinated persons who visit yellow fever-endemic areas pose a high risk of introducing jungle yellow fever cases into nonendemic areas.

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### References

1. Barros MLB, Boecken G. Jungle yellow fever in central Amazon. Lancet 1996;348:969.
2. Ministry of Health. Notified cases of yellow fever by state, Brazil. Brasilia: National Epidemiology Center; 2000.
3. Vasconcelos PFC, Rodrigues SG, Degalier N, Moraes MAP, Rosa JFST, Rosa EST, et al. An epidemic of sylvatic yellow fever in the Southeast Region of Maranhão State, Brazil, 1993-1994: epidemiological and entomological findings. Am J Trop Med Hyg 1997;57:132-7.
4. Deubel V, Huerre M, Cathomas G, Drouet M-T, Wuscher N, Le Guenno B, et al. Molecular detection and characterization of yellow fever in blood and liver specimens of a non-vaccinated fatal human case. J Med Virol 1997;53:212-7.


## Emergence of Metronidazole-Resistant *Bacteroides fragilis*, India

**To the Editor:** Members of the *Bacteroides fragilis* group are the most commonly isolated anaerobic pathogens in humans. Metronidazole has been the drug of choice for preventing and treating such infections for 40 years. Although *B. fragilis* exhibits the broadest spectrum of recognized resistance to antimicrobial agents among anaerobes, the worldwide rate of metronidazole resistance remains low, <5% (1,2). We report here the first metronidazole-resistant strain of *B. fragilis* from India.

A 34-year-old man with myelodysplastic syndrome was admitted to our hospital with a short history of myalgia, general malaise, and bleeding gums. Bone marrow examination showed evidence of severe aplastic anaemia, for which he was treated with cyclophosphamide and blood transfusions. Ceftazidime and amikacin were also administered empirically for febrile neutropenia. The patient remained in the intensive care unit of our medical oncology ward and was given repeated courses of chemotherapy and blood transfusions. He also had repeated episodes of febrile neutropenia, which resolved with a combination of vancomycin, aminoglycosides, and third-generation cephalosporin. After 4 months in the hospital, during an episode of febrile neutropenia, the patient's condition started to deteriorate, and high-grade fever developed. Physical examination showed temperature of 38°C, heart rate 80/min, blood pressure 100/70 mmHg, and marked pallor. Laboratory investigations showed a hemoglobin level of 4g/dL and marked neutropenia (absolute neutrophil count 320/mm$^3$). Liver and renal function test results were within normal limits. Peripheral blood smears were negative for malarial parasites. Culture of urine revealed no growth, and the Widal test was negative. Two blood samples were collected in Wampole isolator tubes (Wampole Laboratories, Cranbury, NJ), for isolation of aerobic and anaerobic bacteria. Subsequently, intravenous antimicrobial therapy with vancomycin, metronidazole, and ceftazidime was started. The patient died a day after collection of blood for culture.

Antemortem blood cultures grew *Pseudomonas aeruginosa* and *B. fragilis.* The isolate of *B. fragilis* was identified by conventional tests and Rap ID ANA II system (Innovative Diagnostic System, Norcross, GA). *P. aeruginosa* was sensitive to piperacillin but resistant to amikacin, ceftazidime, cefotaxime, and ciprofloxacin. *B. fragilis* was resistant to metronidazole (MICs, 256 µg/mL) by both standard broth dilution method and E-test (AB Biodisk, Solne, Sweden). The isolate was also resistant to cefotaxime and ceftazidime. However, it was sensitive to chloramphenicol, clindamycin, and imipenem.

Primary bacteremia caused by anaerobic organisms accounts for <5% of septicemia in cancer patients (3). Chemotherapy is a known predisposing factor for anaerobic bacteremia because it causes gastrointestinal ulceration, which permits anaerobes to enter circulation (4).

Anaerobic bacteremia is usually polymicrobial in etiology and has a high death rate (4). In this case, both bacterial isolates were resistant to the empirical treatment. Delay in initiating appropriate therapy was perhaps a major contributor to the patient's death.

Metronidazole is the drug of choice for empirical coverage of anaerobic infections. The precise incidence of resistance to metronidazole in *B. fragilis* isolates is difficult to estimate (5), since routine antimicrobial sensitivity testing of anaerobes is not being done by most laboratories in the world. Published articles reveal only a few reported cases of *B. fragilis* that were resistant to metronidazole (6-10). Although the incidence of resistance to penicillin, cephalosporins, and clindamycin is increasing dramatically, no resistance to metronidazole in *B. fragilis* was found in some large-scale studies done throughout the world (11,12).

The true incidence of metronidazole resistance in India too is possibly underestimated since antimicrobial sensitivity testing is not being done routinely. However, we are conducting antimicrobial susceptibility testing of all anaerobic isolates in our institute. In a previous study we conducted (13), contrary to this report, none of 32 clinical isolates belonging to the family *Bacteroidaceae* obtained over a 5-year period were resistant to metronidazole.

Recently, the anaerobic reference unit in the UK noted a possible increase in the incidence of metronidazole resistance in *B. fragilis*, an observation that would have major implications for clinical microbiology laboratories, as well as for prophylactic and treatment regimens (5).

There is now a growing debate whether in vitro susceptibility testing should be performed for all *Bacteroides* isolates to guide antimicrobial therapy. The acquisition of metronidazole resistance by *B. fragilis* reported here from India emphasizes the need for a study to assess more accurately the susceptibilities of clinical isolates of *Bacteroides* spp.

Diagnostic microbiology laboratories and clinicians should be aware that the incidence of metronidazole resistance in clinically significant anaerobes may be increasing (5). Since antimicrobial resistance in anaerobes varies from one hospital to another and between different geographic locations, all hospitals should survey their sensitivity patterns and report any emerging resistance.

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### References

1. Finegold SM, Wexler HM. Present status of therapy for anaerobic infections. Clin Infect Dis 1996;23(Suppl 1):S9-14.

2. Falagas ME, Siakavella S. *Bacteroides, Prevotella*, and *Porphyromonas* species. A review of antibiotic resistance and therapeutic options. Int J Antimicrob Agents 2000;15:1-9.
3. Freifeld AG, Walsh TJ, Philip A. Infections in the cancer patient. In: Devita VT Jr, Hellman S, Rosenberg SA, editors. Cancer. Principles and practice of oncology. 5th ed. New York: Lippincott-Raven; 1997. p. 2659-04.
4. Bodey GP. Infections in patients with cancer. In: Holland JF, Frei F, editors. Cancer medicine. 2nd ed. Philadelphia: Lea and Febiger; 1982. p. 1339-72.
5. Brazier JS, Stubbs SL, Duerden BI. Metronidazole resistance among clinical isolates belonging to the *Bacteroides fragilis* group: time to be concerned? J Antimicrob Chemother 1999;44:580-1.
6. O'Donoghue MA, Potter J, Allen KD. Metronidazole-resistant *Bacteroides fragilis* infection. J Infect 1992;25:211-4.
7. Hickey MM, Davies UM, Dave J, Vogler M, Wall R. Metronidazole resistant *Bacteroides fragilis* infection of a prosthetic hip joint. J Infect 1990;20:129-33.
8. Brogan O, Garnett PA, Brow R. *Bacteroides fragilis* resistant to metronidazole, clindamycin and cefoxitin. J Antimicrob Chemother 1989;23:660-2.
9. Lamothe F, Fijalkowski C, Malouin F, Bourgault AM, Delornel L. *B. fragilis* resistant to both metronidazole and imipenem. J Antimicrob Chemother 1986;18:642-3.
10. Turner P, Edward SR, Weston V, Gazes A, Ispaham P, Greenwood D. Simultaneous resistance to metronidazole, co-amoxiclav, and imipenem in clinical isolate of *Bacteroides fragilis*. Lancet 1995;345:1275-7.
11. Snydman DR, Jocubus NV, Dermott LA, Supran S, Cuchural CG, Finegold S. Multicentric study of in vitro susceptibility of the *Bacteroides fragilis* group 1995 to 1996 with comparison of resistance trends from 1990 to 1996. Antimicrob Agents Chemother 1999;2417-22.
12. Aldridge KE, Gelfand M, Reller LB, Ayers CW, Pierson CL, Schoenknecht F, et al. A five year multicentre study of the susceptibility of the *Bacteroides fragilis* group isolates to cephalosporins, cephamicins, penicillin, clindamycin and metronidazole in the United States. Diagn Microbiol Infect Dis 1994;18:235-41.
13. Chaudhry R, Mishra B, Dhawan B, Sharma N. Anaerobic infections in an Indian tertiary care hospital with special reference to Bacteroidaceae. J Infect 1999;38:54-5.

## Proper Nomenclature for the Human Granulocytic Ehrlichiosis Agent

**To the Editor:** In their recent article, "Antigenic variations in vector-borne pathogens," Barbour and Restrepo discuss the outer membrane protein components of *Anaplasma marginale* and related bacteria (1). Citing a reference by Zhi et al. (2), they state that *Ehrlichia granulocytophila* is the agent of human granulocytic ehrlichiosis (HGE).

The use of new names and combinations not widely recognized for genera and species lends increasing confusion to a group of bacteria already in taxonomic disarray. Several other species names have been suggested for the HGE agent since the initial description of the clinical illness caused by this agent and the in vitro technique used to isolate the agent in blood samples (3,4). Both *E. phagocytophila* and *E. equi* are genetically nearly identical to the HGE agent, and the three are probably conspecific. Thus, most scientists in the field today would support use of the name *Ehrlichia phagocytophila* to describe these bacteria.

Recent phylogenetic analyses show that *E. phagocytophila* strains align into a clade that includes *Anaplasma marginale*, the historical precedent in this grouping. Such phylogenetic analyses, which are also supported by comparative antigenic and biological studies, have resulted in a proposal for reclassification of several *Ehrlichia* spp., including *E. phagocytophila*, into the genus *Anaplasma* (5). Until a cogent reclassification based on objective criteria is firmly accepted, the creation and use of new scientific name combinations for a single bacterium yield clinical and laboratory confusion and should be avoided.

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### References

1. Barbour AG, Restrepo BI. Antigenic variations in vector-borne pathogens. Emerg Infect Dis 2000;6:449-57.
2. Zhi N, Ohashi N, Rikihisa Y. Multiple p44 genes encoding major outer membrane proteins are expressed in the human granulocytic ehrlichiosis agent. J Biol Chem 1999;274:17828-36.
3. Bakken JS, Dumler JS, Chen S-M, Eckman MR, VanEtta LL, Walker DH. Human granulocytic ehrlichiosis in the upper midwest United States: A new species emerging? JAMA 1994;272:212-8.
4. Goodman JL, Nelson C, Vitale B, Madigan JE, Dumler JS, Munderloh UG. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. N Engl J Med 1996;334:209-15.
5. Dumler JS, Rihihisa Y, Dasch GA, Barbet AF, Palmer GH, Ray SC. Proposal for taxonomic reorganization of the order *Rickettsiales*, family *Rickettsiaceae*, and tribe *Ehrlichieae*. [abstract 75]. In: Program and Abstracts of the 15th Sesqui-Annual Meeting of the American Society for Rickettsiology; April 30-May 5, 2000; Captiva Island, Florida. American Society for Rickettsiology; 2000.

## Single Nucleotide Polymorphisms in *Mycobacterium tuberculosis* Structural Genes

**To the Editor:** A recent article by Fraser et al. (1) discussed the frequency of single nucleotide polymorphisms (SNPs) in two genomes of *Mycobacterium tuberculosis*, strains H37Rv (2) and CDC1551 (unpublished). The article contains an inaccurate representation of our published *M. tuberculosis* data on SNP frequency. The authors state that "detailed comparison of strains H37Rv and CDC1551 indicates a higher frequency of polymorphism, approximately 1 in 3,000 bp, with approximately half the polymorphism [sic] occurring in the intergenic regions. In other words, 50% of the polymorphisms are in 10% of the genome. While this rate is higher than that suggested (3), it still represents a lower nucleotide diversity than found in limited comparisons from other pathogens."

On the basis of comparative sequence analysis of eight *M. tuberculosis* structural gene loci (open reading frames [orf]), we initially published an estimated average number of synonymous substitutions per synonymous site ($K_s$ value) that indicated that this pathogen had, on average, approximately 1 synonymous difference per 10,000 synonymous sites (4). This finding was unexpected given the relatively large population size of *M. tuberculosis* and paleopathologic

evidence suggesting its presence in humans as early as 3700 B.C. Subsequent sequence analysis of two megabases in 26 structural genes or loci in strains recovered globally confirmed the striking reduction of silent (synonymous) nucleotide substitutions compared with other human bacterial pathogens (3). A large study (approximately 2 Mb of comparative sequence data) of 12 genes potentially involved in ethambutol resistance (5) and 24 genes encoding protein targets of the host immune system (6) provided data consistent with the original estimate of 1 synonymous nucleotide change per 10,000 synonymous sites in structural genes in this pathogen. Our estimate did not include SNPs located in putative regulatory regions of structural genes (intergenic regions), nor did it include nonsynonymous nucleotide changes in structural genes. These classes of polymorphisms were not included in our estimates because of difficulties in ruling out the possibility that they arose as a consequence of selective pressure due to antimicrobial agent treatment or perhaps extensive in vitro passage. Synonymous nucleotide changes (neutral mutations) are commonly used to estimate many values of interest to evolutionary biologists and population geneticists.

The estimate provided by Fraser et al. is based on a genomewide frequency of SNPs (1/3,000 nucleotide sites), 50% of which presumably are located in intergenic regions and 50% in structural genes. On the basis of a genome size of roughly 4.4 Mb, there would be roughly 1,500 total SNPs, with approximately 750 in orfs (90% of genome = 3,960,000 bp) and 750 in intergenic regions (10% of genome = 440,000 bp). On the basis of these estimates, the frequency of all SNPs located in structural genes would be roughly 1/5,280 bp. (An estimate of 1,300 total SNPs [translating to 1/6,000 bp] was presented by the group at a meeting held at the Banbury Center last December.) As expected, these numbers differ from our estimate (1/10,000), in part because they contain both synonymous and nonsynonymous nucleotide polymorphisms.

We analyzed orfs (available in public databases) dispersed around the chromosome of *M. tuberculosis* strains CDC1551 and H37Rv. Surprisingly, the number of nonsynonymous SNPs exceeded the number of synonymous SNPs. We found only approximately 323 synonymous SNPs, yielding a synonymous SNP frequency of roughly 1/12,260 bp in orfs.

*M. tuberculosis*, a pathogen that infects one third of humans, clearly has an unusual if not unique molecular evolution history. Precise data on the frequency of its true SNPs genomewide are critical. At this point, data (3-6) are consistent with our original estimate of 1 synonymous nucleotide change per 10,000 synonymous sites in structural genes in natural populations of this pathogen.

**James M. Musser**
National Institutes of Health, Hamilton, Montana

### References

1. Fraser CM, Eisen J, Fleischmann RD, Ketchum KA, Peterson S. Comparative genomics and understanding of microbial biology. Emerg Infect Dis 2000;6:505-12.
2. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 1998; 393:537-44.
3. Kapur V, Whittam TS, Musser JM. Is *Mycobacterium tuberculosis* 15,000 years old? J Infect Dis 1994;170:1348-9.
4. Sreevatsan S, Pan X, Stockbauer KE, Connell ND, Kreiswirth BN, Whittam TS, et al. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc Natl Acad Sci U S A 1997;94:9869-74.
5. Ramaswamy SV, Amin AG, Goksel S, Stager CE, Dou S-J, El Sahly H, et al. Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2000;44:326-36.
6. Musser JM, Amin A, Ramaswamy S. Negligible genetic diversity of *Mycobacterium tuberculosis* host immune system protein targets: evidence of limited selective pressure. Genetics 2000;155:7-16.

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## Single Nucleotide Polymorphisms in *Mycobacterium tuberculosis* Structural Genes—Response to Dr. Musser

**To the Editor:** In his letter on single nucleotide polymorphisms in *Mycobacterium tuberculosis*, Dr. Musser indicates that genome strain CDC1551 has not been published. Cole et al. (1) described some of the biology of *M. tuberculosis* based on the genome sequence data. The actual sequence, while not published, is in GenBank (Accession NC00962), the sequence data are available at www.sanger.ac.uk, and the annotation is available at http://genolist.pasteur.fr/TubercuList/ . We have a manuscript in preparation using a method of whole genome comparison (2) to evaluate the sequence diversity of strains H37Rv and CDC1551 and applying the information to the analysis of >150 clinical isolates. The complete sequence data and annotation for strain CDC1551 have been available for over a year at www.tigr.org and www.tigr.org/CMR, and periodic updates are provided. In addition, we are preparing to submit the strain CDC1551 sequence and annotation to GenBank (Accession AE000516).

We agree that sequencing accuracy in assessing comparative single nucleotide polymorphism (SNP) data is important. The error frequency suggested by Dr. Weinstock ("Error frequency in a finished sequence has never been precisely measured but is thought to be one error [frameshift or base substitution] in $10^3$ to $10^5$ bases" [3]) is not supported by any evidence. The whole-genome shotgun sequencing method developed by The Institute for Genomic Research (TIGR) (4) and adopted by many others is highly accurate because of the following qualities: 1) high redundancy in shotgun sequencing (average 7.9-fold for the strain CDC1551 project with a minimum of 2-fold coverage for any nucleotide); 2) assignment of quality values to each nucleotide base; 3) adoption of assembly programs that use quality values for consensus building; and 4) manual editing of electropherograms as necessary.

These methods were applied to the *M. tuberculosis* genome sequencing project. In comparing the CDC1551 and H37Rv strains, it is reasonable to suspect that the SNPs also have the potential to be results of sequencing errors. The sequence differences were verified by two independent methods. One hundred SNPs were chosen at random, and the base calls were independently verified by inspection of the original electropherograms at TIGR (CDC1551) and the Sanger Center (H37Rv). A second method, independent of

sequencing, was also used to confirm the base calls of these 100 SNPs. The visual inspection of the electropherograms and the sequencing independent method were in good agreement and indicated that 80 (91%) of 88 successful assays of the nucleotide differences were genuine.

Since our initial report, we have improved our methods for overlaying the annotation of open reading frame coordinates onto our analysis of the coordinates of nucleotide substitutions. Approximately 7% of the genome is noncoding, and approximately 15% of the substitutions are in these regions.

Dr. Musser is correct in pointing out that the substitution frequency expressed in Fraser et al. (5), based on our preliminary annotation of our *M. tuberculosis* sequence data, is not an equivalent comparison to the synonymous substitution frequency derived by his method of sequencing a select set of genes over a wide range of *M. tuberculosis* strains. He uses the methods of Li et al. (6), among the most widely accepted, for the calculation of nucleotide substitution frequencies and derives a $D_s$ value of <0.01 synonymous substitutions per 100 synonymous sites. Our preliminary data presented the frequency of total nucleotide substitutions at all positions (coding [synonymous and nonsynonymous] and noncoding) of the two recently sequenced strains, H37Rv and CDC1551. Our manuscript in preparation comparing the two *M. tuberculosis* strains will contain an analysis of synonymous substitutions. However, while Dr. Musser compared a select group of genes over perhaps several hundred strains, our frequency will be based on a genome-wide comparison between two strains.

**Robert Fleischmann**
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### References

1. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 1998;393:537-44.
2. Delcher AL, Kasif S, Fleischmann RD, Peterson J, White O, Salzberg SL. Alignment of whole genomes. Nucleic Acids Res 1999;27:2369-76.
3. Weinstock GM. Genomics and bacterial pathogenesis. Emerg Infect Dis 2000;6:496-504.
4. Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, et al. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science 1995;269:496-512.
5. Fraser CM, Eisen J, Fleischmann RD, Ketchum KA, Peterson S. Comparative genomics and understanding of microbial biology. Emerg Infect Dis 2000;6:505-12.
6. Li WH, Wu CI, Luo CC. A new method for estimating synonymous and nonsynonymous rates of nucleotide substitution considering the relative likelihood of nucleotide and codon changes. Mol Biol Evol 2000;2:150-512.

## Will Avilamycin Convert Ziracine into Zerocine?

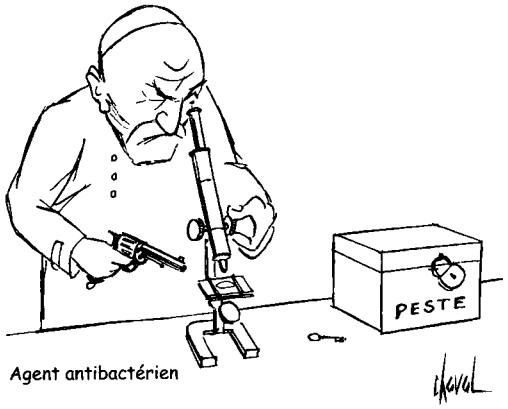
**To the Editor:** Dr. Courvalin urges that avilamycin be prospectively banned as an antibiotic growth promoter to prevent the development of bacteria cross-resistant to the potential human-use product evernimicin (1). Elanco Animal Health, the manufacturer of avilamycin, would like to clarify the situation with respect to avilamycin and everninomicin. It should be noted that there is incomplete cross-resistance in that enterococci resistant to avilamycin exhibit only decreased susceptibility, not complete resistance, to everninomicin (2). Dr. Courvalin's recommendation has become moot, since Schering-Plough has discontinued clinical development of Ziracin, as announced in early May 2000, "because the balance between efficacy and safety did not justify further development of the product" (http://www.sch-plough.com/news/research/2000/050500.html). Thus, avilamycin actually remains in compliance with the Swann principles. In addition, the Scientific Committee on Animal Nutrition, which advises the European Union Commission, released its assessment of the potential impact from cross-resistance in late April 2000 (http://www.europa.eu.int/comm/food/fs/sc/scan/out48_en.pdf) and concluded that, although transfer of resistant bacteria–and presumably resistance genes–from animal to human bacteria is possible, the magnitude of the transfer with avilamycin resistance was not possible to predict. In part, this conclusion reflected the early developmental status of Ziracin and a few reports of clinical experience. An extensive survey of Ziracin showed that 100% of 4,208 enterococcal isolates from patients in 27 European countries were susceptible (3). Another survey of Ziracin showed that 99.5%-100% of 6,030 isolates of methicillin-resistant *Staphylococcus aureus/epidermidis*, enterococci, streptococci, and pneumococci from 33 laboratories around the world were susceptible (4). Avilamycin has been used in animal production in many of the countries from which these clinical isolates originated. To fairly balance a preemptive precautionary action against a currently marketed animal use product and a human clinical candidate, the World Health Organization Global Principles recommended that such an action be initiated only when the human clinical candidate dossier is submitted for regulatory approval, to ensure that the candidate will indeed enter the marketplace. (Use of antimicrobial growth promoters that belong to classes of antimicrobial agents used or submitted for approval in humans and animals should be terminated or rapidly phased out in the absence of risk-based evaluations.) [http://www.who.int/emc/diseases/zoo/who_global_principles.html#Purpose]). This recommendation also acknowledges, in accordance with the Swann Principles, that antimicrobial agents intended for nonhuman use can be used in animal production. The modification by the pharmaceutical industry of older classes of antimicrobials for human clinical use, with counterparts previously developed by animal health companies for use as growth promoters, has become common. Dr. Aarestrup of the Danish Veterinary Laboratory commented that "it will be necessary in the future to either totally avoid the use of antimicrobials for growth promotion or, once antimicrobials have been approved for growth promotion, to reserve these classes for growth promotion and search for therapeutic options among other classes" (2). With respect to avilamycin, this latter option is the better one, now that everninomicin (and perhaps the entire orthosomycin class by extension) has been demonstrated to be unsafe for parenteral or injectable use in humans, because it allows animal producers to use a product that poses no resistance threat to public health. Finally, other unique antibiotics for treatment

of serious gram-positive infections in humans (with no animal use counterparts) are in the pharmaceutical pipeline (e.g., LY333328 and daptomycin) or have recently been approved (e.g., linezolid). We hope that a fair balance can be achieved by the human medical and the animal health and production communities with regard to the types of antimicrobial agents that can be used in each sector.

**Thomas R. Shryock**
Elanco Animal Health, Greenfield, Indiana, USA

### References

1. Courvalin P. Will avilamycin convert ziracine into zerocine? Emerg Infect Dis 2000;6:558.
2. Aarestrup FM. Association between decreased susceptibility to a new antibiotic for treatment of human disease, everninomicin (SCH 27899), and resistance to an antibiotic used for growth promotion in animals, avilamycin. Microb Drug Resist 1998;4:137-41.
3. Schouten MA, Voss A, Hoogkamp-Korstanje JAA, and the European VRE Study Group. Antimicrobial susceptibility patterns of enterococci causing infections in Europe. Antimicrob Agents Chemother 1999;43:2542-6.
4. Jones RN, Hare RS, Sabatelli FJ, Ziracin Susceptibility Testing Group. In vitro gram-positive antimicrobial activity of evernimicin (SCH 27899), a novel oligosaccharide, compared with other antimicrobials: a multicentre international trial. J Antimicrob Chemother 2001;47:15-25.




Cartoon from poster of annual meeting of Société Française de Microbiologie, Section des Agents Antimicrobiens. Used with permission and courtesy of P. Courvalin.

## The Antibiotic Food-Chain Gang

**To the Editor:** In his reply to my letter (1), Dr. Shryock states that use of the growth promoter avilamycin, which confers cross-resistance to other members of the everninomycin class of drugs, was in compliance with the Swann principles. The Swann report, issued in 1969, recommends that antibiotics used to treat infections in humans not be used as animal-food additives (2). The combined efforts of many scientists were needed to bring about the 1999 ban in Europe of spiramycin, tylosin, virginiamycin, and bacitracin, each of which confers resistance to antibiotics used in clinical settings. It appears that more than 30 years was necessary for the animal-food industry to act in accordance with the Swann report.

The reasoning in terms of drug structures can be misleading. The implication is that drugs that are chemically closely related have the same target of action and are therefore subject to cross-resistance, and vice versa. For example, because it has an unusual structure, apramycin (a 4-substituted-2-deoxystreptamin) was used exclusively in animals in the hope that it would not be recognized by any of the known aminoglycoside-modifying enzymes (3). However, enterobacteria of animal origin were resistant to apramycin by synthesis of a plasmid-mediated 3-*N*-aminoglycoside acetyltransferase type IV, which also confers resistance to gentamicin (4). Following spread in animal strains (5), the plasmid was later found in clinical isolates from hospitalized patients (6).

The use of antibiotics in general should be based on the mechanisms of resistance in bacteria, rather than on their chemical makeup. In particular, the concept that resistance was a class phenomenon rapidly lost favor because of the extension of the concept of cross-resistance and the increased occurrence of co-resistance.

In classical cross-resistance, a single biochemical mechanism confers resistance to a single class of drugs: use of a given antibiotic can select resistance to other members of the group but not to drugs belonging to other classes. However, cross-resistance between drug classes can occur by two mechanisms: overlapping targets and drug efflux. An example of target overlap is provided by the macrolides, lincosamides, and streptogramins (MLS), which are chemically distantly related. However, constitutive methylation of a single adenine residue in ribosomal RNA confers high-level resistance to the three classes of antibiotics. This resistance phenotype is due to the fact that all these antibiotics have overlapping targets on the ribosome (7). Active efflux of the drugs outside bacteria has recently been recognized as a common resistance mechanism (8,9). This energy-dependent export confers low-level resistance to a wide variety of antibiotics. The broad substrate specificities of the pumps account for decreased susceptibility to beta-lactams, aminoglycosides, tetracyclines, chloramphenicol, trimethoprim, sulfonamides, fluoroquinolones, and MLS, among others (9).

In contrast to cross-resistance, co-resistance is due to the presence in the same host of several mechanisms, each conferring resistance to a given class of drugs. In addition, the corresponding genes are often adjacent (physically linked) and expressed in a coordinated fashion. One of the most efficient system of this type is represented by the integrons (10) first described in gram-negative bacilli (11,12) and more recently found in gram-positive bacteria (13). Because of the genetic organization resulting in co-expression of the various genes, use of any antibiotic that is a substrate for one of the resistance mechanism will co-select for resistance to the others and thus for maintenance of the entire gene set. Since cross-resistance means cross-selection and co-resistance implies co-selection, the use of any antimicrobial agent is de facto rendered inadequate as a growth promoter.

I also disagree with the notion that because a member of an antibiotic class has been misused as a growth promoter the class should not be used in the future for human

therapy; the hierarchy could conceivably be humans first, animals second, rather than the opposite. For various reasons, the development of daptomycin and ramoplanin has been suspended for several years. If, during this period, these agents had been used as growth promoters, they would not now be under development for humans. I would rather see ramoplanin used for the microbial modulation of the intestinal tract in immunocompromised patients than as an animal-food additive.

During the last 30 years, thanks to molecular biology, enormous progress has been made in understanding the genetics and biochemistry of resistance. Incorporating this knowledge for decision-making in problems of public health importance is timely. I hope that it will not take 30 years for the pharmaceutical industry to act in agreement.

**Patrice Courvalin**
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## References

1. Courvalin P. Will avilamycin convert ziracine into zerocine? Emerg Infect Dis 2000;6:558.
2. Swann MM. Report of the Joint Committee on the use of antibiotics in animal husbandry and veterinary medicine. London: Her Majesty's Stationery Office; 1969.
3. Price KE, Godfrey JC, Kawaguchi H. Effect of structural modifications on the biological properties of aminoglycoside antibiotics containing 2-deoxystreptamine. In: Perlman D, editor. Structure-activity relationships among the semisynthetic antibiotics. New York: Academic Press; 1977. p. 272-4.
4. Chaslus-Dancla E, Martel JL, Carlier C, Lafont JL, Courvalin P. Emergence of 3-N-acetyltransferase IV in *Escherichia coli* and *Salmonella typhimurium* isolated from animals in France. Antimicrob Agents Chemother 1986;29:239-43.
5. Chaslus-Dancla E, Gerbaud G, Lafont JP, Martel JL, Courvalin P. Nucleic acid hybridization with a probe specific for 3-aminoglycoside acetyltransferase type IV: a survey of resistance to apramycin and gentamicin in animal strains of *Escherichia coli*. FEMS Microbiol Lett 1986;34:265-8.
6. Chaslus-Dancla E, Pohl P, Meurisse M, Marin M, Lafont JL. High genetic homology between plasmids of human and animal origins conferring resistance to the aminoglycosides gentamicin and apramycin. Antimicrob Agents Chemother 1991;35:590-3.
7. Fernandez-Munoz R, Monro RE, Torres-Pinedo R, Vasquez D. Substrate- and antibiotic-binding sites at the peptidyl-transferase centre of *Escherichia coli* ribosomes. Studies on the chloramphenicol, lincomycin and erythromycin sites. Eur J Biochem 1971;23:185-93.
8. Nikaido H. Multidrug efflux pumps of gram-negative bacteria. J Bacteriol 1996;178:5853-9.
9. Paulsen IT, Brown MH, Skurray RA. Proton-dependent multidrug efflux systems. FEMS Microbiol Rev 1996;60:575-608.
10. Rowe-Magnus DA, Mazel D. Resistance gene capture. Curr Opin Microbiol 1999;2:483-8.
11. Hall RM. Mobile gene cassettes and integrons: moving antibiotic resistance genes in gram-negative bacteria. Ciba Foundation Symposium 1997;207:192-202.
12. Hall RM, Stokes HW. Integrons: novel DNA elements which capture genes by site-specific recombination. Genetica 1993;90:115-32.
13. Nesvera J, Hochmannova J, Patek M. An integron of class 1 is present on the plasmid pCG4 from gram-positive bacterium *Corynebacterium glutamicum*. FEMS Microbiol Letters 1998;169:391-5.

## Upcoming Events

### Johns Hopkins 19th Annual Graduate Summer Institute of Epidemiology and Biostatistics
**Baltimore, MD**
**June 18-July 6, 2001**

The Johns Hopkins University School of Public Health and School of Medicine Department of Epidemiology and Department of Biostatistics are sponsoring the Graduate Summer Institute of Epidemiology and Biostatistics. The Institute provides an opportunity for physicians, nurses, or health professionals to enroll in courses for either academic or continuing education credit. The deadline for applications is June 1, 2001.

For more information, contact Ayesha Khan, Department of Epidemiology, School of Public Health, Johns Hopkins University, 615 North Wolfe Street, Baltimore, MD 21205, USA. Telephone: 410-955-7158; fax: 410-955-0863; e-mail: akhan@jhsph.edu; or visit http://www.jhsph.edu/summerepi

### 2001 Joint Conference of the American Society for Rickettsiology and the Bartonella as an Emerging Pathogen Group
**Big Sky Resort, Big Sky, Montana,**
**just outside Yellowstone National Park**
**August 17-22, 2001**

Topics will include epidemiology, clinical aspects, immunity, molecular pathogenesis, and genomics of *Ehrlichia*, *Bartonella*, *Coxiella*, and *Rickettsia*.

For more information, contact Michael F. Minnick by telephone: 406-243-5972; fax: 406-243-4184; or e-mail: minnick@selway.umt.edu. Detailed information is also available at the conference website: http://www.cas.umt.edu/asr/.

## Errata

**Vol. 6, No. 5**

In the article, "Prevalence of Non-O157:H7 Shiga Toxin-Producing *Escherichia coli* in Diarrheal Stool Samples from Nebraska," by Paul D. Fey et al., an error occurred in reporting a primer used for amplifying the shiga-toxin gene. The first complete sentence at the top of column 1, page 531, should read, "The following set of primers, which detects both *stx1* and *stx2*, was used: 5' TTTACGATAGACTTCTCGAC 3' and 5' CACATATAAATTATTTCGCTC 3'." We regret any confusion this error may have caused.

**Vol. 7, No. 1**

In the International Update "Emerging Infectious Diseases in Russia, 1990-1999," by Sergey V. Netesov and J. Lyle Conrad, the area of Russia is given incorrectly. The area should be approximately 17,075,000 $km^2$. We regret this error.

**Vol. 7, No. 1**

In the article "Quinolone and Macrolide Resistance in *Campylobacter jejuni* and *C. coli*: Resistance Mechanisms and Trends in Human Isolates," Figure 1, page 25, the genes should have been referred to as follows: *gyrA* and *parC*. In addition, Thr-90 should have been Asp-90. To view the correct figure, see URL: http://www.cdc.gov/ncidod/EID/vol7no3/erratum.htm. We regret any confusion this error may have caused.

## The Cover

**Detail from La Primavera (Spring) (circa 1475-1478).**
**Chloris transformed into Flora, mother of flowers**
**Tempera on panel; 315 cm x 205 cm**
**Sandro Botticelli**

La Primavera was painted for the Villa di Castello, which was owned by a branch of the Medici family. In 1815, the painting was moved to the Uffizi, where it is now. Botticelli's sophisticated understanding of perspective, anatomy, and the humanist debate of the Medici court never overshadowed the poetry of his vision. An allegory of life, beauty, and knowledge united by love, La Primavera captures the freshness of an early spring morning, with the light shining through the tall, straight trees, already laden with their golden fruit.

The meaning of La Primavera has been interpreted in many ways but remains enigmatic. One persisting interpretation suggests that the mythological figures in the painting undergo transformation to show a progressive sublimation of sensual love in intellectual contemplation. This sublimation is in accordance with the harmony that governs the cosmos and is evoked in the figure of Venus.

The transformation of the mythological figures, which is of neoplatonic inspiration, uses symbols and episodes from classical literature, from Hesiod to Ovid, as well as interpretations of classical themes by Botticelli's contemporaries Leon Battista Alberti and Marsilio Ficino. On the right of the painting, Zephyrus (the warm wind of spring) seizes Chloris, the nymph who will be transformed into Flora, mother of flowers. At the center of the painting, through the mediation of Eros and Venus (Ficino's Venus Humanitas, who arouses passion but also converts it into contemplation), the transformation moves on to the Three Graces. The Graces, an ancient symbol of liberality (Aglaia who gives, Euphrosyne who receives, Thalia who returns), in the most properly platonic sense, allude to the relationship between the divine element and the human. On the left, Mercury dispersing clouds marks the definitive arrival of the spiritual moment. Put in these terms, the theme of the painting may appear pedantic; however, Botticelli leaves the extrinsic meaning unresolved and controversial.

Abstracted from Great Museums of the World, Uffizi Florence. Milan: Newsweek, Inc., and Arnold Mondadori Editore; 1968.

## Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or eideditor@cdc.gov (e-mail).

Emerging Infectious Diseases is published in English and features the following types of articles: Perspectives, Synopses, Research Studies, Policy Reviews, and Dispatches. The purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

Chinese, French, and Spanish translations of some articles can be accessed through the journal's homepage at www.cdc.gov/eid. Articles by authors from non-English-speaking countries can be made simultaneously available in English and in the author's native language (electronic version of the journal only).

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# Instructions to Authors

## Manuscript Preparation

Follow "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (Ann Intern Med 1997:126[1]36-47) (http://www.acponline.org/journals/annals/01jan97/unifreqr.htm).

Begin each of the following sections on a new page and in this order: title page, abstract, text, acknowledgments, references, tables, figure legends, and figures.

Title page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Also provide address for correspondence (include fax number and e-mail address).

Abstract and key words. Avoid citing references in the abstract. Include up to 10 key words; use terms listed in the Medical Subject Headings from Index Medicus (http://www.nlm.nih.gov/mesh/meshhome.html).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Type only on one side of the paper and number all pages, beginning with the title page. Indent paragraphs 5 spaces; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use Courier font size 10 and ragged right margins. Italicize (rather than underline) scientific names when needed.

Electronic formats. For word processing, use WordPerfect or MS Word. Send graphics in native format or convert to .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) formats. The preferred font for graphics files is Helvetica. Convert Macintosh files into one of the suggested formats. Submit slides or photographs in glossy, camera-ready photographic prints.

References. Follow the Uniform Requirements style. Place reference numbers in parentheses, not in superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title in full. List the first six authors followed by "et al."

Tables and figures. Create tables within the word processing program's table feature (not columns and tabs within the word processing program). For figures, use color as needed; send files, slides, photographs, or prints. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced. Place figure keys within the figure.

Access the journal's style guide at http://www.cdc.gov/ncidod/EID/style_guide.htm

## Manuscript Submission

Include a cover letter verifying that the final manuscript has been seen and approved by all authors.

Submit three copies of the original manuscript with three sets of original figures and an electronic copy (on diskette or by e-mail) to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D 61, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov.

## Types of Articles

**Perspectives, Synopses, Research Studies, and Policy Reviews**: Articles should be approximately 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

**Perspectives:** Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases or related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change; human demographics and behavior; technology and industry; economic development and land use; international travel and commerce; and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

**Synopses**: This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. Use of subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged.

**Research Studies:** These articles report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (e.g., "Here is what we found, and here is what the findings mean").

**Policy Reviews:** Articles in this section report public health policies that are based on research and analysis of emerging disease issues.

**Dispatches**: These brief articles are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome. Dispatches (1,000 to 1,500 words) need not be divided into sections. Provide a short abstract (50 words); references, not to exceed 10; figures or illustrations, not to exceed two; and a brief biographical sketch.

**Another Dimension:** Thoughtful esays on philosophical issues related to science and human health.

**Book Reviews:** Short reviews (250 to 500 words) of recently published books on emerging disease issues are welcome.

**Letters:** This section includes letters that give preliminary data or comment on published articles. Letters (500 to 1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

**News and Notes**: We welcome brief announcements (50 to 150 words) of timely events of interest to our readers. (Announcements can be posted on the journal web page only, depending on the event date.) In this section, we also include summaries (500 to 1,500 words) of conferences focusing on emerging infectious diseases. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.