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Vol.11, No.7, July 2005

Global Wildlife Trade

EMERGING INFECTIOUS DISEASES

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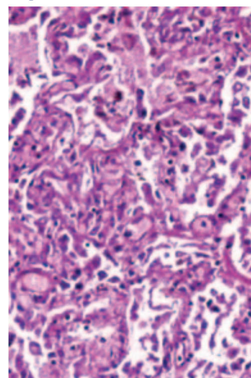

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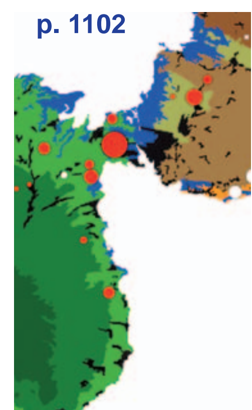

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Attributing Illness to Food

Michael B. Batz,* Michael P. Doyle,† J. Glenn Morris, Jr.,‡ John Painter,§ Ruby Singh,¶ Robert V. Tauxe,§ Michael R. Taylor,* and Danilo M.A. Lo Fo Wong,#
for the Food Attribution Working Group[1]

Identification and prioritization of effective food safety interventions require an understanding of the relationship between food and pathogen from farm to consumption. Critical to this cause is food attribution, the capacity to attribute cases of foodborne disease to the food vehicle or other source responsible for illness. A wide variety of food attribution approaches and data are used around the world, including the analysis of outbreak data, case-control studies, microbial subtyping and source tracking methods, and expert judgment, among others. The Food Safety Research Consortium sponsored the Food Attribution Data Workshop in October 2003 to discuss the virtues and limitations of these approaches and to identify future options for collecting food attribution data in the United States. We summarize workshop discussions and identify challenges that affect progress in this critical component of a risk-based approach to improving food safety.

Foodborne microbiologic hazards may be responsible for as many as 76 million cases of illness in the United States each year (1) and are thus an important food safety challenge. To lower the incidence of foodborne disease, many experts and stakeholders urge the development of a science- and risk-based food safety system, in which decision makers prioritize hazards and interventions using the best available data on the distribution and reduction of risks (2,3). Such a system requires an understanding of the many risk factors between the point of production and the point of consumption and the ability to systematically target intervention efforts along this "farm-to-fork" continuum.

Although the Foodborne Diseases Active Surveillance Network (FoodNet), administered by the Centers for Disease Control and Prevention (CDC), is producing increasingly robust data on the incidence of illness due to specific enteric pathogens, no method exists to categorize these illnesses by mode of transmission, whether drinking water, environmental exposure, or consumption of a specific food. Interventions are almost always food (or process) specific. To design and prioritize effective food safety interventions, we must be able to perform food attribution—that is, identify which foods are vehicles for specific cases of illness. Such data are of particular importance for US government agencies that regulate food and food animals, including the Food Safety Inspection Service (FSIS) of the US Department of Agriculture (USDA), and the Center for Food Safety and Applied Nutrition (CFSAN) and the Center for Veterinary Medicine (CVM) of the Food and Drug Administration (FDA).

Foodborne illnesses can be attributed to foods by using a variety of data sources and analytic approaches; each has its virtues and limitations. In Atlanta on October 31, 2003, the Food Safety Research Consortium (FSRC) sponsored the Food Attribution Data Workshop to explore these approaches in detail. Attendees included representatives from CDC, FSIS, CFSAN, CVM, the Environmental Protection Agency (EPA), consumer advocacy organizations, and member institutions of FSRC, including the University of Maryland at Baltimore, University of Georgia, Iowa State University, University of California at Davis, and Resources for the Future. This article summarizes material discussed at the workshop and identifies challenges that affect progress in this critical component of a risk-based approach to improving food safety.

*Resources for the Future, Washington, DC, USA; †University of Georgia, Griffin, Georgia, USA; ‡University of Maryland School of Medicine, Baltimore, Maryland, USA; §Centers for Disease Control and Prevention, Atlanta, Georgia, USA; ¶Food and Drug Administration, Laurel, Maryland, USA; and #Danish Institute for Food and Veterinary Research, Copenhagen, Denmark

[1]In addition to the authors, the presenters and attendees who constitute the Food Attribution Working Group are Fred Angulo (CDC), Robert Buchanan (FDA), H. Gregg Claycamp (FDA), Caroline Smith DeWaal, Center for Science in the Public Interest (CSPI), Jorge Santo Domingo (EPA), Katherine Field (Oregon State University), David Goldman (USDA), Matthew Moore (CDC), Sarah O'Brien (Communicable Disease Surveillance Centre, England), Efrain Ribot (CDC), Stephen Sundlof (FDA), and Catherine Woteki (Iowa State University).

Food Categorization

For the purposes of attributing illnesses to foods, food vehicles must be grouped into suitable categories. Although the idea may seem simple, the need for a single food categorization scheme has emerged as a critical issue. At a general level, a list of major food commodities might include 11 categories: poultry, eggs, pork, beef, dairy, fish, mollusks, crustaceans, wild game, row crops (e.g., lettuce and corn), and tree crops (e.g., apples and oranges). Each of these commodities could be divided further, leading to such subcategories as broiler chickens and raw oysters. Additionally, classification could include level of processing (raw, fresh-cut, canned, frozen), origin (domestic, imported), and location of preparation (home, food processor, food service). When illnesses are linked to foods with multiple ingredients, such as soups or casseroles, the choices include whether to omit these cases from analysis, to categorize multiple-ingredient foods by "essential" ingredient, or to attribute illnesses by the proportion of individual ingredients.

A common food categorization scheme is essential if different sources of data are to be combined or compared. Because of lack of agreement in categorization, data from CDC, state health departments, and FDA and USDA and their constituent agencies are often not directly comparable. As a first step in any approach to food attribution, food categories need to be standardized across government agencies, with a scheme acceptable to industry, academia, and consumer groups.

Current Approaches to Food Attribution

Approaches to food attribution can be grouped into 2 broad categories, loosely designated as epidemiologic and microbiologic. Epidemiologic approaches are based on public health surveillance and include foodborne outbreak data and case-control studies. Microbiologic approaches rely on data on pathogen samples drawn from human, animal, and food sources and include pathogen subtyping, as used in Denmark's Salmonella Accounts (4) and microbial source tracking (MST) methods, as well as risk assessments of specific pathogens in specific foods.

Danish Experiences

Denmark has an integrated system in which data from public health surveillance and pathogen monitoring of foods and animals are routinely collected, collated, and analyzed by a single coordinating body. Cultures collected from infected persons, animals, and retail food sources are subtyped, allowing for the direct comparison of surveillance and monitoring data and the identification of public health outcomes by food source.

The regular monitoring of food sources is performed on farms, at slaughter, and in retail foods, although the emphasis is on primary production facilities (5). Every flock of egg-laying chickens is regularly tested for salmonellae, as are all flocks of broiler chickens, turkeys, and ducks. Finishing pigs are continually tested, dairy herds are routinely monitored, and poultry, pork, and beef are examined during slaughter processes. Imported meat and poultry products are monitored, as are wild animals, birds, and pets, and retail surveys are performed on raw meat, pork, poultry, shell eggs, fruits, and vegetables.

Salmonella isolates obtained from animal and food sources are subtyped (with serotyping, phage typing, and pulsed-field gel electrophoresis [PFGE]) and compared in a quantitative manner with isolates obtained from human infections (6). A prerequisite of the model is predominance of at least 1 "distinctive" *Salmonella* subtype in each main animal reservoir; human infections of distinctive subtypes are assumed to have originated from that reservoir. Human infections caused by *Salmonella* subtypes found in multiple animal reservoirs are attributed proportionally to the occurrence of the distinctive subtypes. Model results have been corroborated by case-control studies, outbreak reports, time-series analysis, and risk assessments (7). In the past 10 years, the Danish model has proven invaluable for identifying pathogen reservoirs in animal populations, tracking trends of human salmonellosis, and guiding interventions (4).

One weakness of the Danish method is that causation cannot be discerned for cases without distinctive *Salmonella* subtypes; thus, the proportional attribution of such cases across animal reservoirs may not necessarily be accurate. Also, vegetables, fruits, fish, pets, water, and other sources of infection are not directly included in the analysis, under the assumption that the original sources of bacterial infection are animal reservoirs. Furthermore, the model is currently focused on salmonellae and may not be applicable to other pathogens that do not meet certain prerequisites. For example, although extensive subtyping has also been performed on *Campylobacter* isolates, the homogeneous distribution of subtypes across reservoirs makes attribution difficult. Since the Danish model is focused on the major food-animal reservoirs, it cannot identify responsible foods at the point of consumption or at other points along the farm-to-fork continuum.

British Experiences

The United Kingdom uses an integrated systems approach to food safety that includes both epidemiologic and microbiologic methods, with responsibility for foodborne illness consolidated into a single government office. Annual reports on zoonoses, which combine surveillance data with data on food and animal monitoring (8), are produced. In addition, etiologic analyses of foodborne outbreaks, detailing illnesses by pathogen, food source, and

additional risk factors, are performed. UK agencies also perform regular pathogen monitoring and subtyping of animals and retail food (8–10).

The United Kingdom has developed a method for estimating the relative risks associated with specific foods, dividing the number of cases due to a specific food (as derived from their outbreak database) by the estimated total servings of that food consumed in a year. The weaknesses of this system include the assumption that all hospitalizations and deaths are routed through general practitioners and reliance on outbreak data, which may not be representative of sporadic disease. However, the UK outbreak dataset is large, and the food vehicles implicated correlate with findings of local epidemiologic studies. Increasingly, data indicate that interventions guided by the system have been successful in reducing cases and risk for foodborne illness.

US Outbreak Data

Reports of outbreak investigations provide the most comprehensive US data for determining the foods responsible for illnesses. The Foodborne Disease Outbreak Surveillance System contains data on >20,000 US foodborne disease outbreaks reported to CDC since 1973; these reports link specific foods to cases of human illness (11). CDC, the Center for Science in the Public Interest (CSPI), and the FSRC have estimated food attribution using these data (12–16).

Responsibility for investigating foodborne disease outbreaks resides with local and state health departments, which then report these data to CDC. Reported outbreaks represent only a small proportion of those that occur, and the degree of underreporting may vary geographically and temporally. For example, revision of the reporting process and provision of increased resources to CDC and state health departments from the National Food Safety Initiative were associated with a doubling of the number of outbreaks reported annually from 1996 to 1998.

Outbreak data have additional important limitations. Outbreaks that are large, associated with restaurants, have short incubation periods, or cause serious illness are more likely to be investigated and reported. Likewise, illnesses due to pathogens that are difficult to identify or rarely cause outbreaks are underrepresented. For example, the foods most frequently identified as the source of *Campylobacter* outbreaks differ markedly from those identified as sources in community studies of sporadic cases (17).

An approach used by CDC to estimate illness due to a particular food-pathogen combination is to count the number of outbreak-related illnesses due to a particular pathogen and to determine the proportion of these due to each food grouping. These proportions are then applied to estimates of incidence of that pathogen, as reported by CDC (1). This approach is also employed in the Foodborne Illness Risk Ranking Model (FIRRM), an analytic tool developed by FSRC to compare the public health impact of various pathogen-food combinations (14–16). As recent CDC outbreak data were not available when FIRRM was developed, FSRC relied on outbreak data compiled by CSPI. The CSPI database consists primarily (88%) of foodborne outbreak data compiled by CDC and now available on the Internet, but it also includes outbreaks not included in CDC data. CDC may not have received reports on these illnesses from state health departments or may have excluded reports from the database because they did not meet CDC criteria for foodborne outbreaks due to specific pathogens.

Improving food attribution from outbreaks will require improving both the quality and quantity of data. In particular, increased efforts are needed to obtain stool specimens from ill persons early in outbreaks to increase the fraction of outbreaks for which a pathogen is identified to >40% and to trace back foods implicated in outbreaks to their sources. CDC has launched an effort to improve the categorization of food items and ingredients, so outbreaks can be grouped in useful ways for regulatory agencies, industry, and consumers. CDC is also creating new analysis capabilities for the foodborne outbreak surveillance system that will provide data summaries for a variety of purposes, including food attribution.

FoodNet Sporadic Case-Control Studies

FoodNet is an active surveillance program centered at CDC that tracks foodborne illnesses from 9 pathogens in 10 well-defined target populations (18–20). In FoodNet case-control studies, patients reporting through FoodNet are contacted for followup interviews and to complete questionnaires to estimate the proportion of illnesses associated with specific foods, food preparation, handling practices, and such behavior as pet ownership, farm visits, or international travel. FoodNet has performed case-control studies on a variety of pathogens, including *Salmonella* spp., *Escherichia coli* O157:H7, *Campylobacter*, *Cryptosporidium*, and *Listeria monocytogenes* (among others; 17, 21–24).

FoodNet case-control studies are of particular value for assessing food attribution of sporadic illness because they are population based. Because the diseases under investigation are rare in all population subgroups, rate ratios in the data closely approximate risk ratios in the population. Along with case exposure percentages, these risk ratio estimates may be used to calculate the "population attributable fraction," the proportion of new cases occurring during a given period in a particular at-risk population that was attributable to the effect of ≥1 exposures.

FoodNet case-control studies have limitations, primarily due to recall bias, long exposure windows, and immunity (20). First, patients and controls are limited in what they remember and can report in an interview, and the interview format itself has limitations. Second, the periods during which exposures are ascertained for FoodNet case-control studies tend to be long (5–7 days), so the likelihood of detecting a difference in exposure between cases and controls is limited by high exposure frequencies among both cases and controls. Further studies are needed to assess the consequence of using shorter exposure windows. Lastly, if a relatively common infection conveys durable immunity, an important segment of the population may be immune and therefore not susceptible to infection, making the demonstration of an association between exposures and risk for infection more difficult.

Microbial Subtyping and Microbial Source Tracking

CDC's National Molecular Subtyping Network for Foodborne Disease Surveillance (PulseNet) links public health laboratories that use PFGE to routinely fingerprint suspected foodborne bacteria isolates (25). Results of PFGE subtyping of 5 bacteria (*E. coli* O157:H7, nontyphoidal *Salmonella* spp., *Shigella* spp., *L. monocytogenes*, and *Campylobacter* spp.) are stored in the electronic PulseNet database; bacterial strains in the database can be compared quickly and provide an early warning system for emerging outbreaks when related strains appear. PulseNet cannot currently be used for food attribution because it does not include isolates from sporadic cases of human illness or from food or animal sources.

MST refers to a specific application of microbial subtyping in which markers from an isolate are used to trace that isolate to an animal source, similar to what is done for salmonellae in Denmark. If different animal species carry unique, host-specific populations of microorganisms, a subtyped isolate drawn from an infected person could indicate that the isolate originated in 1 species as opposed to another. A large number of MST methods are being researched, most of which were originally developed to trace fecal bacteria in natural waters. Most approaches use genetic or phenotyping fingerprinting methods, although chemical markers, biomarkers, viruses, and bacteriophages are also used as indicators of animal source (26,27). Although MST techniques have potential, no single approach seems ideal for all pathogens and situations. US agencies have begun to research MST specifically for food attribution purposes; CVM, in particular, has investigated methods for *Salmonella* and *Campylobacter* spp. (28). Although results to date are promising, they are only initial steps toward using MST methods to attribute illnesses to food animals. Similarly, data collected on the antimicrobial resistance of bacteria by researchers at CVM, CDC, the Agricultural Research Service (an agency of the USDA), and elsewhere may ultimately prove useful for food attribution purposes.

Risk Assessments

Risk assessments include food contamination data, food storage and consumption patterns, risk behavior, and dose-response functions to predict risks for illness from specific pathogens found in specific foods. If exposure estimates and dose-response functions are sufficiently accurate (a key consideration), risk assessments may produce excellent estimates of the true impact of illness.

Because risk assessments are so resource-intensive, they have been undertaken for only a limited number of pathogen-food combinations. The most comprehensive risk assessments for a single pathogen are those performed for *L. monocytogenes* by CFSAN and FSIS (29,30). This set of 23 individual risk assessments focused on ready-to-eat foods, including deli meats, dairy, produce, and seafood. Risk assessments have also been conducted on *E. coli* O157:H7 in ground beef (31), on *Salmonella enterica* serovar Enteritidis in shell eggs and egg products (32), and on *Vibrio parahaemolyticus* in molluscan shellfish (33).

For risk assessments to be used for food attribution, however, they need to be performed on most food items associated with a particular pathogen. Considering the 3-year duration of the *L. monocytogenes* risk assessments, performing comprehensive risk assessments on a sufficiently large number of pathogen-food combinations for systematic food attribution would be a colossal task.

The other major limitation of using risk assessments for food attribution is that they are inherently predictive. Unlike surveillance data, they do not measure observable public health effects, but rather estimate the impact on the basis of assumptions that are difficult to validate in a dynamic system, in particular dose-response functions, food storage and consumption patterns, and consumer behavior. Furthermore, risk assessments are ill suited for temporal analyses, since they are not routinely updated as new data become available. Risk assessments are most useful for food attribution purposes when compared with other estimates, such as those based on outbreak data or case-control studies.

US Food Monitoring

Various US food safety agencies test for pathogen prevalence in foods through routine monitoring and case studies. FSIS monitoring, focused on the slaughter process, includes regular testing of raw meat and poultry for salmonellae (34), ground beef for *E. coli* O157:H7 (35), and ready-to-eat deli products for multiple pathogens (http://www.fsis.usda.gov/OPHS/rtetest/). FSIS and the Agricultural Research Service have examined pathogen

prevalence in commercial food products, such as *L. monocytogenes* in frankfurters (36). The United States does not have a comprehensive program for monitoring live food animals. Food and animal monitoring data, when not associated with surveillance data, are not applicable for food attribution purposes.

Expert Elicitation

When scientific or epidemiologic data are lacking, sparse, or highly uncertain, expert judgment may be used to fill gaps or combine conflicting estimates into a meaningful solution. Expert judgments derived through formal methods are increasingly used and recommended for assessing risk and the economic impact of regulations (37–39).

FSRC researchers administered an expert elicitation of experienced food safety researchers, public health scientists, and food safety authorities for use in the Foodborne Illness Risk Ranking Model (15). Produced with a standardized, vetted method, the survey asked respondents to estimate the percentage of 11 pathogens caused by each listed food category and included measures of respondent uncertainty and possible biases. Although data need to be analyzed further, initial results are promising and corroborate food attribution percentages derived from other means.

Expert elicitations are limited because they are based on perception, not on observable data. Results may be circular if experts rely on the same sources, or deceptive if experts are similarly misinformed or biased. Expert judgments are thus not an ideal source of food attribution data but may have utility if data are sparse or inconsistent and uncertainty is substantial.

Conclusions

A recent National Academies of Science report, Scientific Criteria to Ensure Safe Food, argues for "the development of a comprehensive national plan to harmonize the foodborne disease surveillance that is conducted by public health agencies with the monitoring of pathogens across the food production, processing, and distribution continuum that is conducted by food safety regulatory agencies" (2). The motivation driving this suggestion is the same as that which motivated FSRC to convene the Food Attribution Data Workshop; to make informed science- and risk-based decisions about food safety interventions, we need to be able to associate foodborne illnesses to specific food vehicles. The goal of the workshop was to review the approaches currently used for food attribution, in the United States and abroad, and to identify future options for the collection of food attribution data in the United States.

Although all workshop attendees or institutions did not reach consensus about the ideal data for food attribution, there was nearly universal agreement that none of the current data sources are sufficient on their own because of methodologic limitations or gaps in available data (see Table). Furthermore, in the United States, data are spread over a wide range of agencies and researchers, resulting in myriad studies covering different aspects of the food attribution problem. These issues make it difficult to accurately and dependably attribute illnesses to the foods responsible as pathogen vehicles—and, in turn, to target appropriate intervention strategies.

Several characteristics should be considered in evaluating and comparing current and future food attribution methods; their relative importance depends on the purpose for which the attribution data are sought. These include scientific accuracy and uncertainty, quality and breadth of data, computational consistency, practical feasibility, cost of implementation, flexibility and scalability, utility for targeting interventions, and congruency with other relevant data sources. Among the critical unresolved issues is how to balance such factors as scientific accuracy and practical feasibility to produce attribution data that will be both useful and affordable.

With so many institutions responsible for various aspects of the food safety system, collaboration is paramount, as is the explicit delineation of responsibilities and powers. Access to these data is a critical issue. Building a system in which data and conclusions are shared in a timely manner among agencies and with industry and academia, and privacy issues with persons and industry participants have to be addressed. Creation of an open searchable database of outbreaks would greatly expand the opportunities for research and collaboration.

As described here, a variety of approaches have been used to better define the source of foods responsible for human infections. However, none of these approaches is likely to be sufficient on its own. The implicit conclusion, therefore, is that the scientific and accurate attribution of foodborne illnesses to specific foods means developing a comprehensive program that combines many of the discussed methods and data. Such a system can be achieved with increased resources and cooperation among food safety institutions.

The Food Attribution Data Workshop was sponsored by the Food Safety Research Consortium, a multidisciplinary collaboration to improve public health; members include the University of Maryland, Baltimore; University of Georgia; Iowa State University; University of Massachusetts; University of California, Davis; Michigan State University; and Resources for the Future. The workshop grew out of an FSRC project funded by the Robert Wood Johnson Foundation and received generous support from the Joint Institute for Food Safety and Applied Nutrition, the Center for Food Safety and Applied Nutrition and Center for Veterinary Medicine within the FDA, and the Office of

Table. Current approaches to food attribution

Approach	Primary advantages	Primary limitations	Refs
Denmark Salmonella Accounts	Microbial subtyping provides direct link between public health endpoint and animal	Difficult to expand to other pathogens; requires distinctive subtypes across reservoirs	5,6
	High reporting of illnesses (social health care)	Focus on animals ignores nonanimal sources	
	National, temporal coverage for both illnesses and animal/product monitoring	Focus on reservoirs, not food products at point of consumption	
UK outbreak data	Large dataset: national, temporal coverage	May not correlate with sporadic case data	8
	Results correlate with local epidemiologic findings	Not all pathogens well represented	
		Dependence on general practitioners	
US outbreak data	National and temporal coverage	May not correlate with sporadic case data	11,12
	Large common dataset	Geographic and temporal inconsistencies (local reporting) and biases towards certain foods	
	Straightforward, uses existing data	Not all pathogens well represented	
	Outbreaks and outbreak cases can be aggregated into food categories		
Case-control studies	Population-based studies	Survey format has recall bias and other limits	17, 20–24
	Captures risk factors not included in most surveillance data (travel, food preparation questions)	Long exposure windows (problems with common exposures)	
	Can implicate risks missed by laboratory testing	Durable immunity in population can impede associating exposures with illnesses	
		No laboratory verification	
Microbial subtyping	Subtyping of illnesses and foods can provide direct link between public health endpoint and source of infection	For animal sourcing, subtypes must be distinctive across species (see Danish Salmonella Accounts)	25–28,5,6
	Can be used to identify specific foods (outbreak investigations) or animal reservoirs (source tracking by species)	Utility may be limited to certain pathogens	
	Many different techniques, growing fast	Resource intensive; requires human surveillance, extensive monitoring of food and animals, plus laboratory testing, data storage, analysis	
Risk assessments	Can estimate cases not captured by surveillance methods (not limited by underreporting or biases in epidemiologic methods)	Predictive; cannot be verified	29–33
	Uses consumption and contamination data ignored by surveillance-based approaches	Large uncertainties in dose-response models and exposure estimates	
		Resource- and time-intensive (each pathogen-food combination requires its own exhaustive study)	
Food monitoring data	Captures upstream contamination (avoids environmental and cross-contamination after purchase)	Not usable for food attribution unless made compatible (through subtyping or other means) with public health data	34–36
Expert elicitation/ judgment	Useful when data are sparse or conflicting	Respondents can be similarly biased	15, 37–39
	Formal methods increase utility	Requires some level of consensus for reasonable error bounds	
		Based on perception, not data	

Risk Assessment and Cost-Benefit Analysis and the Economic Research Service within USDA.

Mr. Batz is a research associate with Resources for the Future, with technical responsibility for the FSRC's foodborne illness risk ranking model. He served as the primary author for this workshop summary.

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Address for correspondence: Michael B. Batz, Resources for the Future, 1616 P St NW, Washington, DC 20036-1434, USA; fax: 202-939-3460; email: mbatz@rff.org

Wildlife Trade and Global Disease Emergence

William B. Karesh,* Robert A. Cook,* Elizabeth L. Bennett,* and James Newcomb†

The global trade in wildlife provides disease transmission mechanisms that not only cause human disease outbreaks but also threaten livestock, international trade, rural livelihoods, native wildlife populations, and the health of ecosystems. Outbreaks resulting from wildlife trade have caused hundreds of billions of dollars of economic damage globally. Rather than attempting to eradicate pathogens or the wild species that may harbor them, a practical approach would include decreasing the contact rate among species, including humans, at the interface created by the wildlife trade. Since wildlife marketing functions as a system of scale-free networks with major hubs, these points provide control opportunities to maximize the effects of regulatory efforts.

Threats to global health and risk factors for emerging infectious diseases run the gamut from climate change to poverty to security issues, but few are as immediately manageable as the global trade in wildlife. Trade in wildlife provides disease transmission mechanisms at levels that not only cause human disease outbreaks but also threaten livestock, international trade, rural livelihoods, native wildlife populations, and the health of ecosystems. Quantifying the global wildlife trade is almost impossible since it ranges in scale from local barter to major international routes, and much is conducted illegally or through informal networks. Some estimates indicate that ≈40,000 live primates, 4 million live birds, 640,000 live reptiles, and 350 million live tropical fish are traded globally each year (1). Live wildlife in markets in Guangzhou, China, trade in masked palm civets, ferret badgers, barking deer, wild boars, hedgehogs, foxes, squirrels, bamboo rats, gerbils, various species of snakes, and endangered leopard cats, along with domestic dogs, cats, and rabbits (2). After the outbreak of severe acute respiratory syndrome (SARS) in 2003, 838,500 wild animals were reportedly confiscated from the markets in Guangzhou (3). Wild mammals, birds, and reptiles flow daily through trading centers, where they are in contact with persons and with dozens of other species before they are shipped to other markets, sold locally, or even freed and sent back into the wild as part of religious customs such as merit release (4) or because they become unwanted pets. In a single market in North Sulawesi, Indonesia, up to 90,000 mammals are sold per year (5). In a survey conducted at 1 market in Thailand for 25 weekends, >70,000 birds, representing of 276 species, were sold (6). A similar survey of 4 markets in Bangkok in 2001 found that of 36,537 observed birds; only 37% were native to Thailand, while 63% were non-native species (7).

In lieu of precise trade data, we conservatively estimated that in East and Southeast Asia, tens of millions of wild animals are shipped each year regionally and from around the world for food or use in traditional medicine. The estimate for trade and local and regional consumption of wild animal meat in Central Africa alone is >1 billion kg per year (8), and estimates for consumption in the Amazon Basin range from 67 to 164 million kilograms annually (9,10); for mammals alone, this consumption consists of 6.4 million to 15.8 million individual animals (11). In Central Africa, estimates of the number of animals consumed by humans annually vary, but 579 million has been proposed (12).

Hunters, middle marketers, and consumers experience some type of contact as each animal is traded. Other wildlife in the trade is temporarily exposed, and domestic animals and wild scavengers in villages and market areas consume the remnants and wastes from the traded and potentially traded wildlife. These numbers combined suggest that at least some multiple of 1 billion direct and indirect contacts among wildlife, humans, and domestic animals result from the wildlife trade annually. The increasingly global scope of this trade, coupled with rapid modern transportation and the fact that markets serve as network hubs rather than as product endpoints, dramatically increases the movement and potential cross-species

*Wildlife Conservation Society, Bronx, New York, USA; and †Bio-Economic Research Associates, Boulder, Colorado, USA

Figure. Vendor selling wild-caught birds for release at a religious shrine in Thailand. (Photo by W.B. Karesh.)

transmission of the infectious agents that every animal naturally hosts.

Since 1980, >35 new infectious diseases have emerged in humans (13), ≈1 every 8 months. The origin of HIV is likely linked to human consumption of nonhuman primates (14). Recent Ebola hemorrhagic fever outbreaks in humans have been traced to index patient contact with infected great apes that are hunted for food (15). SARS-associated coronavirus has been associated with the international trade in small carnivores (16), and a study comparing antibody evidence of exposure to this coronavirus demonstrated a dramatic rise from low or zero prevalence of civets at farms to an approximately 80% prevalence in civets tested in markets (17).

The inadvertent movement of infectious agents due to the wildlife trade is not limited to human pathogens but also affects pathogens of domestic animals and native wildlife. H5N1 type A influenza virus was recently isolated from 2 mountain hawk eagles illegally imported to Belgium from Thailand (18). A paramyxovirus highly pathogenic for domestic poultry entered Italy through a shipment of parrots, lovebirds, and finches imported from Pakistan for the pet trade (19). Monkeypox was introduced to a native rodent species and subsequently to humans in the United States by importing wild African rodents from Ghana for the US pet trade (20). Chytridiomycosis, a fungal disease now identified as a major cause of the extinction of 30% of amphibian species worldwide, has been spread by the international trade in African clawed frogs (21). Merit release of wild birds and reptiles that have passed through markets provides another avenue for introducing novel infectious agents into the wild (4) and warrants further attention (Figure).

Many diseases are transmitted through the same species of parasites carried by imported animals. For example, from November 1994 to January 1995, US Department of Agriculture personnel inspected 349 reptile shipments from 22 countries containing 117,690 animals. Ticks were removed from animals in 97 shipments, and infested shipments included 54,376 animals (22). Ticks carry many diseases that threaten livestock and human health, including heartwater disease, Lyme disease, and babesiosis.

The possibility of emerging infectious diseases spreading between persons and animals is rising, fueled by human activities ranging from the handling of bushmeat and the trade in exotic animals to the destruction or disturbance of wild habitat (23–25). In a list of 1,415 human pathogens, 61% are known to be zoonotic, and multiple host pathogens are twice as likely to be associated with an emerging infectious disease of humans (26). Seventy seven percent of pathogens found in livestock are shared with other host species (27).

In addition to the direct health effects of the pathogens on persons and animals, animal-related disease outbreaks have caused hundreds of billions of dollars of economic damage globally, destabilizing trade and producing devastating effects on human livelihoods. The rash of emerging or reemerging livestock disease outbreaks around the world since the mid 1990s, including bovine spongiform encephalopathy, foot-and-mouth disease, avian influenza, swine fever, and other diseases, has cost the world's economies $80 billion (28). In early 2003, the United Nation's Food and Agriculture Organization reported that more than one third of the global meat trade was embargoed as a result of mad cow disease, avian influenza, and other livestock disease outbreaks. Efforts to control the spread of avian influenza in Asian countries since 2003 have required the culling of >140 million chickens (29). The projected growth of industrial livestock production in nonindustrialized countries to meet global protein demand will increase the impact of future disease outbreaks on economic and food supply security. Some of these outbreaks will inevitably be linked to the trade in wildlife.

Rather than attempting to eradicate pathogens or the wild species that may harbor them, a practical approach to decrease the risk for the spread of infectious diseases would include decreasing contact among species. Closing down retail poultry markets in Hong Kong for 1 day per month reduced the rate of H9N2 avian influenza virus in market birds (30). Little equivalent research has been

conducted in market systems that sell wildlife, but an analogous approach to the precautionary principle (31) would be an appropriate action to take before the next outbreak or pandemic. Since wildlife markets are a system of networks with major hubs, these trading points provide practical control opportunities to maximize the effects of regulatory efforts (32). Focusing efforts at markets to regulate, reduce, or in some cases, eliminate the trade in wildlife could provide a cost-effective approach to decrease the risks for disease for humans, domestic animals, wildlife, and ecosystems.

Dr. Karesh is the director of the Wildlife Conservation Society's Field Veterinary Program and also serves as the co-chair of the World Conservation Union Veterinary Specialist Group. His projects and programs focus on the links between wildlife, domestic animals, and human health and well-being in nonindustrialized countries around the world.

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Address for correspondence: William B. Karesh, Wildlife Conservation Society, 2300 Southern Blvd, Bronx, NY 10460, USA; fax: 718-220-7126, email: wkaresh@wcs.org

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Occupational Deaths among Healthcare Workers

Kent A. Sepkowitz* and Leon Eisenberg†

Recent experiences with severe acute respiratory syndrome and the US smallpox vaccination program have demonstrated the vulnerability of healthcare workers to occupationally acquired infectious diseases. However, despite acknowledgment of risk, the occupational death rate for healthcare workers is unknown. In contrast, the death rate for other professions with occupational risk, such as police officer or firefighter, has been well defined. With available information from federal sources and calculating the additional number of deaths from infection by using data on prevalence and natural history, we estimate the annual death rate for healthcare workers from occupational events, including infection, is 17–57 per 1 million workers. However, a much more accurate estimate of risk is needed. Such information could inform future interventions, as was seen with the introduction of safer needle products. This information would also heighten public awareness of this often minimized but essential aspect of patient care.

The fundamental ethic of health care is that sick persons must receive care (1). This premise carries an unstated consequence: an occupational risk to healthcare workers who respond to the needs of contagious patients. This predicament was shown yet again during the severe acute respiratory syndrome (SARS) epidemic. As often occurs when infectious disease outbreaks are caused by an emerging agent, healthcare workers were the group most affected. According to the World Health Organization, 8,098 cases occurred during the outbreak, and 774 (9.6%) persons died (2). Healthcare workers accounted for 1,707 (21%) of the cases (2).

More specific information from outbreak hospitals in Hong Kong (3), Singapore (4), Guangdong Province (5), and Toronto (6,7) showed that 378 (57%) of 667 cases occurred in healthcare workers or medical students. The higher proportion in these reports may be attributable to the availability of more detailed site-specific information. The number of fatal infections in healthcare workers is not known, but deaths have been reported.

Of course, SARS is not the only infection that presents an occupational risk to healthcare workers. During the past 2 decades, occupationally acquired hepatitis B, HIV infection, multidrug-resistant tuberculosis, and viral hemorrhagic fevers, among others, have killed healthcare workers. In earlier generations, diseases such as occupationally acquired tuberculosis, measles, diphtheria, and scarlet fever posed substantial risk (8,9). In response, the Centers for Disease Control and Prevention (CDC) and other organizations have promulgated guidelines for healthcare worker protection, recommending vaccination, early patient screening, isolation precautions, and use of personal protective equipment (10). Perhaps the most successful is the 1991 Occupational Safety and Health Administration (OSHA) bloodborne pathogen standard, which contributed to reduction of hepatitis B among healthcare workers (11).

Despite this recognized risk, no country has a system in place to track fatal, occupationally acquired infections in their entirety. In this article, we examine occupational death rates for healthcare workers by using currently available US federal data sources. To provide more inclusive rates, we also estimate the number of annual deaths from occupationally acquired infections.

Methods

Available Data: Numerator

The US Department of Labor, through the Bureau of Labor Statistics, maintains an annual "census of fatal occupational injuries" across a wide range of occupations and exposures as part of its injuries, illness, and fatalities program (12). Federal law compels employers to notify OSHA of any occupational death within 8 hours of the death by telephone or in person at a local OSHA office (13). OSHA then reports the data in 2 ways: by occupation or by industry. When classified by "occupation," healthcare workers

*Memorial-Sloan-Kettering Cancer Center, New York, New York, USA; and †Harvard Medical School, Boston, Massachusetts, USA

are placed into any of 7 broad groups. Physicians and nurses, for example, are categorized as "managerial and professional specialty," while health technologists and technicians are grouped under "technical, sales, and administrative support," and nursing aides, orderlies, and attendants are considered "service occupations."

In contrast, the "industry" classification classifies all healthcare workers into "health services" without additional job-specific information. The annual death totals derived from "occupation" and from "industry" classifications differ by ≈15%–20%.

In either approach, OSHA places all deaths into 1 of 6 distinct categories: transportation accidents, assaults and violent acts, contact with objects and equipment, falls, exposure to harmful substances or environments, and fires and explosions. Because deaths from occupationally acquired diseases such as tuberculosis or hepatitis are not routinely captured in this system, the occupational risk of healthcare work is underestimated (12).

Although no national agency systematically tracks deaths due to occupationally acquired infection, both percutaneous injuries and tuberculin skin test conversions are reported to OSHA by completing the OSHA Form 300 (Log of Work Related Injuries and Illnesses), OSHA Form 301 (Injury and Illness Incident Report), or both. The latter requires more specific information about how the injury or illness occurred. The number of unreported events is not known; however, an institution may be cited or fined for incomplete records, which probably improves compliance.

Needlestick-related deaths are only occasionally reported through this system. According to OSHA data, from 1992 to 2002, a total of 67,363 workers died of occupational injuries, including 28 healthcare workers who died of complications related to needlestick exposures. OSHA cautions, however, that they collect and report fatal work injuries; needlestick data therefore reflect only those cases that fall within the 6 defined injury definitions (K. Loh, pers. comm.).

The National Institute for Occupational Safety and Health (NIOSH), a branch of CDC, is charged with providing leadership and conducting research to prevent workplace illness and injury. They regularly publish the Worker Health Chartbook, which reports fatal occupational illnesses (14). Infectious diseases, however, are not included in the illness report. Instead, data are focused on occupational pneumoconiosis, mesothelioma, and hypersensitivity pneumonitis.

NIOSH information regarding occupational infection is derived from 4 federal health databases as "nonfatal illnesses" (14). These databases include the National Surveillance System for Healthcare Workers, which obtains information from 60 hospitals that voluntarily submit needlestick and tuberculin conversion data on a regular basis. The Viral Hepatitis Surveillance Program and the Sentinel Counties Study of Acute Viral Hepatitis track incident cases of hepatitis, including those occurring in healthcare workers. Cases of AIDS and HIV infection among healthcare workers are gathered from several sources, including the CDC HIV/AIDS Surveillance Reporting System. Finally, staffTRAK-TB is used by tuberculosis control programs to monitor skin test conversion rates.

These data sources, although useful, have substantial limitations. First, they measure only the initial injury or exposure and not the consequent disease. Most needlesticks and tuberculin conversions do not result in disease; rarer yet are those that lead to fatal infection. Thus, rates of needlestick and tuberculin conversions, although meaningful, may not accurately reflect the outcomes of greatest interest: disease and death. Further complicating this problem, the latent period from initial infection to disease for HIV, tuberculosis, and other infections is measured in years to decades. For example, a worker may sustain a needlestick, become infected with HIV, but not develop clinical symptoms for several years. In the interval, the worker may have changed jobs several times, making linking the exposure to the disease difficult.

In addition, the tuberculin skin test is notoriously difficult to interpret, with suboptimal sensitivity and specificity, and so may distort the actual trend in tuberculosis infection rates. Finally, as many as 50%, and possibly more, of all percutaneous injuries are not reported, which complicates tracking by the current passive surveillance system (15).

Available Data: Denominator

To calculate an annual occupation-specific fatality rate, we determined the number of persons at risk per occupation by using 2 datasets from the Department of Labor: the 2001 National Occupational Employment and Wage Estimates (16) and the 2002 Current Population Survey (CPS) (17). A major difference between these 2 data sources is the inclusion of self-employed workers in the CPS report. In addition, only the CPS counts experienced but unemployed workers.

Results

The US labor force is composed of 136 million persons, 6 million of whom are healthcare workers with potential patient contact (16,17). Approximately half of these are registered or licensed practical nurses. An additional 3 million persons work in healthcare-support occupations and may have patient contact, including nursing aides, orderlies, and attendants (1.3 million); home health aides (560,000); and medical or dental assistants (600,000) (16,17). These estimates do not include persons without routine patient contact employed in such occupations as

healthcare administrators, medical secretaries, and other clerical staff (16,17).

From 2000 to 2002, the Department of Labor reported an annual average of 77 healthcare worker deaths with the "industry" categorization versus 93 deaths with the "occupation" category (12). Deaths from transportation accidents and assaults and violent acts accounted for most. To address the latter problem, NIOSH recently published the monograph Violence: Occupational Hazards in Hospitals (http://www.cdc.gov/niosh/2002-101.html).

Specific data are reported for some but not all healthcare worker occupations in the "occupation" classification (Table 1). For example, an annual average of 10 doctors, 18 registered nurses, and 18 health technologists/technicians died. In addition, statistics maintained by the National EMS (Emergency Medical Services) Memorial Service show that ≈12 emergency medical service workers are killed annually, including 13 in 2002 (18). The EMS deaths are not specifically noted in the Department of Labor statistics; therefore, whether these deaths are included in the overall number is uncertain.

Estimated Deaths from Specific Infections

To estimate the contribution of occupationally acquired infection, we examined the effects of hepatitis B, hepatitis C, HIV infection, and tuberculosis by using available information on disease incidence and natural history. Table 2 combines both injury-related data reported to the Department of Labor (shown in Table 1) and our estimates from specific infections, detailed below. Overall, we estimate that 9–42 healthcare workers per million die annually from occupational infection.

Hepatitis B

CDC estimates that, in 1983, 10,000 healthcare workers became infected with hepatitis B through occupational exposure (M.J. Alter, pers. comm.). The natural history of hepatitis B infection indicates that chronic infection developed in 5%–10% (500–1,000) of these persons. Although estimates vary, as many as 15%–25% (75–250 persons) of those with chronic infection will die from a hepatitis B–related complication, including cirrhosis or hepatocellular carcinoma (19). Since the time from infection to serious medical disease in the subset with these complications typically is about 20 years, most of these deaths can be expected to occur during this decade.

The risk of hepatitis B has diminished by >90% since the introduction of standard precautions and a recombinant vaccine (11). Despite vaccine availability, however, coverage is incomplete because >30% of workers refuse to be vaccinated (11). As a consequence, CDC estimates that, in 2002, another 400 healthcare workers became infected with hepatitis B virus, a number that has been stable since 1995 (M.J. Alter, pers. comm.).

Hepatitis C

CDC estimates that 3.9 million persons in the United States, or 1.8% of the population, have been infected with hepatitis C virus and that 2.7 million (1.3%) are chronically infected (20,21). Healthcare workers as a group have the same hepatitis C virus seroprevalence as the rest of the US population (20). However, transmission from a hepatitis C–infected patient to a healthcare worker occurs in 1%–3% of percutaneous exposures (22). With an estimated 380,000 percutaneous injuries annually (23), 50–150 transmissions would be expected, assuming that hospitalized patients have the same hepatitis C virus seroprevalence as the rest of the US population. Our understanding of the natural history of hepatitis C virus (HCV) continues to evolve; however, as many as 5% of those infected, or 3–8 healthcare workers annually, can be expected to die of liver disease.

This estimate may be low because hospitalized patients in some regions may have rates of HCV infection well above that of the US population, raising the likelihood of exposure to a positive source case. HCV seroprevalence of at least 5% has been reported in several groups who are frequently hospitalized. These groups include patients requiring dialysis (20), intravenous drug users with or without HIV infection (20), and perhaps patients in psychiatric hospitals and outpatient facilities (24).

Human Immunodeficiency Virus

CDC distinguishes between "documented" and "possible" occupational transmission of HIV. Documented infection refers to documented seroconversion in healthcare workers after occupational exposure or other laboratory evidence of occupational infection. Possible infection refers to history of occupational exposure to infected blood

Table 1. Occupation-specific death rates for US healthcare workers*

Occupation	Number employed (× 10^3)	Total deaths	Death rate
Emergency medical services	116–170	11	64–95
Physicians	340–820	10	12–29
Registered nurses	2,300	18	8
Technologists and technicians	650	18	28
Home nursing aides, orderlies, and attendants	1,700	13	8

*Rates expressed per 1 million workers. Numbers reflect 3-year average (2000–2002) of violent deaths and do not include infectious causes. Emergency medical services deaths reflect 4-year average (1999–2002) and exclude deaths sustained in the collapse of the World Trade Center towers in 2001. Range of number employed reflects 2 different federal databases (see text) (12,13,16,17).

Table 2. Occupational deaths among US healthcare workers (HCW), 2002*

Cause of death	No. deaths	HCW death rate, excluding support occupations (N = 6.2 million)	HCW death rate, including support occupations (N = 9.1 million)
Injury	77–93	12–15	8–10
Infection-related†	80–260	13–42	9–29
Total	157–353	25–57	17–39

*Rates expressed per 1 million workers. Estimates based on incidence and natural history of specific infections. Number of deaths by injury reflect 3-year average (2000–2002) (12,13,16,17).
†Includes deaths from hepatitis B virus (75–250) and hepatitis C virus, HIV, and tuberculosis (5–10 total).

or other fluid in healthcare workers without identifiable behavioral or transfusion risks, but for whom seroconversion specifically resulting from an occupational exposure was not documented, i.e., a baseline, postexposure test for HIV was not performed (25).

To date, 26 (46%) of 57 US healthcare workers with voluntarily reported, documented, occupationally acquired HIV infection have progressed to AIDS, as have 121 (88%) of 138 healthcare workers with possible occupational transmission (25). Job-specific information is available for persons with either documented or possible disease. Twenty-four (42%) of 57 proven transmissions have occurred in nurses, 16 (28%) in clinical laboratory technicians, and 6 (11%) in nonsurgical physicians. Among the 138 persons with possible occupational acquisition, in addition to the occupations above, cases were noted among 12 emergency medical technicians (9%), 6 surgeons (4%), 15 health aide/attendants (11%), and 13 housekeepers and maintenance workers (9%). This distribution by occupation may be applicable to other infections transmitted by percutaneous injury, such as hepatitis B and hepatitis C, but comparable information from recent studies of these infections is not available.

Antiviral therapy to manage an occupational exposure to HIV has resulted in severe hepatitis requiring liver transplant, though no therapy-related deaths have been reported (26). The number of healthcare workers who have died from proven or probable occupationally acquired HIV infection has not been reported, but some have died and risk for serious complication persists (27).

Tuberculosis

Tuberculosis has long represented an occupational threat to healthcare workers (28). This risk became particularly evident during the late 1980s and early 1990s, when several nosocomial outbreaks of multidrug-resistant tuberculosis occurred in the United States. During these years, workers experienced a tuberculin conversion, and several developed active drug-resistant disease (29). The infection was fatal in at least 9 immunocompromised healthcare workers (30). Treatment for occupationally acquired resistant tuberculosis also has resulted in death (31). Healthcare workers who became latently infected with multidrug-resistant tuberculosis strains at this time remain at risk for disease reactivation (32).

In 2003, CDC and the American Thoracic Society revised the recommendation for treatment of latent *Mycobacterium tuberculosis* infection with pyrazinamide and rifampin because of an unexpectedly high rate of hepatotoxicity with this regimen (33). According to CDC, 6 healthcare workers were included in the 49 persons in whom severe hepatitis developed or death occurred. One healthcare worker died from this complication (K. Ijaz, pers. comm.).

Occupational Death Rate among Other Workers

To place healthcare worker risk into context, we applied the same approach to derive average annual death rates among several worker groups for the 3-year period 2000–2002 (Table 3). The US workforce has a rate of ≈41 occupational fatalities per million workers. Fishermen and construction workers have the highest rate (>1,000 deaths per million workers annually). Members of the military (361–671 per million), police and related protective service workers (108 per million), and firefighters (93 per million) also have markedly elevated rates. Lawyers (7–14 per million) and waiters (5 per million) have relatively low rates of occupational death.

Infectious Risk to Healthcare Workers Internationally

The risk of acquiring a work-related fatal infection represents a substantial risk to healthcare workers in developing countries (34). In addition to viral hemorrhagic fevers, occupationally acquired tuberculosis in Africa is increasingly recognized. Reports from Malawi (35), Ethiopia (36), and South Africa (37) describe substantial rises in active cases of tuberculosis among healthcare workers, many of whom die of the disease (25% in a series from Malawi). Few reports have examined the occupational risk for HIV infection in disease-endemic, resource-poor countries, but transmission is likely (38).

Summary and Recommendations

We estimate that 17–57 US healthcare workers per million employed die annually from occupational infections and injuries (Table 2). However, the number of deaths that results from occupationally acquired infection is an educated guess at best. We projected the potential consequences of only 4 diseases by relying on the published prevalence, transmission rate, and natural history of these infections.

Table 3. Occupational death rate for various jobs*

Occupation	No. employed (× 10^3)	Total deaths	Death rate
Fisherman	39	46	1,179
Construction worker	825–1,108	1,198	1,081–1,452
Pilot	107–129	102	791–953
Military (active and reserve)	2,600	94	361
Truck driver	2,544–3,365	530	157–208
Protective service	2,000	219	108
Firefighter	1,100	102	93
US workforce	136,000	5,780	42.5
Healthcare worker	6,200–9,100	157–353	17–57
Sheetmetal worker	172–207	8	39–46
Bartender	339–427	10	23–29
Lawyer	490–920	6	7–14
Waiter	1,893–1,981	9	5

*Numbers represent average of annual deaths during 3-year period, 2000–2002. Range of number employed reflects 2 different federal databases (see text). Rates expressed per 1 million workers (12,13,16,17).

Our results therefore may underestimate the actual occupational death rate for these diseases. Furthermore, these estimates do not account for deaths from other infections, which demonstrates the problems engendered by the current lack of a national tracking system. This finding stands in contrast to the rigorous approach used to track occupational deaths of various other workers, such as police officers and firefighters (39,40).

The recent experiences with SARS and smallpox vaccination have demonstrated the vulnerability of healthcare workers to occupationally acquired infections. In addition, these events have served as a reminder of the critical societal responsibility of the healthcare worker. Although not as central to a national disaster response as protective service workers, healthcare workers are a critical component and in this capacity may incur risk to their health.

We recommend that national organizations assume responsibility for accurately tracking deaths caused by occupationally acquired infections. A nationwide tracking system will accomplish several important goals. First, it will determine the magnitude of the problem and inform future interventions. This approach has been used successfully with needlestick injuries: a problem was identified and quantified, then assessable preventive measures (e.g., the safer needle system) were put into place. This system could also lead to appropriate financial compensation. In 1976, the Public Safety Officers' Benefits Program was initiated to provide a 1-time financial benefit to survivors of police officers, firefighters, and emergency workers killed in the line of duty. The benefit also extends to those who become permanently and totally disabled as the result of trauma sustained in the line of duty.

This approach was invoked recently as the national smallpox vaccination plan was initiated. Because of healthcare worker reluctance to accept a vaccine known to cause fatal reactions, if only rarely, the government opted to extend this program to cover healthcare workers for this complication. This decision was a good start in acknowledging the unique occupational hazards of healthcare but should not remain an isolated decision made at a politically charged moment.

Most of all, a national registry would provide an ongoing reminder of the risk of caring for others, by raising awareness among laypersons and professionals alike. The 9 million persons employed in the healthcare industry and their families merit better protection for their health and greater recognition for their contributions.

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Dr. Sepkowitz is director of infection control at Memorial Sloan-Kettering Cancer Center and professor of medicine at Weill Medical College of Cornell University. He has a longstanding interest in the risk of occupationally acquired infections in healthcare workers.

Dr. Eisenberg is Presley Professor of Social Medicine Emeritus at Harvard Medical School. His current scholarly interest is the environmental determinants of gene expression.

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Address for correspondence: Kent A. Sepkowitz, Memorial-Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021, USA; fax: 212-717-3021; email: sepkowik@mskcc.org

Drug-resistant *Neisseria gonorrhoeae* in Michigan

Kathryn E. Macomber,* Martha S. Boehme,* James T. Rudrik,* Dara Ganoczy,† Erin Crandell-Alden,* William A. Schneider,* and Patricia A. Somsel*

The increasing prevalence of quinolone-resistant *Neisseria gonorrhoeae* (QRNG) in the United States is a cause for concern. Detecting resistance is complicated by the widespread use of molecular tests that do not provide isolates for susceptibility testing. The Michigan Department of Community Health developed a sentinel surveillance program to detect antimicrobial drug resistance in *N. gonorrhoeae*. Sentinel surveillance from 11 laboratories submitted 1,122 isolates for antimicrobial drug susceptibility testing and detected 2 clusters of QRNG from January 2003 to September 2004. These clusters were epidemiologically distinct: one involved young, heterosexual youth, and the other involved older men who have sex with men. This finding led to changes in local treatment recommendations that limited spread of resistant strains. Development of the sentinel program, collection of data, and epidemiologic analysis of the clusters are discussed.

Since the 1970s, the treatment and control of gonorrhea have been complicated by the ability of *Neisseria gonorrhoeae* to develop resistance to a variety of antimicrobial agents, including penicillin, tetracycline, and fluoroquinolones. Recent limitations in oral treatments for gonorrhea resulting from the discontinued manufacturing of cefixime, as well as decreases in the availability of isolates for susceptibility testing as culture methods are replaced by nucleic acid amplification tests, pose additional challenges for successfully treating patients and identifying resistant organisms.

In 1986, the then-Centers for Disease Control (CDC) established the Gonococcal Isolate Surveillance Project (GISP) to monitor changes in antimicrobial drug–susceptibility patterns. Twenty-five isolates are collected monthly from participating sexually transmitted disease (STD) clinics across the United States (30 cities represented in 2003) and are sent to CDC for susceptibility testing (1).

In the early 1990s, fluoroquinolone-resistant *N. gonorrhoeae* (QRNG) was reported from a number of areas outside of the United States, and resistant strains became well established in Thailand, Hong Kong, Japan, and the Philippines. Sporadic reports of QRNG in the United States at that time were usually associated with travel to Asia. Prevalence of QRNG in Hawaii steadily increased from 1997 to 2001 (2,3). In 2000, California reported QRNG in San Francisco, San Diego, and Orange County. In 2001, 33 (2.5%) of 1,311 of isolates tested in California were resistant to fluoroquinolones; this increase continued in 2002 (4,5). As a result of the increasing prevalence of QRNG, the Hawaii Department of Health and California Department of Health Services recommended that clinicians avoid using fluoroquinolones when treating gonorrhea (6). Because of QRNG prevalence variation in countries outside of the United States, CDC recommended in its 2002 STD Treatment Guidelines that fluoroquinolones not be used to treat gonorrhea acquired in Asia, the Pacific Islands, Hawaii, California, or other areas with an increased prevalence of QRNG (6–9).

In addition to Hawaii and California, low numbers of QRNG-resistant isolates had been reported from cities in the United States before 2000 (4,5). GISP data for 2003 showed resistant isolates from Cleveland, Baltimore, Chicago, Dallas, and Kansas, with a significant increase in QRNG in Seattle, New York City, Massachusetts, California, and Michigan, and smaller increases in Phoenix, Minneapolis, Chicago, Las Vegas, and Portland (1).

The primary therapies currently recommended by CDC for uncomplicated gonococcal infections of the cervix, urethra, and rectum include ceftriaxone, cefixime, or a fluoroquinolone (ciprofloxacin, levofloxacin, or ofloxacin) (6). In July of 2002, Wyeth Pharmaceuticals stopped manufacturing cefixime, the only recommended oral cephalosporin; company inventories were fully depleted by October 2002 (10). Michigan used only cefixime

*Michigan Department of Community Health, Lansing, Michigan, USA; and †Department of Veterans Affairs, Ann Arbor, Michigan, USA

tablets, although other states may have used cefixime oral suspension, which may have been available longer. Although the US Food and Drug Administration has approved cefpodoxime and cefuroxime axetil to treat uncomplicated gonococcal infections, CDC has not recommended either of these oral cephalosporins to replace cefixime because they fail to meet CDC's efficacy standards (10).

CDC recommended, in 2002, that state health departments monitor local antimicrobial drug–susceptibility patterns to guide local treatment recommendations (3). In response, the Michigan Department of Community Health (MDCH) established a sentinel surveillance system to monitor the prevalence of drug-resistant gonorrhea, characterize patients with drug-resistant infections, and provide local treatment recommendations in Michigan. Before 2003, only sporadic cases of QRNG were detected in Michigan; all of these patients acquired their infections during foreign travel. However, resistant strains might have gone undetected, as an estimated 97% of genital gonorrhea testing in Michigan is performed by nucleic acid testing, from which viable isolates for susceptibility testing cannot be obtained (MDCH, unpub. data). To augment routine susceptibility studies performed at MDCH, a special surveillance project to collect gonococcal isolates from clinical laboratories across the state was initiated in July 2002 and continued through September 2004. We describe the development of this surveillance project, discuss the challenges of maintaining surveillance on a voluntary basis, and present data collected from the project.

Methods and Materials

Selection of Participating Sites

Although ≈110 clinical laboratories in Michigan offer comprehensive microbiology services, many have switched to nucleic acid methods. To determine the number of laboratories that perform genital gonorrhea cultures as their routine detection method, positive gonorrhea case reports submitted to the state health department during a representative 3-month period (August–October 2001) were examined to obtain a convenience sample of laboratories that culture genital specimens for gonorrhea. Nineteen laboratories reported ≥1 culture-based positive results during that time. These laboratories were contacted to determine the average number of cultures positive for gonorrhea per year, whether cultures were performed on genital specimens, whether genital gonorrhea cultures were expected to continue to be collected for the next 6 months, and whether the laboratory was willing to submit isolates for surveillance. Of the 19 laboratories, 5 clinical (hospital) laboratories were identified that recovered ≥10 genital gonorrhea cultures each month; all 5 agreed to participate as sentinel sites. These 5 laboratories were located in 5 different counties (Table 1). The state health department laboratory also received routine gonorrhea cultures from a local health department STD clinic and occasionally referred *N. gonorrhoeae* isolates for identification or susceptibility testing. These cultures were also included in the surveillance system. After the identification of QRNG cases, surveillance was expanded to include 4 additional STD clinics in 4 counties (Table 1).

Isolation, Identification, and Susceptibility Testing

Laboratories were provided with chocolate agar slants (Remel, Lenexa, KS, USA), International Air Transport Association–compliant mailing containers, and specific instructions for packaging and shipping. Courier pickup of isolates on an on-call basis was also arranged. Isolates were accepted on chocolate agar slants or frozen in Trypticase soy broth with glycerol.

Gonococcal isolates from genital and nongenital cultures either were recovered from cultures collected by the local health department STD clinic or were referred from the sentinel sites or other clinical laboratories. Cultures obtained from the local STD clinic were plated onto Modified Thayer-Martin medium (Becton Dickinson, Cockeysville, MD, USA) and incubated for 18 to 24 h at 35°C in a candle jar before transport to MDCH. At MDCH, the plates were incubated an additional 48 h at 35°C in 5% to 10% CO_2 and examined daily. Suspect colonies grown on Thayer-Martin medium and referred isolates were presumptively identified by Gram stain and oxidase reaction. The isolates were subcultured on chocolate agar (Remel) for further testing. All isolates were frozen in skim milk at –70°C.

Isolates were identified by using the apiNH system (bioMérieux, Inc., Durham, NC, USA). If an isolate was not identified by apiNH, conventional biochemical tests were performed, including cystine tryptic agar sugar fermentation test with glucose, sucrose, maltose, and lactose.

Antimicrobial drug susceptibility for ciprofloxacin, spectinomycin, tetracycline, ceftriaxone, and cefixime or cefpodoxime was determined by disk diffusion on gonococcal (GC) agar base supplemented with 1% GCHI enrichment (Remel) according to the Clinical and Laboratory Standards Institute (formerly NCCLS) procedure (11). Any isolate categorized as repeatedly resistant to or intermediately resistant to ciprofloxacin was tested to determine MIC (12). MIC was determined by Etest (AB BIODISK North America, Piscataway, NJ, USA) on GC Agar Base supplemented with 1% GCHI enrichment, according to the manufacturer's instructions (13). *N. gonorrhoeae* ATCC 492226 was used as the quality control strain for both disk diffusion and Etest. Beginning January 1, 2004, MDCH added cefpodoxime and deleted

Table 1. Characteristics of submitting providers, gonorrhea testing, Michigan

Laboratory	Location	Types of sites*	Location	2003 participants N = 564 (%)	2004 participants N = 510 (%)
967-bed teaching hospital	Southeast	ER, PMD	Southeast	45 (8)	32 (6)
181-bed community hospital	West	ER, PMD	West	104 (18)	N/A
438-bed teaching hospital	Mid-state	ER, PMD	Mid-state	57 (10)	N/A
County A	Mid-state	STD and teen clinic	Mid-state	136 (24)	104 (20)
County B	West	STD clinic	West	22 (4)	112 (22)
360-bed community hospital	Southwest	ER, PMD	Southwest	84 (15)	N/A
County C	Southeast	STD clinic	Southeast	54 (10)	174 (34)
County D	East	STD clinic	East	17 (3)	33 (6)
County E	East	STD clinic	East	N/A	23 (5)
478-bed community hospital	Southeast	ER, PMD	Southeast	13 (2)	9 (2)
University student health center	Southeast	Student health	Southeast	15 (3)	9 (2)
Referred from other sites	Varied	ER, PMD, STD clinic,	Varied	17 (3)	14 (3)

*ER, emergency room; PMD, primary medical doctor; STD, sexually transmitted disease; N/A, not available.

cefixime from its routine susceptibility-testing panel for gonorrhea.

Epidemiologic Analysis

Final reports of susceptibility results were distributed to the submitting laboratory and to the MDCH Bureau of Epidemiology. For cases with susceptibility testing results reported from January 2003 to September 2004, provider information was obtained from the submitting laboratory. Demographic, behavioral, and clinical data were solicited from the patients' healthcare providers. For all gonorrhea patients, each provider was contacted to give permission to receive a data collection form by secure fax. The form collected information on reason for visit, zip code, age, race, ethnicity, sex, sexual orientation, prior gonorrhea infection, primary therapies for gonorrhea and chlamydia, and the reason a culture was performed. The completed forms were faxed back to the Bureau of Epidemiology, where epidemiologic and laboratory data were entered into a Microsoft Access database (Microsoft Corp., Redmond, WA, USA). Patients with QRNG were interviewed (by phone or in person) by MDCH disease intervention specialists, and additional information was collected, including that on illicit drug use, recent use of antimicrobial agents, sexual partner risk factors, HIV status, and travel history. Prevalence ratios were used to examine the associations between QRNG and demographic, behavioral, and clinical characteristics. Data were analyzed with SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA). The project was determined to be routine surveillance activity and thus exempt from human subjects review by the MDCH Institutional Review Board.

Results

From January 1, 2003, to September 30, 2004, susceptibility testing results for 1,122 isolates (from 1,074 patients) were reported. Patient and specimen characteristics for QRNG and non-QRNG isolates are shown in Table 2, stratified by year. A total of 582 isolates (from 564 patients) obtained by disk diffusion were reported during calendar year 2003. All isolates were susceptible to cefixime, ceftriaxone, and spectinomycin; 43 (7.4%) were resistant to tetracycline. Seventeen (2.9%) isolates were resistant to ciprofloxacin (MIC ≥1 µg/mL), and 1 (0.2%) showed intermediate resistance (MIC 0.12–0.5 µg/mL). The 17 ciprofloxacin-resistant isolates in 2003 represented 14 individual cases of QRNG. Table 3 lists 2003 and 2004 QRNG cases with relevant risk factors and demographics. In 2003, 11 patients were male. Patients ranged in age from 16 to 45 years (median 26). Nearly half of the QRNG patients were white (43%). Four of the 11 male patients were men who have sex with men (MSM). A large number of cases (57%) were detected at public STD clinics in county A.

From January 1 to September 30, 2004, a total of 540 (510 patients) isolates whose susceptibilities were measured by disk diffusion showed no resistance to cefpodoxime, ceftriaxone, and spectinomycin. Eight isolates (1.5%) were resistant to ciprofloxacin, and 1 (0.2%) isolate had intermediate resistance. Resistance to tetracycline was similar to that seen in the previous year. All 8 cases of QRNG detected in the 2004 study period were in MSM. Patients ranged from 20 to 43 years of age (median 26). Most were white (88%), and more than half (63%) were detected at clinic B, a public STD clinic in a county not contiguous to clinic A.

Cumulative distribution, for the entire study period, of the submissions by site are shown in Table 1. Fifteen percent (158/1,074) of all gonococcal isolates were submitted by emergency rooms, 19% (205/1,074) were from primary medical doctors, and 64% (692/1,074) were from STD clinics. Although 35% of isolates were submitted by private providers, only 23% of QRNG cases were identified through those venues. Most QRNG cases were identified through public STD clinics (77%). The overall return rate of the data collection form from healthcare providers was

Table 2. Patient and specimen characteristics, gonorrhea study, Michigan*

	2003 non-QRNG N = 550 (%)	2003 QRNG N = 14 (%)	2004 non-QRNG N = 502 (%)	2004 QRNG N = 8 (%)
Median age, y	23	26	24	26
Sex				
Male	267 (49)	11 (79)	411 (82)	8 (100)
Female	281 (51)	3 (21)	91 (18)	0
Unknown	2 (0)	0	0	0
Race				
Black	323 (59)	7 (50)	313 (62)	0
White	76 (14)	6 (43)	89 (18)	7 (88)
Other	9 (1)	0	6 (1)	1 (12)
Unknown	142 (26)	0	94 (19)	0
Asian	0	1 (7)	0	0
Hispanic				
Yes	11 (2)	1 (7)	16 (3)	0
No	204 (37)	12 (86)	283 (57)	7 (88)
Unknown	335 (61)	1 (7)	203 (40)	1 (12)
Site of specimen				
Cervix/vagina	260 (47)	2 (21)	74 (15)	0
Eye	1 (1)	1 (7)	1 (0)	0
Urethra	244 (44)	7 (43)	392 (78)	6 (75)
Throat	16 (3)	1 (7)	16 (3)	0
Rectum	17 (3)	1 (7)	11 (3)	1 (12)
Urine	6 (1)	2 (15)	5 (1)	1 (12)
Other	6 (1)	0	3 (0)	0
Symptoms				
Discharge/dysuria	338 (61)	10 (72)	373 (74)	6 (75)
None	72 (13)	3 (21)	56 (11)	1 (12)
Unknown	140 (26)	1 (7)	73 (15)	1 (12)

*QRNG, quinolone-resistant *Neisseria gonorrhoeae*.
†Intermediate QRNG grouped with non-QRNG cases.

84%. Providers varied significantly in their response to a request for patient information; STD clinics and private medical doctors had higher response rates (93% and 72%, respectively) than emergency rooms (55%).

Although most persons with gonorrhea in our sentinel surveillance system are African American (76% of those with known race), the prevalence of QRNG was higher among whites, 7% versus 1% among non-whites. The prevalence ratio was 1.07 (p<0.0001, 95% confidence interval 1.02–1.11) (Table 4). The prevalence of QRNG was higher among those ≥40 years of age, 4% versus 2% among those <40 years of age. MSM constituted 11% of all gonorrhea patients in the surveillance system, but they accounted for 63% of male QRNG patients; heterosexuals comprised 37% of the male QRNG patients. The prevalence of QRNG was highest for MSM (14%) and was relatively low for heterosexual men (1%) and women (1%). The presence of symptoms was not associated with quinolone-resistant infections, as the prevalence of QRNG was similar between both those with and without symptoms. Prior history of gonorrhea was not significantly associated with QRNG; the prevalence of QRNG among those with a history of gonorrhea was 2%, versus 3% among those without a history of gonorrhea. Table 5 shows enhanced variables, such as drug use, travel history, and HIV status, collected from interviews conducted with QRNG patients. Most QRNG patients reported no recent use of antimicrobial agents, no illicit drug use, and no recent travel. These findings also held true for sex partner characteristics, although sex partners had an increased percentage of illicit drug use and a higher percentage of unknown responses. Three of the 22 QRNG patients during the study period were HIV-positive.

Discussion

Michigan has seen a higher prevalence of QRNG in recent years among heterosexuals, especially men in county A (3.4%), compared to other surveillance sites, such as New York and Massachusetts (1.6% and 1.8%, respectively) (14). Shortly after sentinel surveillance was instituted, a geographic cluster of QRNG cases was discovered among a group of heterosexual teenagers in clinic A. A sexual link from 1 QRNG patient to another was discovered in only half of the patients. How QRNG initially emerged in this population is still unclear, as none of these heterosexual patients had a travel history. In cooperation with MDCH, the local health departments in county A (where the patients resided) and in the 5 contiguous coun-

Table 3. Quinolone-resistant gonorrhea cases, Michigan, 2003–2004

Collection date	Sex	Race	Age	Previous gonorrhea*	Sexual orientation	Site	Provider†
1/6/03	M	Black	16	No	Heterosexual	Urethra	Clinic A
3/5/03	M	Black	28	Yes	Heterosexual	Urethra	Clinic A
3/10/03	M	White	24	No	Heterosexual	Urethra	Clinic A
3/20/03	F	White	18	Yes	Bisexual	Cervix	Clinic A
5/19/03	M	Black	30	Yes	Heterosexual	Urethra	Clinic A
5/21/03	M	White	42	No	Heterosexual	Eye	PMD
7/5/03	M	Asian	31	No	Heterosexual	Urethra	PMD
7/8/03	M	White	19	No	Homosexual	Rectum	Clinic B
7/24/03	M	White	33	No	Homosexual	Throat	Clinic B
8/25/03	M	Black	36	Yes	Homosexual	Urethra	Clinic A
8/29/03	F	Black	18	No	Heterosexual	Cervix	Clinic A
8/26/03	M	Black	19	No	Heterosexual	Urine	Clinic A
9/26/03	F	Black	45	No	Heterosexual	Urethra	PMD
10/27/03	M	White	24	Unknown	Homosexual	Urine	PMD
1/22/04	M	White	43	Yes	Homosexual	Urethra	Clinic B
2/23/04	M	Other	39	Yes	Bisexual	Urine	PMD
3/15/04	M	White	20	No	Homosexual	Urethra	Clinic B
4/7/04	M	White	24	No	Homosexual	Urethra	Clinic B
5/14/04	M	White	26	No	Homosexual	Urethra	Clinic
6/28/04	M	White	21	Yes	Homosexual	Urethra	Clinic B
7/9/04	M	White	28	No	Homosexual	Rectum	Clinic B
8/18/04	M	White	26	No	Homosexual	Urethra	Clinic C

*Has the patient had gonorrhea (ever)?
†PMD, primary medical doctor; clinic, county sexually transmitted disease clinic.

ties issued a ban on using ciprofloxacin to treat gonorrhea. Before this recommendation, 71% of clinic A patients were treated with ciprofloxacin. After the recommendation was implemented, only 7% received ciprofloxacin as their primary treatment, according to clinic records.

A quick response to this geographic cluster may have halted the spread of QRNG in the community. However, at clinic A the direct costs of treatment increased, since 250 mg of intramuscular ceftriaxone costs nearly 3 times more than a 500-mg dose of ciprofloxacin (US $12.85 vs. $4.14). Indirect costs associated with staffing and increased amounts of time spent per patient (since all were treated with ceftriaxone and all had isolates submitted for culture) also increased but were not calculated. After 18 months without any cases, despite continued surveillance, the quinolone use ban on all patients (excluding MSM, per the revised STD treatment guidelines) was lifted, and no additional cases have since been reported. In addition, a cluster of cases in MSM in county B led local officials to make several recommendations for MSM,

Table 4. Prevalence of ciprofloxacin-resistant gonorrhea infection among surveillance population

	% patients with QRNG (n/total)	Prevalence ratio (95% confidence interval)	p value
Sex		1.02 (1.00–1.04)	0.03
Male	3 (19/697)		
Female	1 (3/375)		
Men who have sex with men		1.14 (1.05–1.24)	<0.0001
Yes	14 (12/87)		
No	1 (7/488)		
White		1.07 (1.02–1.11)	<0.0001
Yes	7 (13/178)		
No	1 (9/896)		
Discharge and/or dysuria		0.991 (0.960–1.02)	0.57
Yes	2 (16/724)		
No	3 (4/131)		
History of gonorrhea		0.991 (0.967–1.02)	0.52
Yes	2 (7/285)		
No	3 (14/425)		
>40 y of age		1.02 (0.975–1.06)	0.30
Yes	4 (3/84)		
No	2 (19/990)		

Table 5. Characteristics of quinolone-resistant *Neisseria gonorrhoeae* patients, Michigan

	2003 N = 14 (%)	2004 N = 8 (%)
Primary therapy for gonorrhea		
Ciprofloxacin	0	2 (25)
Cefixime	3 (21)	0
Ceftriaxone	9 (64)	5 (63)
Other	2 (14)	0
None	0	1 (12)
Primary therapy for chlamydia		
Azithromycin	10 (72)	7 (88)
Doxycycline	1 (7)	1 (12)
None	3 (21)	0
Recent use of antimicrobial agent*		
Yes	2 (14)	1 (12)
No	9 (64)	6 (76)
Unknown	3 (21)	1 (12)
Illicit drug use		
Yes	3 (21)	1 (12)
No	8 (57)	6 (76)
Unknown	3 (21)	1 (12)
Recent travel†		
Yes	2 (14)	0
No	12 (86)	7 (88)
Unknown	0	1 (12)
HIV status		
Positive	1 (7)	2 (25)
Negative	13 (93)	6 (75)
Sex partner illicit drug use		
Yes	3 (21)	2 (25)
No	5 (36)	3 (38)
Unknown	6 (43)	3 (37)
Sex partner history of travel†		
Yes	1 (7)	0
No	10 (72)	5 (63)
Unknown	3 (21)	3 (37)
Sex partner recent antimicrobial use*		
Yes	1 (7)	0
No	7 (50)	5 (63)
Unknown	6 (43)	3 (37)

*Has the patient/sex partner used antimicrobial agents in the last 60 days?
†Has the sex partner traveled outside the United States (or to Hawaii) in the last 60 days?

which included increasing provider awareness about the importance of asking about patients' sexual orientation, avoiding quinolone use, and using culture to diagnose gonorrhea.

During this surveillance project, 2 clusters were identified in 2 counties. However, QRNG surveillance is limited and not optimally representative: during the study period, it only operated in 9 of Michigan's 83 counties. This surveillance system captured only 4.4% of the total gonorrhea cases in Michigan; however, the counties represented in this surveillance system, in addition to the surveillance in Detroit City for the GISP project, report 38% of Michigan's gonorrhea cases. Those patients in the surveillance system are more likely to be seen at a public STD clinic (67% of those in the surveillance system vs. 23% of those in statewide morbidity reports), be African American (76% of the surveillance system vs. 40% of statewide morbidity), and male (67% of those in surveillance system vs. 44% of statewide gonorrhea case-patients). The Detroit City Health Department joined the GISP project in 2003, and no isolates collected from that site have been resistant to fluoroquinolones. This finding suggests that surveillance isolates should be collected from multiple geographic sites, ideally with demographic diversity, to demonstrate emerging resistance.

The fastidious nature of *N. gonorrhoeae* presented some challenges for the submitting laboratories. Approximately 10% of the total isolates received were either nonviable on subculture or overgrown with other organisms and were reported as unsatisfactory. At the end of December 2003, three of the 5 clinical laboratories stopped sending isolates to MDCH, citing economic and staffing barriers. Since QRNG prevalence was highest among patients seeking care from public STD clinics, MDCH asked additional public clinics to collect cultures. At the end of the study period, cultures were submitted by 5 local health departments and 2 private healthcare providers (a student health center and a major urban hospital laboratory).

MDCH will continue to provide routine *N. gonorrhoeae* susceptibility testing to monitor the emergence of resistance when isolates are available. Clinical laboratories are also encouraged to submit positive cultures to MDCH for susceptibility testing. To improve surveillance efforts, MDCH has recommended that clinicians culture specimens from patients with persistent symptoms. Clinical laboratories are asked to assist by submitting isolates for susceptibility testing from patients who are repeatedly culture-positive. However, as nucleic acid amplification tests replace culture-based methods, molecular techniques to demonstrate resistance and identify clusters will need to be developed.

Although Michigan's data are not geographically representative, the state's sentinel surveillance system is strong because numerator and denominator data are collected, allowing for the calculation of true prevalence ratios. The continued emergence of QRNG among gonorrhea cases will be a major financial limitation to state STD programs. Quinolones are currently the only oral treatment for gonorrhea recommended by CDC, and intramuscular ceftriaxone costs nearly 3 times more than a dose of ciprofloxacin. Since approximately one third of Michigan's estimated 17,000 gonorrhea patients in 2004 were treated in public clinics, the cost of QRNG will substantially limit the services the Michigan STD program can provide to residents. This study illustrates that, although a local ban on ciprofloxacin use in response to a QRNG cluster demanded more intensive resources for 1 Michigan county, the

response may have been more timely, effective, and less costly than a statewide reaction.

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Ms. Macomber is a HIV/STD epidemiologist in the Michigan Department of Community Health. She conducts STD data analyses, oversees the quinolone-resistant *N. gonorrhoea* project, and is the principal investigator for a project collecting enhanced behavioral surveillance data for gonorrhea.

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Address for correspondence: Kathryn E. Macomber, 3423 N. MLK Blvd, P.O. Box 30195, Lansing, MI 48909, USA; fax: 517-335-8121; macomberk@michigan.gov

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SARS Vaccine Development

Shibo Jiang,* Yuxian He,* and Shuwen Liu*

Developing effective and safe vaccines is urgently needed to prevent infection by severe acute respiratory syndrome (SARS)–associated coronavirus (SARS-CoV). The inactivated SARS-CoV vaccine may be the first one available for clinical use because it is easy to generate; however, safety is the main concern. The spike (S) protein of SARS-CoV is the major inducer of neutralizing antibodies, and the receptor-binding domain (RBD) in the S1 subunit of S protein contains multiple conformational neutralizing epitopes. This suggests that recombinant proteins containing RBD and vectors encoding the RBD sequence can be used to develop safe and effective SARS vaccines.

Severe acute respiratory syndrome (SARS) is a newly emerged infectious disease caused by SARS-associated coronavirus (SARS-CoV) (1). It originated in the Guangdong province of China in late 2002, spread rapidly around the world along international air-travel routes, and resulted in 8,450 cases and 810 deaths in 33 countries and areas on 5 continents (www.cdc.gov/mmwr/mguide_sars.html). The global outbreak of SARS seriously threatened public health and socioeconomic stability worldwide. Although this outbreak was eventually brought under control in 2003, several isolated outbreaks of SARS subsequently occurred because of accidental releases of the SARS-CoV isolates from laboratories in Taiwan, Singapore, and mainland China (http://www.who.int/csr/sars/en). In late 2003 and early 2004, new infections in persons who had contact with animals infected with SARS-CoV strains significantly different from those predominating in the 2002–2003 outbreak were reported in Guangdong, China (1). These events indicate that a SARS epidemic may recur at any time in the future, either by the virus escaping from laboratory samples or by SARS-CoV isolates evolving from SARS-CoV–like virus in animal hosts.

*New York Blood Center, New York, New York, USA

Origin and Evolution of SARS-CoV

Coronaviruses of the genus *Coronavirus* can be divided into 3 antigenic groups. Group 1 consists of human coronavirus 229E (HCoV-229E), porcine epidemic diarrhea virus, and feline infectious peritonitis virus (FIPV). Group 2 includes bovine coronavirus, murine hepatitis virus, and human coronavirus OC34 (HCoV-OC43). Group 3 contains avian infectious bronchitis virus. SARS-CoV is a new member of the genus *Coronavirus*, but it does not belong to any of the 3 antigenic groups, although some reports suggest that it most resembles the group 2 coronavirus (2). SARS-CoV may have originated in animals. SARS-CoV–like virus with >99% nucleotide homology with human SARS-CoV was identified in palm civets and other animals found in live animal markets in Guangdong, China (3). The SARS-CoV–like virus that exists in animals does not cause typical SARS-like disease in the natural hosts and is not transmitted from animals to humans. Under certain conditions, the virus may have evolved into the early human SARS-CoV, with the ability to be transmitted from animals to humans or even from humans to humans, resulting in localized outbreaks and mild human disease. Under selective pressure in humans, the early human SARS-CoV may further evolve into the late human SARS-CoV, which can cause local or even global outbreaks and typical SARS in humans with high death rates. Early human SARS-CoV is closer genetically to animal SARS-CoV–like virus than to late human SARS-CoV, which has a 29-nucleotide (in some isolates a 415-nucleotide) deletion in open reading frame 8 (3,4). The characteristics of these viruses are summarized in the Table (4–6).

SARS-CoV can be efficiently grown in cell culture (1) and rapidly spread from person to person (7). It can survive in feces and urine at room temperature for >2 days (http://www.who.int/csr/sars/en) and may cause serious, even fatal, disease. SARS-CoV, a National Institute of Allergy and Infectious Diseases Biodefense Category C priority pathogen (http://www2.niaid.nih.gov/Biodefense/bandc_priority.htm) could be used by bioterrorists as a biological weapon. Therefore, development of effective and

Table. Comparison of civet SARS-CoV-like virus and the early and late human SARS-CoV*

Characteristics	SARS-CoV–like virus	Early human SARS-CoV	Late human SARS-CoV
Transmission	Animal-to-animal	Animal/human-to-human	Human-to-human
Outbreak	No	No/local	Local/global
Causes disease	No	Mild	Severe
Representative strains	SZ3, SZ16	GD03T0013	Tor2, Urbani, BJ01, GZ02
Source	Palm civets	SARS patients during 2003–2004 epidemic	SARS patients during 2002–2003 outbreak
29-nucleotide deletion	No	No	Yes (some have a 415–nucleotide deletion)
Properties of spike protein			
Genetic homogenicity	Low	Low	High
Rate of nonsynonymous mutation	High	High	Low
Binding affinity to ACE2	Low	Low	High

*Information was obtained from references 4–6. SARS-COV, severe acute respiratory syndrome–associated coronavirus; ACE2, angiotensin-converting enzyme 2.

safe vaccines is urgently needed to prevent a new SARS epidemic and for biodefense preparedness. Currently, 3 major classes of SARS vaccines are under development: 1) inactivated SARS-CoV (Figure 1), 2) full-length S protein (Figure 2A), and 3) those based on fragments containing neutralizing epitopes (Figure 2B).

Inactivated SARS-CoV–based Vaccines

SARS-CoV expresses several structural proteins, including nucleocapsid, membrane, envelope, and spike (S) proteins (1). All may serve as antigens to induce neutralizing antibodies and protective responses. In general, prior to identification of the protein that contains the major neutralizing epitopes, the inactivated virus may be used as the first-generation vaccine because it is easy to generate whole killed virus particles. However, once the neutralizing epitopes are identified, the inactivated virus vaccine should be replaced by vaccines based on fragments containing neutralizing epitopes since they are safer and more effective. Several reports have showed that SARS-CoV inactivated with formaldehyde, UV light, and β-propiolactone can induce virus-neutralizing antibodies in immunized animals (8–11), and the first inactivated SARS-CoV vaccine is being tested in the clinical trials in China. However, safety of the inactivated vaccine is a serious concern; production workers are at risk for infection during handling of concentrated live SARS-CoV, incomplete virus inactivation may cause SARS outbreaks among the vaccinated populations, and some viral proteins may induce harmful immune or inflammatory responses, even causing SARS-like diseases (12,13).

S Protein–based Vaccines

The S protein of SARS-CoV, a type I transmembrane glycoprotein, is responsible for virus binding, fusion, and entry and is a major inducer of neutralizing antibodies (1,14). S protein consists of a signal peptide (SP: amino acids [aa] 1–12) and 3 domains: an extracellular domain (aa 13–1193), a transmembrane domain (aa 11194–1215), and an intracellular domain (aa 1216–1255). Its extracellular domain consists of 2 subunits, S1 and S2 (14), although the cleavage site between these subunits has not been clearly defined. The S1 subunit is responsible for virus binding to the receptor, angiotensin-converting enzyme 2 (ACE2) (15,16). A fragment located in the middle region of the S1 subunit (aa 318–510) is the receptor-binding domain (RBD) for ACE2 (17–19). SARS-CoV may also bind to cells through the alternative receptors DC-SIGN or L-SIGN (20,21), but the binding sites for these alternative receptors have not been defined. The S2 subunit, which contains a putative fusion peptide and 2 heptad repeats (HR1 and HR2), is responsible for fusion between the viral and target cell membranes. Infection by SARS-CoV is initiated by binding of RBD in the viral S protein S1 subunit to ACE2 on target cells. This forms a fusogenic core between the HR1 and HR2 regions in the S2 domain that brings the viral and target cell membranes into close proximity, which results in virus fusion and entry (22–24). This scenario indicates that the S protein may be used as a vaccine to induce antibodies for blocking virus binding and fusion.

Several recombinant vector-based vaccines expressing SARS-CoV S protein have been assessed in preclinical studies. Yang et al. (25) reported that a candidate DNA vaccine encoding the full-length S protein induced neutralizing antibodies (neutralizing titers ranging from 1:50 to 1:150) and protected mice from SARS-CoV challenge. Using DNA vaccines encoding the full-length and segments of S protein to immunize rabbits, Wang et al. have produced higher titers of neutralizing antibodies and demonstrated that major and minor neutralizing epitopes are located in the S1 and S2 subunits, respectively (26). Other groups also found neutralizing epitopes in the S2 subunit (27,28). Bisht et al. (29) have shown that intranasal or intramuscular inoculations of mice with highly attenuated modified vaccinia virus Ankara (MVA) vaccines encoding full-length SARS-CoV S protein also produce neutralizing antibodies with mean neutralizing titers of

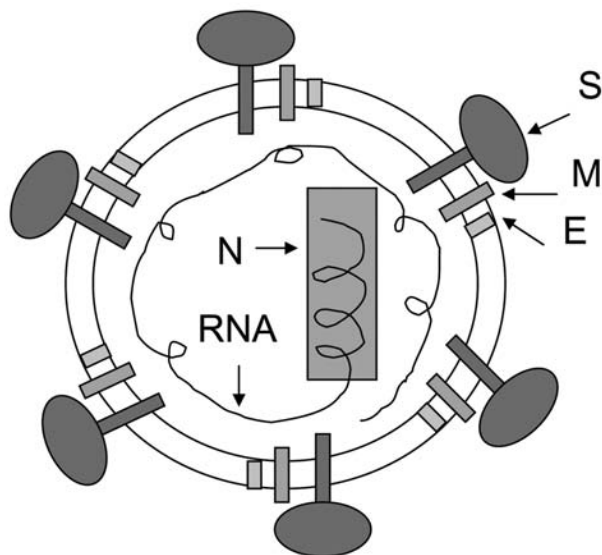

Figure 1. Strategy for designing vaccines for severe acute respiratory syndrome (SARS) using inactivated SARS-associated coronavirus. This virus expresses several structural proteins, including nucleocapsid (N), membrane (M), envelope (E), and spike (S).

1:284. Bukreyev et al. (30) reported that mucosal immunization of African green monkeys with an attenuated parainfluenza virus expressing S protein resulted in production of neutralizing antibodies and protected animals from infection by challenge with SARS-CoV. These data suggest that the S protein can induce neutralizing antibodies and protective responses in immunized animals.

Using convalescent-phase sera from SARS patients and a set of peptides spanning the entire sequence of the SARS-CoV S protein, we have identified 5 linear immunodominant sites (IDS) in the S protein (Figure 2A). IDS I, II, III, and V reacted with >50% of the convalescent-phase sera from SARS patients, while IDS IV was reactive with >80% of SARS sera, suggesting that IDS IV is the major immunodominant epitope on the S protein (31). Synthetic peptides corresponding to IDS could induce high titers of S protein–specific antibodies, but none of these antibodies possesses neutralizing activity. These findings suggest that the IDS in S protein may not induce neutralizing antibodies. Whether these antibodies enhance infection by heterologous SARS-CoV strains or mediate harmful immune responses is unclear. The S protein of FIPV expressed by recombinant vaccinia can cause antibody-dependent enhancement of disease if vaccinated animals are subsequently infected with wild-type virus (32). Our previous studies on HIV-1 showed that antibodies against some immunodominant epitopes in the HIV-1 envelope glycoprotein could enhance infection by heterologous HIV-1 strains (33). Most recently, Yang et al. (6) demonstrated that the polyclonal and monoclonal antibodies against S protein of the late SARS-CoV (Urbani strain) could neutralize infection by the relevant late SARS-CoV strains. However, these antibodies enhanced infection by an early human SARS-CoV isolate (GD03T0013) and the civet SARS-CoV–like viruses. These investigators have shown that the ACE2-binding domain mediates the antibody-dependent enhancement of civet SARS-CoV–like virus entry (6). Theoretically, some antibodies to the ACE2-binding domain may enhance infection if these antibodies closely mimic the receptor ACE2 and induce similar conformational changes, as the receptor likely does. The S protein with truncation at aa 1153 failed to cause antibody-dependent enhancement of infection, although it still induced neutralizing antibodies. This finding suggests that removal of the aa 1153–1194 region may abrogate induction of virus infection–enhancing antibodies (6). Vaccination of ferrets with MVA-based SARS vaccine expressing full-length S protein caused liver damage after animals were challenged with SARS-CoV (34). These findings raised concerns about the efficacy and safety of the vaccines containing or expressing full-length S protein.

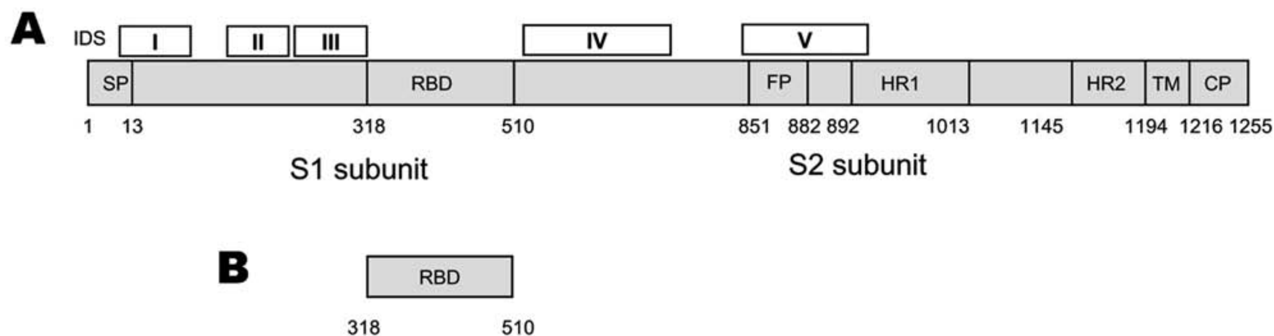

Figure 2. Strategies for designing vaccines for severe acute respiratory syndrome (SARS) using A) spike (S) protein and B) fragments containing neutralizing epitopes. SP, signal peptide; RBD, receptor binding domain; FP, fusion peptide; HR, heptad repeat; TM, transmembrane domain; CP, cytoplasm domain. IDS, immunodominant sites I to V corresponding to the sequences of amino acid residues 9-71, 171–224, 271–318, 528–635, and 842–913, respectively. The residue numbers of each region correspond to their positions in the S protein of SARS-associated coronavirus (SARS-CoV) strain Tor2. RBD contains the major neutralizing epitopes in the S protein. The recombinant RBD may be used as an efficacious and safe vaccine for preventing infection by SARS-CoV strains with distinct genotypes.

Vaccines Based on Fragments Containing Neutralizing Epitopes

RBD, a fragment (≈193 aa residues) in the middle of S1 subunit of S protein (Figure 2B), is responsible for virus binding to the receptor on target cells. We have demonstrated that the antisera from SARS patients and from animals immunized with inactivated SARS-CoV reacted strongly with RBD (9,35). Absorption of antibodies by RBD from these antisera results in the removal of most of the neutralizing antibodies, and RBD-specific antibodies isolated from these antisera have potent neutralizing activity (35,36). We have also shown that rabbits and mice immunized with RBD produced high titers of neutralizing antibodies against SARS-CoV with 50% neutralizing titers at a >1:10,000 serum dilution (37). The immunized mice were protected from SARS-CoV challenge (unpub. data). The antibodies purified from the antisera against SARS-CoV significantly inhibited RBD binding to ACE2 (9,36–38). Using spleen cells from mice immunized with RBD, we have generated a panel of 25 monoclonal antibodies (MAbs) that recognize different conformational epitopes on RBD and possess potent neutralizing activity (38). Our result is in agreement with the report by van den Brink et al. (39), who identified 3 human neutralizing anti-S MAbs from antibody phage display libraries by using inactivated SARS-CoV as the target. These researchers also found that all of these MAbs specifically bound to RBD and blocked interaction between RBD and ACE2. These findings suggest that RBD contains the major neutralizing epitopes in the S protein and is an ideal SARS vaccine candidate because RBD contains the receptor-binding site, which is critical for virus attachment to the target cell for infection (15,17–19). Antibodies specific for RBD are expected to block binding of virus to the target cell. RBD induces higher titers of neutralizing antibodies than those vaccines expressing the full-length S protein (25,26,29,30,37,38). RBD sequences among the late SARS-CoV strains are highly conserved. When the early and late SARS-CoV strains are compared, only 3 to 5 aa residues are variable among the 193 residues in RBD and most of the isolates vary by only 1 residue (4). van den Brink et al. (39) showed that 1 human MAb (CR3014) specific for RBD of SARS-CoV strain FM1 can effectively bind to most RBDs of the early and late SARS-CoV strains. These data suggest that antibodies directed against RBD of a SARS-CoV isolate may neutralize infection by a broad spectrum of SARS-CoV strains. Therefore, recombinant proteins containing RBD or vectors encoding RBD may be used as vaccines for preventing infection by SARS-CoV with distinct genotypes.

Conclusions

An ideal SARS vaccine should 1) elicit highly potent neutralizing antibody responses against a broad spectrum of viral strains; 2) induce protection against infection and transmission; and 3) be safe by not inducing any infection-enhancing antibodies or harmful immune or inflammatory responses. Currently, an inactivated SARS-CoV vaccine is in clinical trials in China. Safety is the major concern for this type of vaccine (12). The S protein is the major inducer of neutralizing antibodies. Recombinant vector-based vaccines expressing full-length S protein of the late SARS-CoV are under development. These vaccines can induce potent neutralizing and protective responses in immunized animals but may induce antibodies that enhance infection by early human SARS-CoV and animal SARS-CoV–like viruses (6). Recent studies have demonstrated that recombinant RBD consists of multiple conformational neutralizing epitopes that induce highly potent neutralizing antibodies against SARS-CoV (9,26,35–38). Unlike full-length S protein, RBD does not contain immunodominant sites that induce nonneutralizing antibodies. RBD sequences are relatively conserved. Thus, recombinant RBD or vectors encoding RBD may be used as safe and efficacious vaccines for preventing infection by SARS-CoV with distinct genotypes.

Dr. Jiang is associate member and head of the Viral Immunology Laboratory, Lindsley F. Kimball Research Institute, New York Blood Center. His primary research interests include development of vaccines and therapeutic agents against SARS-CoV and HIV.

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Address for correspondence: Shibo Jiang, Lindsley F. Kimball Research Institute, New York Blood Center, 310 East 67th St, New York, NY 10021, USA; fax: 212-570-3099; email: SJiang@NYBloodcenter.org

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West Nile Virus–associated Flaccid Paralysis

James J. Sejvar,* Amy V. Bode,† Anthony A. Marfin,† Grant L. Campbell,† David Ewing,‡ Michael Mazowiecki,‡ Pierre V. Pavot,§ Joseph Schmitt,¶ John Pape,# Brad J. Biggerstaff,† and Lyle R. Petersen†

The causes and frequency of acute paralysis and respiratory failure with West Nile virus (WNV) infection are incompletely understood. During the summer and fall of 2003, we conducted a prospective, population-based study among residents of a 3-county area in Colorado, United States, with developing WNV-associated paralysis. Thirty-two patients with developing paralysis and acute WNV infection were identified. Causes included a poliomyelitis-like syndrome in 27 (84%) patients and a Guillain-Barré–like syndrome in 4 (13%); 1 had brachial plexus involvement alone. The incidence of poliomyelitislike syndrome was 3.7/100,000. Twelve patients (38%), including 1 with Guillain-Barré–like syndrome, had acute respiratory failure that required endotracheal intubation. At 4 months, 3 patients with respiratory failure died, 2 remained intubated, 25 showed various degrees of improvement, and 2 were lost to followup. A poliomyelitislike syndrome likely involving spinal anterior horn cells is the most common mechanism of WNV-associated paralysis and is associated with significant short- and long-term illness and death.

Acute paralysis associated with West Nile virus (WNV) infection (1–8) has been attributed to Guillain-Barré syndrome (3), a poliomyelitislike syndrome (2,4–6,8), and a generalized myeloradiculitis (1,7). Several reports have described acute respiratory failure occurring with WNV-associated paralysis (5,7). However, the frequency of acute paralysis in WNV neuroinvasive disease remains unknown, and the clinical features of WNV-associated respiratory weakness have not been characterized.

During 2003, Colorado experienced an epidemic of human WNV disease; 2,947 cases were reported to the US Centers for Disease Control and Prevention (CDC) that included 621 neuroinvasive cases and 63 deaths. Acute paralysis was seen in many patients, and in several, acute respiratory failure developed that required emergent intubation. We conducted a population-based assessment of WNV-infected persons in whom acute paralysis developed to describe the clinical features, mechanisms, and short-term outcomes.

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Centers for Disease Control and Prevention, Fort Collins, Colorado, USA; ‡Centennial Neurology, Greeley, Colorado, USA; §Longmont Clinic, Longmont, Colorado, USA; ¶McKee Hospital, Loveland, Colorado, USA; and #Colorado Department of Health and Environment, Denver, Colorado, USA

Methods

During the summer and fall of 2003, we identified patients with acute weakness and WNV infection from among the populations of Boulder, Larimer, and Weld counties (combined population ≈724,000) in northern Colorado by active case-finding. We were notified of suspected cases by infection control practitioners and health professionals at 8 hospitals in and around the catchment area and through ongoing state-based surveillance. A suspected case of WNV-associated weakness was defined as muscle weakness in a person of at least 1-point decrement on manual muscle testing by using the Medical Research Council (London, UK) 1–5 scale (see online Appendix Figure 1, available at http://www.cdc.gov/ncidod/EID/vol11no07/04-0991-appG1.htm), respiratory failure requiring intubation that developed <48 hours after presentation, or both. All patients had IgM antibodies detected in acute-phase serum samples by IgM antibody-capture enzyme-linked immunosorbent assay at the Colorado Department of Health Services and Environment (9,10). Remaining acute-phase serum samples from 26 patients were tested by plaque-reduction neutralization assays for antibodies to WNV and St. Louis encephalitis virus at CDC (9,10). All had WNV-specific neutralizing antibody titers ≥1:10, which were at least 4-fold greater than those for St. Louis encephalitis virus.

Patients were approached under the auspices of a public health event, and oral consent was obtained. Results of

an initial neurologic examination were recorded, and standardized demographic, clinical, and medical history data were obtained from patient interviews and medical records. Results of serial neurologic examinations were documented on a standardized form.

Four months after initial assessment, we repeated the neurologic examinations, and patients or family members completed a self-administered questionnaire that gathered information on functioning in daily activities. Strength scores at 14 locations (see online Appendix Figure 2, available at http://www.cdc.gov/ncidod/EID/vol11no07/04-0991-appG2.htm) were evaluated at nadir and followup by using manual muscle testing scores. A proportional odds model for the strength scores was used to evaluate improvement; anticipated correlation was incorporated by estimating model parameters with generalized estimating equations. Within-patient correlations of the scores were estimated, and all pairwise differences were evaluated for significance by using bootstrap methods (11); adjustment for multiple comparisons was made by using the Bonferroni adjustment. Statistical analyses were performed with SAS version 8.2 (SAS Institute, Cary, NC, USA), S-Plus version 6.2 (Insightful Corp., Seattle, WA, USA), Sudaan version 8.0.2 (Research Triangle Institute, Research Triangle Park, NC, USA), and EpiInfo version 6.04d (CDC, Atlanta, GA, USA).

Results

Two hundred nineteen cases of WNV neuroinvasive disease were identified by state-based surveillance in our catchment area; among these, we identified 32 patients with acute paralysis and WNV infection. Eighteen (56%) were male; the median age was 56 years (range 15–84, Figure 1). All but 1 were Caucasian, and 3 (9%) were Hispanic. Sixteen patients (50%) had concomitant encephalitis, 10 (31%) had meningitis, and 6 (19%) had paralysis alone. Those 26 patients with concomitant meningitis or encephalitis represented 12% of the patients identified as having neuroinvasive disease.

Twenty-nine patients were examined by study neurologists within 7 days of weakness onset; 2 patients were evaluated on days 11 and 18 after weakness onset, respectively, and initial neurologic findings for 1 patient were obtained by personal communication (T. Clark, Colorado Pulmonary Associates, Denver, CO, USA). All but 1 of the patients were hospitalized. The median length of stay in the hospital was 17 days (range 2–87 days). Five patients (16%) with encephalitis were immunocompromised: 2 had prior liver transplants, 2 had hematologic malignancies, and 1 was receiving immunosuppressive medication for rheumatoid arthritis. One patient had insulin-dependent diabetes mellitus. Twenty-six (81%) had no prior medical problems.

Twenty-seven patients (84%) had asymmetric weakness consistent with a poliomyelitislike syndrome, 4 (13%) had symmetric ascending weakness with sensory abnormalities consistent with the acute inflammatory demyelinating polyradiculoneuropathy form of Guillain-Barré syndrome, and 1 had scapular winging and shoulder abduction weakness consistent with a long thoracic nerve paralysis.

Poliomyelitislike Syndrome

The incidence of poliomyelitislike syndrome was 3.7/100,000. Associated signs and symptoms are shown in Table 1. Two patients had weakness in the absence of other systemic features of infectious illness. Among 25 patients with systemic signs or symptoms listed in Table 1, including 4 in which weakness was concurrent with illness onset, the median interval between illness onset and weakness onset was 3 days (range 0–18). All but 1 patient had other neurologic features suggestive of acute WNV infection (Table 1). Patterns of weakness (Appendix Figure 1) included acute monoplegia (weakness or paralysis of 1 limb, n = 5); asymmetric upper (n = 1) or lower (n = 5) extremity weakness; and generalized asymmetric tetraplegia or quadriplegia (asymmetric weakness in ≥3 limbs, n = 16). Deep tendon reflexes in affected limbs were diminished or absent. Nineteen patients (70%) with poliomyelitislike syndrome had cranial nerve involvement, which included unilateral (n = 2) or bilateral (n = 8) facial paralysis, extraocular muscle weakness (n = 4), dysphagia (n = 13), dysarthria (n = 6), and vocal cord paralysis (n = 2). Limb weakness by strength testing or subjective patient interpretation progressed to its lowest point (nadir) within 24 hours in 24 patients (88%), with a range of <6 hours to 3 days. Two patients reported sensory deficits (subjective numbness or paresthesias), and 16 (60%) reported pain in affected limbs preceding onset of weakness. Paralysis due to WNV infection in 2 patients occurred exclusively in limbs with prior lower motor neuron dysfunction due to lumbar disc herniation (previously resolved with discectomy).

Electromyography/nerve conduction studies performed on 14 patients with poliomyelitislike syndrome suggested a motor axonopathy and/or an anterior horn cell process. Preservation of voluntary motor unit potentials was observed in all tested myotomes in 10 patients, and voluntary motor unit potentials were absent in some myotomes in 4 patients. Fourteen patients with poliomyelitislike syndrome underwent magnetic resonance imaging (MRI) of the brain (n = 6), spine (n = 2), or both (n = 6). Two displayed focal lesions of the basal ganglia, thalami, and brainstem bilaterally on T2- and diffusion-weighted sequences, and 4 (including 3 who did not undergo electromyography/nerve conduction studies) had signal abnor-

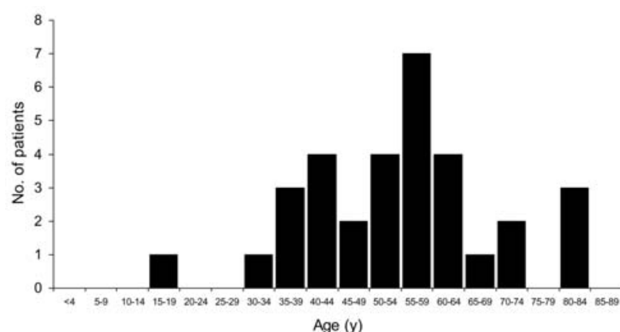

Figure 1. Age distribution of 32 patients with West Nile virus–associated paralysis.

malities in the anterior cord and ventral roots on T2- and diffusion-weighted sequences, suggesting anterior horn cell involvement (Figure 2).

Guillain-Barré–like Syndrome

Four patients had symmetric, ascending weakness with sensory symptoms suggestive of the acute inflammatory demyelinating polyradiculoneuropathy form of Guillain-Barré syndrome (Appendix Figure 1). All 4 had symptoms listed in Table 1 before weakness onset (range 2–12 days), 2 had meningitis before onset of weakness, and 2 had tremors. Initial deep tendon flexes were diminished or absent in all patients. Pain in affected limbs preceded weakness in 2 patients. The median interval between weakness onset and nadir was longer in patients with Guillain-Barré–like syndrome (4 days) than those with poliomyelitislike syndrome (1 day) ($p<0.005$, Wilcoxon rank sum test). One patient with Guillain-Barré–like syndrome had facial diplegia.

Electromyography/nerve conduction studies were carried out in 3 patients with Guillain-Barré–like syndrome, and all showed findings consistent with a predominantly demyelinating sensorimotor neuropathy. In patients with Guillain-Barré–like syndrome, voluntary motor unit potentials were preserved in tested myotomes.

Brachial Plexus Neuropathy

One patient with meningitis exhibited apparent, isolated, long thoracic nerve weakness with scapular winging and shoulder abduction weakness but without additional limb involvement. This patient also had pain before onset of weakness.

Respiratory Involvement

Twelve patients (39%), including 1 patient with Guillain-Barré–like syndrome, developed acute respiratory weakness that required endotracheal intubation and eventual tracheostomy (Table 2). In 10 patients, diaphragmatic paralysis, shown by hemidiaphragmatic elevation on chest radiograph, or pulmonary function test results with a restrictive pattern and retention of carbon dioxide, suggested neuromuscular respiratory failure. These studies were not available for the remaining 2 patients. Five additional patients with poliomyelitislike syndrome had dyspnea and showed evidence of diaphragmatic weakness by chest radiograph and pulmonary function test results, but were not intubated. Three patients died of respiratory failure 7, 27, and 35 days after intubation, after ventilatory support was withdrawn. All patients except the one with Guillain-Barré–like syndrome had encephalitis. Patients in whom dysarthria, dysphagia, or both developed were more likely to experience subsequent respiratory failure (odds ratio [OR] 62, $p<0.0001$, Fisher exact test). The interval between onset of these bulbar symptoms and intubation was $\leq$1 day in 9 of 12 patients (range <1–6 days). Facial nerve paralysis without other cranial nerve abnormalities was not associated with subsequent respiratory failure (Table 2).

Immunocompromised patients and patients with encephalitis were more likely to experience respiratory

Table 1. Signs and symptoms in 32 patients with West Nile virus (WNV)–associated paralysis

Systemic sign or symptom	Acute infection, N = 32 n(%)	4-month followup, N = 27 n(%)
Fever (temperature $\geq$38°C)	29(91)	0
Nausea with or without vomiting	26(81)	0
Headache	28(88)	5(19)
Altered mental status	16(50)	0
Meningismus	10*(31)	0
Rash	4(13)	0
WNV-associated neurologic features		
Tremor	21†(66)	8(25)
Myoclonus	15(47)	2(6)
Parkinsonism	8(25)	2(6)
Cerebellar ataxia	3(9)	2(6)
Limb atrophy	0	17(53)

*Includes 2 patients with Guillain-Barré–like syndrome and 1 patient with long thoracic nerve weakness.
†Includes 2 patients with Guillain-Barré–like syndrome.

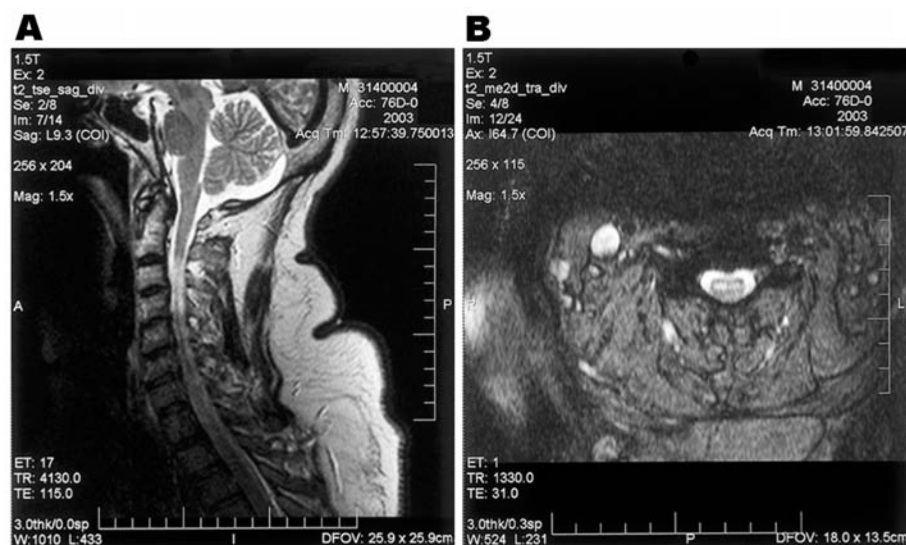

Figure 2. Saggital (A) and axial (B) T2-weighted magnetic resonance images of the cervical spinal cord in a patient with acute asymmetric upper extremity weakness and subjective dyspnea. A shows a diffuse cervical cord signal abnormality, and B shows an abnormal signal in the anterior horn region.

failure (Table 2). Pattern of limb weakness was not predictive of respiratory failure; 3 patients had initial upper extremity involvement, 4 had initial lower extremity involvement, and 5 had more diffuse generalized involvement before respiratory failure.

Cerebrospinal Fluid (CSF) Parameters

Lumbar puncture was performed on 30 patients. They had a median CSF leukocyte count of 108 cells/mm^3 (range 0–740 cells/mm^3), a median CSF protein level of 98 mg/dL (range 27–138 mg/dL), and a median CSF glucose level of 55 mg/dL (range 36–135 mg/dL). Pleocytosis was lymphocytic in 14 patients, neutrophilic in 13, and unknown in 3. Among recorded cell differentials (n = 27), neutrophils were predominant in CSF collected on or before the day of weakness onset (10/14, 71%). CSF collected after the time of weakness onset was more often lymphocytic (10/13, 77%, OR 8.3, p = 0.03). Three of 4 patients with Guillain-Barré–like syndrome had lumbar punctures; 2 had cytoalbuminologic dissociation (elevated protein levels without pleocytosis) commonly seen in Guillain-Barré syndrome, and 1 had pleocytosis with an elevated protein level. The median CSF leukocyte count and protein level (64 cells/mm^3 and 100 mg/dL, respectively) in patients with respiratory failure were not significantly different from those without respiratory failure (117 cells/mm^3 and 96 mg/dL, each p = 0.5, Wilcoxon rank sum test).

Short-term Outcomes

Three patients with poliomyelitislike syndrome died in the hospital after withdrawal of ventilatory support. Of the surviving patients, 13 were discharged and 15 (including 1 with Guillain-Barré–like syndrome) were admitted to long-term care facilities. The patient with long thoracic nerve palsy was not hospitalized. At 4 months, 2 patients with poliomyelitislike syndrome were lost to followup. Of 15 patients sent to long-term care facilities, 5 with poliomyelitislike syndrome and 1 with Guillain-Barré–like syndrome remained in these facilities, and the remaining 9 had been discharged (median length of stay 40 days, range 17–106). No persons initially discharged were readmitted. Of 21 patients employed or in school before illness, 9 were working full-time or part-time at the 4-month followup. Seventeen required continuing physical therapy.

Four of the 27 reassessed patients (patients 4, 18, 20, and 43), all with poliomyelitislike syndrome, showed almost no improvement in strength of involved muscles. Two patients with Guillain-Barré–like syndrome regained baseline strength. Although the remainder showed general improvement by the proportional odds model (p<0.001), none had returned to baseline status (Appendix Figure 1).

Table 2. Presence of dysarthria/dysphagia, facial weakness, encephalitis, and immunocompromised status among patients with and without respiratory failure due to West Nile virus infection

Variable	Respiratory failure (N = 12*)	No respiratory failure (N = 20)	Odds ratio	p value†
Dysarthria/dysphagia	11	3	62	<0.0001
Immunocompromised status	4	0	Undefined	0.01
Encephalitis	12	5	Undefined	<0.0001
Facial nerve weakness	6	5	3	0.25

*Includes 1 patient with respiratory weakness secondary to Guillain-Barré–like syndrome.
†Fisher exact test.

In patients with poliomyelitislike syndrome undergoing electromyography studies, the absence of voluntary motor unit potentials was associated with lack of strength improvement in involved myotomes. Neurologic signs at 4 months are shown in Table 1. Three patients (all with respiratory paralysis) were nonambulatory; 12 of 24 ambulatory patients, including 2 with Guillain-Barré–like syndrome, required a wheelchair (n = 4), walker (n = 3), or canes or crutches (n = 5).

Pairwise comparisons of the correlations among patients with poliomyelitislike syndrome showed that at both times, weakness was more likely to be of greater severity in comparable limbs across the body (e.g., both arms, both legs) than in limbs on the same side (e.g., right arm and leg); facial weakness was strongly correlated with ipsilateral arm weakness (Appendix Figure 2). Limbs improved distally to a greater extent than proximally.

Of 9 surviving patients who had respiratory failure, 2 remained intubated at 4 months, and 7 displayed various degrees of improvement (Appendix Figure 1); however, none had returned to baseline strength or level of function. The median duration of intubation for the 7 extubated patients was 49 days (mean 66 days, range 21–135).

Discussion

The overall incidence of WNV-associated paralysis in this population was 4.3/100,000, with an incidence of poliomyelitislike syndrome of 3.7/100,000, which is comparable to that of paralysis seen during epidemics of poliovirus disease (12). During 2003, 219 cases of neuroinvasive disease were reported in this 3-county area of Colorado (13), of whom we estimate that flaccid paralysis developed in 12%. A total of 2,773 cases of WNV neuroinvasive disease were reported in the United States during the 2003 epidemic (13); ≈330 cases of WNV-associated neuromuscular weakness may have occurred in the United States during this period.

In contrast to persons typically considered at risk for WNV encephalitis alone, our patients were relatively young, with a third-quartile age of 61 years (Figure 1), and healthy, with >80% having no prior medical conditions. Many patients required prolonged hospitalization and time in rehabilitation facilities and had severe disabilities, which suggests that paralysis caused by WNV infection is associated with considerable lost productivity and incurred healthcare costs. Thus, the long-term economic impact of WNV paralysis needs assessment.

Twenty-seven patients with WNV-associated paralysis had electrophysiologic or neuroimaging evidence of a poliomyelitislike syndrome or a clinical syndrome compatible with this diagnosis. These patients had asymmetric, acute weakness, pleocytosis, and in 14 patients with studies performed, results of electromyography/nerve conduction studies were consistent with motor axonopathy, anterior horn cell involvement, or both. However, 4 patients had clinical, electrophysiologic, and laboratory findings more suggestive of a Guillain-Barré–like syndrome. These patients had characteristic bilateral, symmetric, ascending weakness with associated sensory symptoms, and 2 of 3 patients with available CSF data showed characteristic cytoalbuminologic dissociation. The 3 patients who underwent electrodiagnostic studies had findings consistent with a demyelinating sensorimotor neuropathy typical of Guillain-Barré–like syndrome (3,14). Further electrophysiologic and pathologic characterization of this syndrome is needed because its management may differ from that of poliomyelitis.

Although WNV-associated weakness may occur without other findings suggesting acute WNV disease (14), all but 6 of our patients had meningitis or encephalitis and displayed other WNV-associated neurologic signs, including tremors, myoclonus, and parkinsonism (15–18). A neutrophilic, rather than the more typical lymphocytic, pleocytosis may be seen soon after onset of WNV disease (17) or other viral infections of the central nervous system (19–21). Our patients who had CSF obtained on the day of or shortly after weakness onset were more likely to have a neutrophilic pleocytosis than those with CSF obtained later in their illness. Clinicians should recognize the potential for a neutrophilic predominance in CSF obtained early in the course of WNV neuroinvasive disease.

A generalized asymmetric tetraplegia or quadriplegia was the most common weakness pattern, followed by monoplegia. Consistent with previous reports (22), all patients except 2 with Guillain-Barré–like syndrome demonstrated continued weakness at 4 months, although nearly all had some improvement in strength as indicated by manual muscle testing scores. All 10 patients with a poliomyelitislike illness who had even minimal preservation of motor unit potentials on initial electromyogram improved in strength in associated myotomes at 4 months; 4 patients with no motor unit potentials on initial electromyogram did not improve in strength in these myotomes. Complete destruction of large spinal motor neurons correlating with a completely paralyzed muscle has been observed in poliovirus-associated paralysis (23). Absence of motor unit potentials on electromyography may reflect this loss and may have prognostic value for future strength recovery. However, independent predictors of strength outcome remain unknown.

The facial weakness observed in 40% of patients with poliomyelitislike syndrome and in 1 patient with Guillain-Barré–like syndrome nearly or completely resolved, which is consistent with observations of patients with poliovirus disease and suggests a favorable outcome of this manifestation (24,25). Weakness was more severe in congruent

limbs across the body than in ipsilateral limbs, and facial weakness was associated more strongly with arm than with leg weakness, which is consistent with the patchy focal cell destruction demonstrated pathologically in WNV poliomyelitis (26). Improvement in limb strength tended to occur distally to proximally.

Thirty-eight percent of the patients, including all 3 who died, had respiratory failure requiring intubation, and 16% of the patients had dyspnea and diagnostic evidence of neuromuscular respiratory failure but were not intubated. Respiratory failure has been described with WNV-associated paralysis (6–8) but has not been characterized in detail. In 1 patient with Guillain-Barré–like syndrome, respiratory failure developed, a common complication of this syndrome (27). However, the association with limb weakness of a motor neuron or anterior horn cell type, presence of an elevated hemidiaphragm on chest radiograph, and a restrictive pattern of respiratory failure by pulmonary function testing suggest a central, poliomyelitislike etiology for respiratory failure in all other patients. This etiology is supported by the electrodiagnostic findings of lower motor neuron involvement in affected limbs in 5 patients with respiratory failure. Additionally, histopathologic findings in patients with respiratory weakness and WNV infection have demonstrated neuronophagia and leukocytic inflammation of the dorsal motor nuclei of the vagus and glossopharyngeal nerves, which is similar to that seen in the spinal anterior horns (26). Disease due to poliovirus infection has been associated with diaphragmatic, intercostal muscle, and bulbar weakness with respiratory failure (28–31), and poliomyelitislike respiratory insufficiency has been described in infections with other flaviviruses (32).

Lower bulbar dysfunction, specifically dysarthria and dysphagia, was more frequent in patients with respiratory failure; in most, lower bulbar dysfunction followed or was concurrent with limb weakness and preceded respiratory failure by less than a day. Patients with lower bulbar signs and acute limb paralysis require monitoring for respiratory failure. Facial paralysis was not associated with increased risk of respiratory failure, which possibly reflects the neuroanatomic separation of the involved cranial nerve nuclei. Although results of MRI are frequently reported as normal in patients with WNV neuroinvasive disease, detailed images of brainstem and cervical spine are frequently not obtained. Although 4 patients displayed spinal cord and ventral root lesions at involved levels, and 1 patient with respiratory failure had lower brainstem signal abnormalities, detailed brainstem and spinal cord images in our patients were generally not obtained. In patients with acute weakness and bulbar signs, MRI should include the lower brainstem and spinal cord. Although 2 patients remained intubated at 4 months, all other surviving patients with respiratory involvement were extubated, although duration of ventilatory support was often prolonged.

In summary, our findings suggest that involvement of WNV with anterior horn cells, which resulted in a poliomyelitislike syndrome, represents the most common underlying cause of paralysis with WNV infection. In the population assessed by our study, the incidence of paralysis was comparable to that seen during large epidemics of poliovirus infection. Respiratory involvement was a frequent and severe manifestation of this syndrome, with a high degree of illness and death. Thus, early and prominent dysarthria and dysphagia may be predictors of subsequent respiratory failure in this group.

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Dr. Sejvar is a neurologist and medical epidemiologist with the Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention. His primary research interests are surveillance and epidemiology of encephalitis, Creutzfeldt-Jakob disease, and other infectious diseases of the nervous system.

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Address for correspondence: James J. Sejvar, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd, Mailstop A39, Atlanta, GA 30333, USA; fax: 404-639-3838; email: JSejvar@cdc.gov

Past Issues on West Nile Virus

Primate-to-Human Retroviral Transmission in Asia

Lisa Jones-Engel,* Gregory A. Engel,† Michael A. Schillaci,‡ Aida Rompis,§ Artha Putra,§ Komang Gde Suaryana,§ Agustin Fuentes,¶ Brigitte Beer,# Sarah Hicks, Robert White,** Brenda Wilson,** and Jonathan S. Allan****

We describe the first reported transmission to a human of simian foamy virus (SFV) from a free-ranging population of nonhuman primates in Asia. The transmission of an exogenous retrovirus, SFV, from macaques (*Macaca fascicularis*) to a human at a monkey temple in Bali, Indonesia, was investigated with molecular and serologic techniques. Antibodies to SFV were detected by Western blotting of serum from 1 of 82 humans tested. SFV DNA was detected by nested polymerase chain reaction (PCR) from the blood of the same person. Cloning and sequencing of PCR products confirmed the virus's close phylogenetic relationship to SFV isolated from macaques at the same temple. This study raises concerns that persons who work at or live around monkey temples are at risk for infection with SFV.

Recent epidemics such as HIV and severe acute respiratory syndrome (SARS) have changed the way we view emerging infectious diseases; these epidemics show that animal reservoirs are important sources of new infectious threats to humans. Contact between humans and animals is a crucial rate-limiting step in this process, although data describing the variables that influence animal-to-human transmission are relatively scarce. Nonhuman primates, by virtue of their genetic, physiologic, and sometimes social similarities to humans, are particularly likely sources of infectious agents that pose a threat to humans (1,2). Data on simian immunodeficiency virus (SIV)/HIV dramatize this point; scientists now theorize that SIVs were transmitted from primates to humans on several occasions (3,4). As a result, concern is increasing that other infectious agents enzootic in primate populations may endanger humans (5).

The family of SIV is 1 of 4 primateborne retroviruses known to infect humans (6). Simian T-cell lymphotropic viruses, enzootic in both Asian and African Old World monkeys and apes, may have repeatedly crossed the species barrier (7,8). The resulting human form of the virus, HTLV, is the etiologic agent of 2 human diseases, adult T-cell leukemia and tropical spastic paresis (9). Serologic studies have demonstrated evidence of primate-to-human transmission of simian retrovirus (SRV), a retrovirus enzootic among Old World monkeys, in laboratory workers exposed to captive primates (10). To date, no disease has been linked to human infection with SRV. Finally, in the past decade, evidence of infection with simian foamy virus (SFV) has been identified in 1% to 4% of persons who come into frequent contact with primates in zoos and primate laboratories and among 1% of bushmeat hunters in Central Africa (11–15).

SFV

SFVs are exogenous retroviruses enzootic in both New and Old World primates (16–18). Phylogenetic analyses of SFVs indicate a species-specific distribution of virus strains not unlike that of SIV among some African primate species (19–23). Among captive primate populations, seroprevalence of antibodies to SFV may reach 100% in adults, with many animals seroconverting before the onset of sexual maturity (19,24; J. Allan, unpub. data). Fewer data are available on the seroprevalence of antibodies to SFV among free-ranging populations of primates. SFV is present in highest concentrations in the saliva of infected laboratory macaques (*Macaca mulatta* and *M. fascicularis*) and African green monkeys (*Cercopithecus aethiops*), which suggests that bites, scratches, and mucosal splashes with

*University of Washington National Primate Research Center, Seattle, Washington, USA; †Swedish/Providence Hospital Family Practice Residency, Seattle, Washington, USA; ‡University of Toronto at Scarborough, Toronto, Ontario, Canada; §Udayana University, Denpasar, Bali, Indonesia; ¶University of Notre Dame, Terre Haute, Indiana, USA; #Southern Research Institute, Frederick, Maryland, USA; and **Southwest Foundation for Biomedical Research, San Antonio, Texas, USA

saliva from primates are likely mechanisms of transmission (25–27).

Because SFV is not known to occur naturally in humans, detecting serologic or molecular evidence, or both, of infection in a human, along with a history of close contact with primates, constitutes strong evidence for primate-to-human transmission, i.e., a marker for cross-species transmission (28). Existing serologic and molecular techniques can sensitively and specifically detect human SFV infections (29).

Primates and humans come into contact in a variety of contexts in Asia, including owning primate pets, observing performance monkeys, participating in ecotourism activities, hunting primates for bushmeat, and visiting monkey temples (30,31). Monkey temples are religious sites that have, over time, become associated with populations of free-ranging primates. Monkey temples are common throughout southern and Southeast Asia, where primates play an important role in culture. Asia's monkey temples annually bring millions of people, including hundreds of thousands of tourists, from around the world into close proximity with free-ranging primates (32–34). Worldwide, monkey temples may account for more human-primate contact than any other context.

In Bali, ≈45 temple sites contain substantial populations of free-ranging macaques (33). Those who spend the most time in or around monkey temples include workers who maintain the temples; nuns, monks, and others who live on or around temple grounds; merchants who sell a variety of goods to tourists; and farmers whose fields are raided by macaques. Worshippers and tourists may also come into contact with temple macaques. These temples are thus an important context in which to investigate cross-species transmission of infectious agents.

Serologic data on free-ranging Southeast Asian macaques, though incomplete, suggest that SFV is enzootic among these macaque populations and that corresponding rates of SFV infection are high (80%–100%) (L. Jones-Engel, unpub. data). We hypothesized that humans who come into contact with these macaques might similarly show evidence of infection with SFV and investigated this proposition among a group of persons who worked at or around the Sangeh monkey temple in Bali, Indonesia.

Methods

Study Site: Sangeh Monkey Temple

The Sangeh monkey temple is in central Bali, Indonesia, ≈20 km north of Denpasar, Bali's most populous city. The 17th-century Hindu temple complex at Sangeh serves the surrounding community and is a popular domestic and international tourist destination. Approximately 200 free-ranging *M. fascicularis* roam throughout the 6-hectare temple complex and into the surrounding rice fields and farms. Most of the macaques' caloric intake is from daily provisions provided by temple workers and food given to them by visitors.

Human Participant Sampling

In July 2000, as part of a larger study on the epidemiology of exposure to primateborne viruses at the Sangeh monkey temple, 82 workers from Sangeh agreed to participate in the present study (32). After informed consent was obtained, a questionnaire designed to elicit demographic data as well as data describing the frequency and type of exposure to Sangeh's macaques was administered in Bahasa Indonesia, the national language of Indonesia. Subsequently, 10 mL of blood was withdrawn from each participants antecubital vein, 6 mL was centrifuged to extract serum, and the remainder was mixed with EDTA. Serum specimens and whole blood were then stored at –20°C.

Macaque Sampling

In July 2000, 38 macaques within the Sangeh monkey temple area and surrounding forest were darted opportunistically and sedated with 3 mg/kg of Telazol (tiletamine HCl/zolazepam HCl). Following universal precautions, researchers withdrew 10 mL of blood from each macaque's femoral vein. The macaques were closely monitored during anesthesia and recovery. Six milliliters of blood was placed in a serum separator tube and centrifuged in the field to extract the serum. The remaining blood was placed in a tube containing EDTA. Sera and whole blood were frozen and stored at –20°C.

Western Blot Analysis

Western blot immunoassays were performed with a few modifications (21). Briefly, human foreskin fibroblast cells were infected with SFVbab1 (an isolate from a baboon) and maintained until notable cytopathologic changes were observed (19). Culture supernatant fluid containing virus was harvested, and SFV was purified through a 20% sucrose cushion, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the antigens were blotted onto nitrocellulose sheets. The nitrocellulose paper was blocked with 3% bovine serum albumin and subsequently incubated with serum at a dilution of 1:40. Viral proteins were detected with the streptavidin-biotin system (Amersham Inc., Arlington Heights, IL, USA) by using diaminobenzidine as the substrate for color development. The criterion used for a positive sample was antibody reactivity to both p70 and p74 *gag*-related proteins (19).

PCR Detection of SFV

DNA was purified from whole blood by using the QIAamp DNA Blood Mini Kit (Qiagen, Inc., Valencia,

CA, USA). Briefly, 20 µL protease and 200 µL buffer AL were combined with 200 µL whole blood and incubated at 56°C for 10 min. After incubation, 200 µL ethanol was added, and the entire mixture was applied to a QIAamp spin column. The purified DNA was eluted from the column with 70 µL nuclease-free water, and concentration was determined spectrophotometrically at optical density 260 nm.

The presence of SFV DNA was determined by using nested PCR. Five hundred ng purified DNA was combined with a PCR reaction mixture with a final concentration of 10 mmol/L Tris (pH 9.0), 50 mmol/L KCl, 0.1% Triton X-100, 2 mmol/L $MgCl_2$, 200 µmol/L each dNTP, 0.15 mg/mL BSA, 1 µm Taq polymerase, and 400 nmol/L of each primer in a total volume of 50 µL. The following primer pairs were used: first round, forward 5′ CAG TGA ATT CCA GAA TCT CTT C 3′, reverse 5′ CAC TTA TCC CAC TAG ATG GTT C 3′; and second round, forward 5′ CCA GAA TCT CTT CAT ACT AAC TA 3′, reverse 5′ GAT GGT TCC CTA AGC AAG GC 3′ (29). "Touchdown PCR" was used for both rounds with reaction conditions of initial denaturation at 94°C for 2 min, followed by 7 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, with a 2°C decrease in annealing temperature per cycle to 48°C, followed by 33 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 45 seconds with a final extension at 72°C for 2 min. Second-round conditions were the same, except 19 cycles were used instead of 33.

Twenty µL of the PCR reaction underwent electrophoresis on a 1% low melting point agarose gel. DNA bands (365-bp product) were excised from the gel and purified by using Wizard PCR Preps DNA Purification System (Promega Corp., Madison, WI, USA). The PCR product was ligated into the pCR 2.1-TOPO vector by using the TOPO TA Cloning Kit (Invitrogen Corp., Carlsbad, CA, USA.). An SFV DNA plasmid (pSFV-1Lgp), representing long terminal repeat, *gag*, and *pol* of SFV-1, was included as a positive PCR control and for determining sensitivity of detection by serial dilution (provided by A. Mergia). TOP10 cells were transformed with the ligation reaction, plated onto Luria broth agar plates containing 50 µg/mL kanamycin, and incubated overnight at 37°C. Miniscreen DNA was purified by using Wizard Plus Minipreps DNA Purification System (Promega). Samples were sequenced with the ABI 373 automated fluorescent sequencer using BigDye Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, USA).

Amplification of Mitochondrial Sequences

Five hundred ng purified DNA from whole blood was combined in a PCR reaction mixture with a final concentration of 10 mmol/L Tris (pH 9.0), 50 mmol/L KCl, 0.1% Triton X-100, 2.5 mmol/L $MgCl_2$, 200 µmol/L each dNTP, 0.15 mg/mL BSA, 1 U Taq polymerase, and 400 nmol/L of each primer in a total volume of 50 µL. The following primers were used: forward, 12SA, 5´ CTG GGA TTA GAT ACC CAC TAT 3´, and reverse, 12SO, 5´ GTC GAT TAT AGG ACA GGT TCC TCT A 3´ (35). Cycling conditions were the following: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 5 min, with a final extension at 72°C for 5 min. The 101-bp product underwent electrophoresis and was processed for DNA sequencing essentially as described for SFV.

The alignments were made in Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and ClustalX 1.81 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). Columns in the alignment in which gaps had been inserted in regions with insertions, and deletions were stripped before the analyses. DNA trees were created with the neighbor-joining method by using the Phylip program (DNAdist; Neighbor), and the output was generated with Treeview (http://taxonomy. zoology.gla.ac.uk/rod/treeview.html). The GenBank accession numbers for the SFV and mitochondrial DNA sequences reported here are AY628152-69 and AY633510-39, respectively.

Results

Seroprevalence of SFV among Macaques

The seroprevalence of SFV among the Sangeh macaques is presented in Table 1. Thirty-eight macaques (29 males and 9 females; 4 juveniles, 6 subadults, 28 adults) were sampled. Thirty-four (89.5%) of the 38 macaques were seropositive for SFV by Western blot; 2 (50%) of 4 juveniles, all 6 (100%) subadults, and 26 (93%) of the adults were antibody positive. All 9 females were SFV seropositive. SFV seroprevalence in this free-ranging macaque population was consistent with seroprevalence studies done in captive (25) and other free-ranging macaque populations (L. Jones-Engel, unpub. data).

Table 1. Seroprevalence of antibodies to simian foamy virus among Sangeh macaques (*Macaca fascicularis*)

Age group*	No. Western blot positive/total (%)
Juvenile	2/4 (50.0)
Male	2/4 (50.0)
Subadult	6/6 (100.0)
Male	4/4 (100.0)
Female	2/2 (100.0)
Adult	26/28 (92.9)
Male	19/21 (90.5)
Female	7/7 (100.0)
All ages	34/38 (89.5)
Male	25/29 (86.2)
Female	9/9 (100.0)

*Juveniles are defined as 1–3 years of age, subadults as 3–5 years of age, adults as >5 years of age.

Human Participant Exposure Data

Nearly two thirds of the study sample was male (62.2%), and the mean age of the study participants was 35 years (Table 2). Of participants who reported being exposed, 23 (28.0%) of 82 persons reported having been bitten by the temple's macaques (Table 3). Five of the 23 reported being bitten more than once. Thirty-one (37.8%) of 82 reported having been scratched, including 9 persons who had been scratched more than once. In total, 37 (45.1%) of 82 reported having been either bitten or scratched.

Of the 82 persons whose serum was tested for antibodies to SFV, 81 had negative results by Western blot. Serum from a 47-year-old farmer (BH66) was reactive to SFV gag proteins, p70/p74 (Figure 1). This man reported that he visited the monkey temple every day and had previously been bitten once and scratched on more than one occasion by macaques there. He reported that the bite and scratches, which occurred on his hands and toes, had bled and that he had washed the wounds and applied traditional medicines. He denied owning or coming into contact with a pet primate. He also denied hunting primates or consuming primate meat.

Detection of SFV DNA in a Person from Bali

To determine whether SFV was present in humans and macaques, we performed nested PCR amplification of SFV by using conserved primers designed to detect macaque SFV (29). SFV was detected in the macaques tested, as well as in the 1 human participant (BH66), whose serum contained antibodies to SFV. Blood samples from all 81 human participants that were seronegative for antibodies to SFV were also negative for SFV by PCR. The limits of sensitivity for nested PCR were 1–10 SFV DNA copies per 500 ng cellular DNA, as determined by dilution of a positive control plasmid.

Quantitative PCR products of BH66, 5 of the Sangeh macaques (*M. fascicularis*), and 13 pet macaques (*M. tonkeana*, *M. maura*, and *M. fascicularis*) from Sulawesi, Indonesia, were cloned at least twice, sequenced, and compared with published sequences from a rhesus macaque (*M. mulatta*) (SFV-1mac, M55279), an African green monkey (*Cercopithecus aethiops*) (SFV-3agm, M74895), and a chimpanzee (*Pan troglodytes*) (SFVcpz, U04327). As shown in Figure 2, SFV from BH66 was most closely related to an SFV sequence amplified from 1 of the macaques at the Sangeh Monkey Temple (BP6). To verify that the BH66 sample was of human origin and not a mislabeled sample from an SFV-infected monkey, we amplified a small fragment from the 12S ribosomal mitochondrial DNA from 2 healthy unexposed humans, BH66, several macaque species, and African green monkeys; cloned the products; and derived DNA sequences. Phylogenetic analysis (Figure 3) showed that the human DNA sample grouped with mitochondrial DNA sequences from humans, confirming that the BH66 sample was from a human. Lymphocytes from BH66 were not available for isolation of SFV directly.

Table 2. Human study population, demographic characteristics

Demographic characteristic	No. (%)
All participants	82 (100.0)
Age group (y)	
<20	9 (11.0)
20–29	23 (28.0)
30–39	20 (24.4)
40–49	22 (26.8)
>49	8 (9.7)
Sex	
Male	51 (62.2)
Female	31 (37.8)

Discussion

Human Infection with SFV at the Sangeh Monkey Forest

This report documents the first case of SFV infection in a person with known exposure to free-ranging Asian primates. Antibodies to SFV were detected in serum, and SFV genomic segments were amplified from blood of the infected person. Because PCR is prone to contamination, which leads to false-positive results, PCR products were sequenced and compared to SFV from other macaque species. Phylogenetic analysis showed that SFV amplified from the infected human was most similar to SFV from *M. fascicularis* at the Sangeh monkey temple. Although ascertaining exactly how or when the person acquired his infection was not possible, he did report having been bitten once and scratched on a number of occasions by macaques at Sangeh. He denied other past contacts with primates.

Because only 1 infected person was detected and our sample size was limited (82 persons), we cannot estimate the prevalence of SFV infection in this human sample. Previous research on naturally acquired SFV infection among bushmeat hunters in Central Africa found 10 who seroconverted in a population of 1,099 (1%), of which 3 also had positive results by PCR. Surveillance of larger

Table 3. Prevalence of reported bite and scratch injuries

Descriptor	No. (%)
Bitten	23 (28.0)
Bitten more than once	5 (6.1)
Scratched	31 (37.8)
Scratched more than once	9 (11.0)
Bitten or scratched	37 (45.1)
Possessed food at time of injury	35 (94.6)
All participants	82 (100.0)

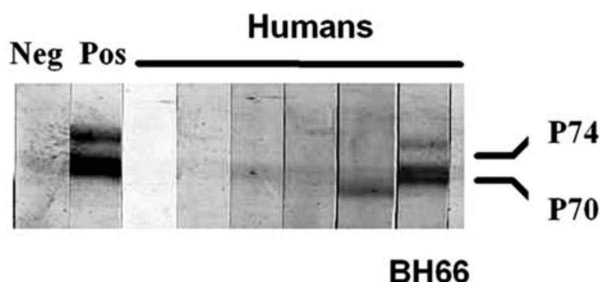

Figure 1. Western blot analysis of human serum samples for evidence of simian foamy virus (SFV) antibodies. Antibodies to the gag precursor proteins (p70/p74) were apparent from the human BH66 blood sample, which indicated infection with SFV. Positive control is an SFV-infected baboon.

numbers of persons exposed to primates at monkey temples is necessary to estimate the risk of SFV infection in this context.

Monkey Temples in Bali as Contexts for Primate-Human Contact

Human and macaque sympatry in Southeast Asia dates back as far as 25,000 years, as evidenced by remains at Niah Cave in Borneo (37). Hindu and Buddhist temples are a relatively recent addition to the landscape, first appearing 1,000 to 4,000 years ago. Contemporary human-macaque commensalism at each monkey temple is shaped by the behavioral characteristics of the particular monkey population as well as the community's unique geographic, social, religious, and economic factors. Human-macaque contact differs at the various monkey temples. At Sangeh, where tourism has become an important economic resource, macaques depend on visitor feeding for most of their nutrition and have learned to climb on visitors' heads and shoulders to obtain food. Local photographers, who make a living photographing visitors with monkeys, encourage this behavior. Macaques, sometimes provoked by visitors, can become aggressive and will bite or scratch people.

The Sangeh Temple Committee, which manages the Sangeh Monkey Forest, estimates ≈250 persons work in and around the monkey forest. Of the 82 persons who participated in the present study, 28% had been bitten by a macaque, and 6% had been bitten more than once. In comparison, 700,000 tourists visit the 4 main monkey temples on the island of Bali (Padangtegal/Ubud, Sangeh, Alas Kedaton, and Uluwatu) annually and as many as 5% of these visitors are bitten by macaques (A. Fuentes, unpub. data). These data suggest that visitors receive the preponderance of macaque bites.

Importance of SFV Infection in Humans

The literature describes ≈40 cases of human infection with SFV. Long-term follow-up data are unavailable for many of these cases. However, no disease has been linked to SFV among infected humans. Furthermore, no cases of human-to-human transmission have been described, even among those receiving transfusions of blood products from a worker at a primate center who was later shown to be infected with SFV (38).

Notwithstanding, more data are needed before SFV can be proclaimed a "virus without a disease." First, SFV infection has not been extensively studied in immunocompromised persons, so whether SFV has a more aggressive course in an immunologically "permissive" environment is unknown. Two persons died shortly after receiving baboon

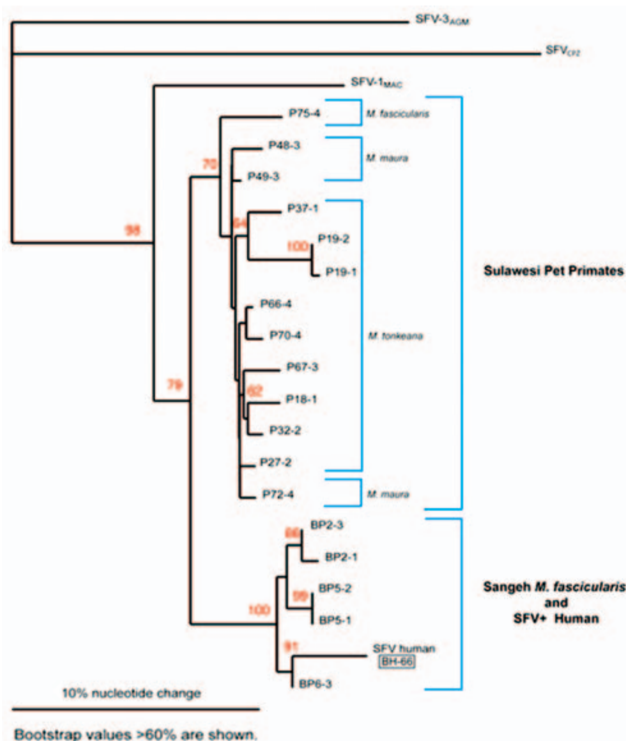

Figure 2. Phylogenetic analysis of simian foamy virus (SFV) DNA from several species of Indonesian primates and an infected human. BP 2, 5, and 6 represent Sangeh monkey temple macaques (*Macaca fascicularis*). P 18, 19, 27, 32, 37, 66, 67, and 70 are pet macaques (*M. tonkeana*) from Sulawesi, Indonesia. P48, 49 and 72 are pet macaques (*M. maura*) from Sulawesi, Indonesia. P75 is a pet *M. fascicularis* macaque from Sulawesi, Indonesia. All Sulawesi pet primate samples were collected during 2000. SFV-1mac represents a published sequence from a rhesus macaque (*M. mulatta*), and SFV-3agm is a published sequence from an African green monkey (*Cercopithecus aethiops*). SFVcpz is a published sequence from a chimpanzee (*Pan troglodytes*) and was used an outgroup for this tree. The SFV human strain (BH66) clustered with an SFV sequence amplified from BP6 one of the macaques at the Sangeh monkey temple. The SFV DNA tree was created with the neighbor-joining method by using the PHYLIP program (DNAdist; Neighbor). Bootstrap replicates were 1,000. Bootstrap values were calculated by using Seqboot, DNAdist, Neighbor, and Consense (PHYLIP programs). Bootstrap values 60% are shown. The SFV tree was plotted in Treeview.

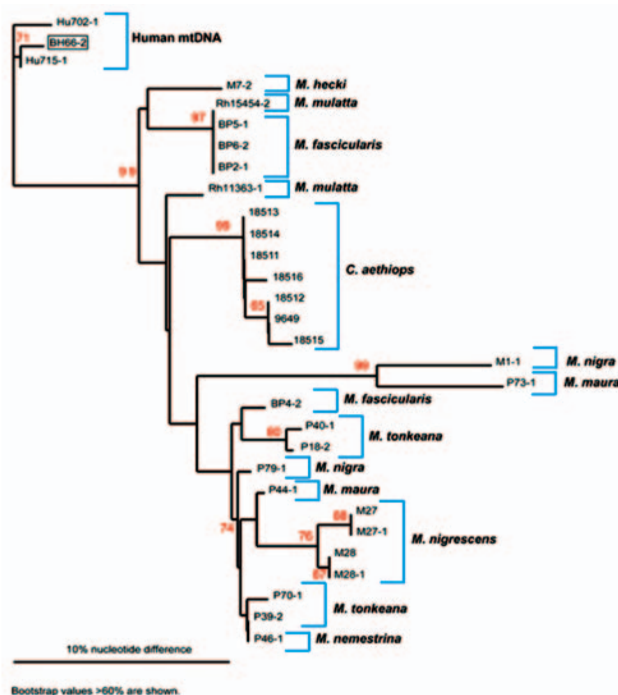

Figure 3. Phylogenetic analysis of mitochrondrial (mt) DNA from nonhuman primates and humans. mtDNA was amplified and sequenced from the simian foamy virus-infected person (BH66), 2 human controls (Hu702 and Hu715), *M. mulatta* (Rh15454, 18511,18512, 18513,18514,18515, 11363, 9649), *M. fascicularis* (BP2, 4, 5, 6), *M. nemestrina* (P46), *M. tonkeana* (P18,39,40), M. maura (P44, 73), *M. nigra* (P79, M1); *M. nigrescens* (M27, 28), and *M. hecki* (M7). The mtDNA tree was created with the neighbor-joining method with the Phylip program (DNAdist; Neighbor). Bootstrap replicates were 1,000. Bootstrap values were calculated by using Seqboot, DNAdist, Neighbor, and Consense (PHYLIP programs). Bootstrap values >60% are shown. The mtDNA tree was plotted in Treeview. This analysis suggests that BH66 was of human origin. Although the phylogenetic tree constructed with mtDNA from a variety of monkey samples can be used to distinguish human from monkey mtDNA, a large number of nuclear mtDNA sequences, have evolved as pseudogenes (36). These sequences can be highly divergent from mtDNA and resulted in some ambiguity as mtDNA amplified from several monkeys did not group with other members of the same species. Because of the nature and variability of these sequences, definitive conclusions about mtDNA phylogenies could not be determined; however, mtDNA trees were still useful for determining the origin of mtDNA material.

liver transplants in 1994, and SFV was detected in the blood and tissues of both at autopsy (39). More research in this area is needed. Moreover, little is known about the epizootiology of SFV among wild primates. Although SFV infection in humans has not culminated in any observable symptoms, SFV strains may differ in their capacity to infect or cause disease in humans. Finally, whether SFV can adapt in humans after transmission and potentially lead to disease needs to be examined.

Implications and Recommendations for Public Health

The recent SARS epidemic vividly demonstrates how the economic infrastructure and dense population of Asia facilitate the rapid international spread of disease. The combination of large primate reservoirs, prevalent human-primate contact, a growing immunocompromised population, and advanced infrastructure in Asia increases the likelihood of a primateborne zoonosis emerging on this continent.

The demonstration of SFV transmission in the context of a monkey temple in Bali points to a broad public health concern: other enzootic primate infectious agents may cross the species barrier and cause significant morbidity and mortality in human populations. Given our lack of knowledge of the effects of SFV, as well as the poorly defined risk of other primateborne zoonoses, steps should be taken to decrease the risks of cross-species transmission among the many persons who visit these sites. Previous data on both worker and visitor exposure to macaques at monkey temples suggest that human behaviors, especially the practice of feeding macaques, is a risk factor for being bitten or scratched (33,34). We have recommended that monkeys be fed only by specially trained personnel who minimize physical contact with monkeys. Such restrictions have been successfully employed at other monkey forests in Asia. For example, Singapore's Bukit Timah Nature Reserve has nearly 200 free-ranging macaques (*M. fascicularis*), and in 2002, an estimated 380,000 visitors made use of the reserve's trail system, yet contact between monkeys and visitors at Bukit Timah is rare. This finding may be ascribed to the park's policy, enforced by stiff fines, of prohibiting visitors from feeding macaques.

Conclusions

This study reports the first case of human SFV infection in Asia and also, for the first time, links natural transmission of SFV to a person to a specific population of primates. Our findings suggest that workers in and around monkey temples can become infected with SFV. By implication, visitors to monkey temples, especially those who are bitten by a macaque, may also be at risk for SFV infection. Because many visitors to monkey temples are international travelers, these findings have ramifications for the potential global spread of primateborne infectious agents. Demonstrating natural cross-species transmission in a context that does not involve hunting for bushmeat implies that other contexts of primate-human contact may also facilitate the transmission of simian pathogens. These data point to the need for further research into SFV transmission in other contexts, including pet ownership and performance animals, as well as in the diverse geographic areas where humans and primates come into contact. Such research will help describe the overall picture of the

emergence of primateborne pathogens such as HIV/SIV and will form a scientific basis for guiding policies and programs to deter the spread of emerging zoonotic pathogens.

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Dr. Jones-Engel is a research scientist at the University of Washington's National Primate Research Center. Her research focuses on bidirectional pathogen transmission between humans and primates in Asia and its implications for public health and primate conservation.

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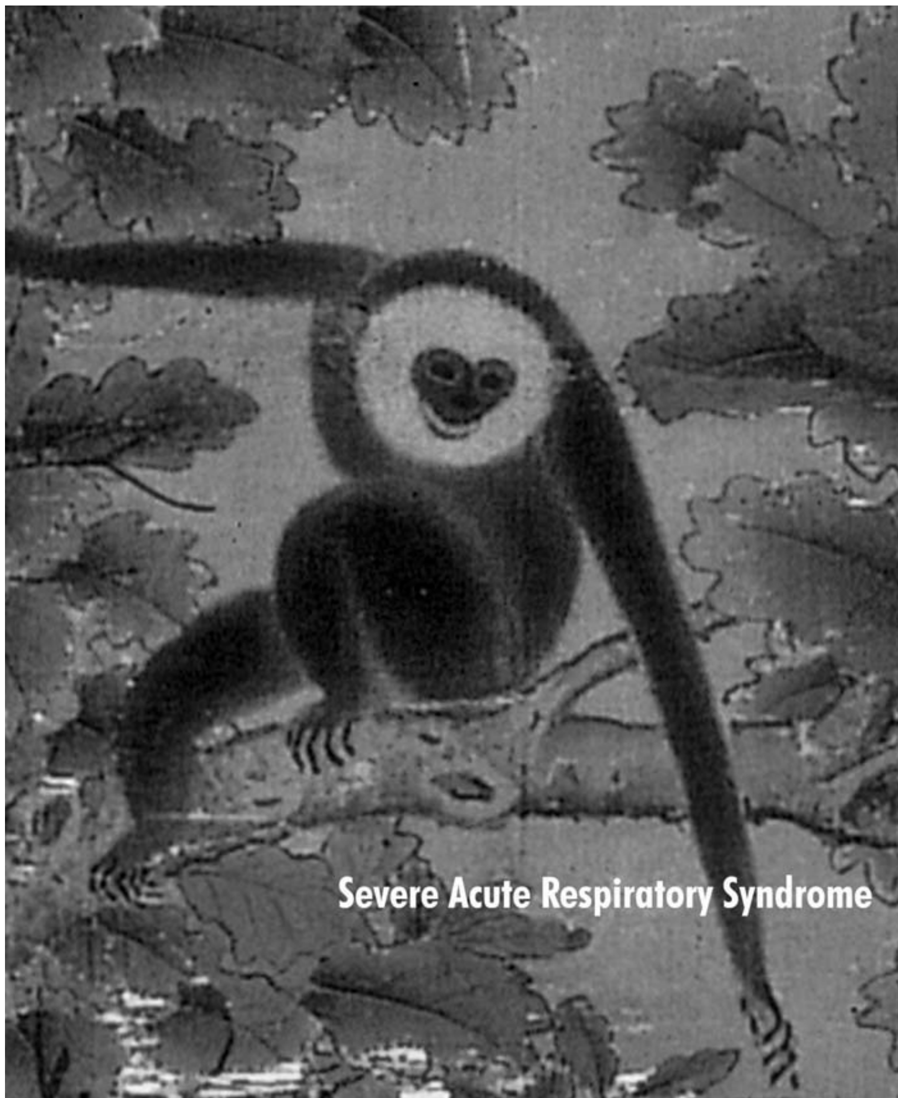
Address for correspondence: Lisa Jones-Engel, University of Washington, National Primate Research Center, HSB I-039, Box 357330, Seattle, WA 98195, USA; fax: 206-543-7959; email: jonesengel@bart.rprc.washington.edu



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Influenza A H5N1 Replication Sites in Humans

Mongkol Uiprasertkul,* Pilaipan Puthavathana,* Kantima Sangsiriwut,* Phisanu Pooruk,* Kanittar Srisook,* Malik Peiris,† John M. Nicholls,† Kulkanya Chokephaibulkit,* Nirun Vanprapar,* and Prasert Auewarakul*

Tissue tropism and pathogenesis of influenza A virus subtype H5N1 disease in humans is not well defined. In mammalian experimental models, H5N1 influenza is a disseminated disease. However, limited previous data from human autopsies have not shown evidence of virus dissemination beyond the lung. We investigated a patient with fatal H5N1 influenza. Viral RNA was detected by reverse transcription–polymerase chain reaction in lung, intestine, and spleen tissues, but positive-stranded viral RNA indicating virus replication was confined to the lung and intestine. Viral antigen was detected in pneumocytes by immunohistochemical tests. Tumor necrosis factor-α mRNA was seen in lung tissue. In contrast to disseminated infection documented in other mammals and birds, H5N1 viral replication in humans may be restricted to the lung and intestine, and the major site of H5N1 viral replication in the lung is the pneumocyte.

Highly pathogenic avian influenza virus H5N1 is the first avian influenza virus that was documented to cause respiratory disease and death in humans (1–3). In 2004, it caused widespread disease in poultry in Asia (4) and led to human disease in Thailand and Vietnam, with reported fatality rates of 66% and 80%, respectively (5,6). With the emergence of a second wave of disease outbreaks in poultry in Thailand, Vietnam, and Indonesia, this disease poses a global threat to human health (4). Additional human cases have been reported since August 2004. The high pathogenicity of this virus in avian species is associated with readily cleavable hemagglutinin (HA), but other amino acid residues in HA and neuraminidase have been recently reported to be involved in avian pathogenicity (7). In mice, some H5N1 virus strains cause a disseminated infection and death, and this phenotype was associated with specific amino acid substitutions in PB2 and the multibasic cleavage site in HA (8). Natural infection of felines with H5N1 viruses also resulted in disseminated infection (9). However, the pathogenesis of H5N1 disease in humans is more obscure. Despite severe and generalized clinical manifestations, the result of multiple organ dysfunction, previous limited autopsy data failed to show evidence of viral replication beyond the respiratory tract (10,11). The tissue tropism of the virus in humans has also not been clearly established by immunohistochemical analyses (10,11). The absence of detectable viral antigen–positive cells in previous reports may relate to the fact that the patients died during the late phase of the disease after intensive treatment with antiviral drugs. In this report, we investigated a case of fatal H5N1 disease in a child for tissue tropism caused by the virus in the lungs and other organs.

*Mahidol University, Bangkok, Thailand; and †University of Hong Kong, Hong Kong Special Administrative Region, People's Republic of China

Methods

Patient and Virologic Diagnosis

Detailed clinical description of the patient is reported elsewhere (12). The patient was a 6-year-old boy who had a progressive viral pneumonia that led to acute respiratory distress syndrome and death 17 days after onset of illness. He was initially treated with multiple broad-spectrum antimicrobial agents. Virologic diagnosis of H5N1 infection was made on day 7 of illness. After oseltamivir became available in Thailand, he was treated on day 15 of his illness with this agent until he died. He was also treated with methylprednisolone on day 15 until death and with granulocyte colony-stimulating factor for leukopenia from day 5 to day 10 of illness.

Virologic diagnosis was made by antigen detection, viral culture, and reverse transcription–polymerase chain

reaction (RT-PCR) on a nasopharyngeal wash specimen as described (12) and was confirmed by seroconversion of neutralizing antibody against H5N1 virus. The virus was identified as avian influenza virus (H5N1) by whole genome sequencing. The virus was an avian virus with no evidence of genetic reassortment with human influenza viruses. Phylogenetic analysis showed that the viral genomic sequence formed a distinct cluster with other H5N1 viruses isolated from humans and poultry in Thailand and Vietnam, but it was still related to the previously described H5N1 viruses circulating in southern China. As with other viruses isolated from poultry in Vietnam, Thailand, and Indonesia, this virus was also a genotype Z virus (4).

Pathologic Examination

Autopsy was carried out by standard techniques, and precautions were taken to minimize risk of transmission of infection. The tissue obtained was prepared for routine histologic analysis, and a portion was stored at –70°C for further study. For RT-PCR, fresh unfixed specimens were minced into small pieces in lysis buffer of an RNA extraction kit (RNA Wizard, Ambion, Austin TX, USA). Total RNA was then extracted according to the manufacturer's protocol. RNA was also extracted from paraffin-embedded tissues by sequential extraction with TriZol reagent (Invitrogen, Carlsbad, CA, USA) and the RNAEasy kit (Qiagen, Valencia, CA, USA) after digestion with proteinase K. RT-PCR for H5 was then conducted on extracted RNA by using One Step RT-PCR kit (Qiagen) with the H5 specific primer pairs H5F (5′-ACTCCAATGGGGGC-GATAAAC-3′) and H5R (5′-CAACGGCCTCAAACT-GAGTGT-3′) (13). An RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was done in parallel to control for the amount and quality of RNA as described (14). Strand-specific RT-PCR was carried out by a method similar to RT-PCR for viral RNA detection, except that only 1 primer was added at the reverse transcription step.

For immunohistochemical analysis, sections were deparaffinized and rehydrated. Antigenic site retrieval was accomplished by heating each slide in a microwave oven at 700 W for 15 min in 0.05 mol/L citric acid buffer, pH 6.0, and cooling for 20 min at room temperature. Endogenous peroxidase activity was blocked by incubating the slides in 0.3% H_2O_2 for 30 min at room temperature. Sections were incubated with 20% normal goat serum (Dako, Glostrup, Denmark) for 20 min at room temperature and then with an anti-influenza A nucleoprotein monoclonal antibody at a 1:100 dilution (B.V. European Veterinary Laboratory, Woerden, the Netherlands) for 1 h at room temperature. Slides were rinsed 3 times in 0.05 mol/L Tris-buffer, pH 7.6, 0.1% Tween 20 and incubated with horseradish peroxidase–conjugated goat anti-mouse immunoglobulin at a 1:400 dilution (Dako) for 30 min at room temperature. The slides were washed as above, developed with diaminobenzidine (Dako), and counterstained with hematoxylin. Some slides of lung tissue were double-stained with a monoclonal antibody (1:50 dilution) against surfactant (Dako).

Cytokine Expression

Tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-6 (IL-6) mRNA were detected in the extracted RNA by an RT-PCR with previously described primer pairs (15–17). Plasma levels of TNF-α and IFN-γ were measured by enzyme-linked immunosorbent assay (Pierce Endogen, Rockford, IL, USA) and compared with samples from 3 H3 influenza–infected patients and 5 healthy persons.

Results

The autopsy showed proliferative phase of diffuse alveolar damage, interstitial pneumonia, focal hemorrhage, and bronchiolitis. The pneumocytes showed reactive hyperplasia without virus-associated cytopathic changes (Figure 1). Superimposed infection by fungus, morphologically consistent with aspergillosis, was seen in some areas of the lung. The lymph nodes, spleen, and bone marrow showed slight histiocytic hyperplasia. No evidence of hemophagocytic activity was seen. The liver had mild fatty changes, activated Kupffer cells, and slight lymphoid infiltration in the portal areas. The brain was edematous, and small foci of necrosis were found. Intestines, kidneys, heart, and other organs showed no remarkable changes.

H5-specific RNA was detected in the lung, spleen, and small and large intestines by RT-PCR (Figure 2A). Control reactions without the reverse transcription step were negative, confirming that the PCR amplicon was not contaminated. The successful extractions of RNA from all organs were confirmed by the amplification of GAPDH mRNA (data not shown). We also tested whether the RNA was genomic RNA from virion or replicating RNA and mRNA from productively infected cells. To determine this, we conducted strand-specific RT-PCRs. Positive- and negative-stranded viral RNA was found in the lung, small intestines, and large intestines, but only negative-stranded RNA was detected in the spleen (Figure 2B). Because of the absence of positive-stranded RNA, which would serve as mRNA and the template for genome replication, we concluded that viral replication was absent or very low in the spleen and that the viral RNA detected in the spleen was probably nonreplicating virion RNA. No evidence of viral RNA was seen in the adrenal glands, brain, bone marrow, kidneys, liver, or pancreas. Results of the RT-PCR for viral RNA in plasma were also negative.

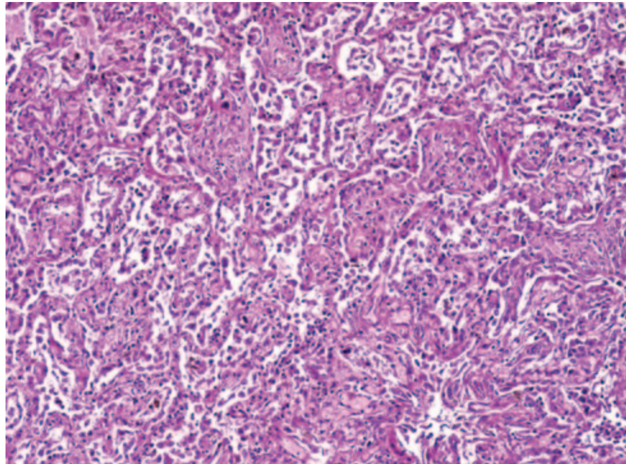

Figure 1. Microscopic shape of the lung showing proliferative phase of diffuse alveolar damage and interstitial pneumonia with reactive hyperplasia of pneumocytes (magnification x100).

Immunohistochemical analysis detected influenza A virus antigen-positive cells in lung tissue. The staining was localized in nuclei of alveoli-lining cells. Positive cells were found in 4 of 9 blocks of lung tissue. The shape and location of the antigen-positive cells indicated that they were type II pneumocytes. To confirm this, we used surfactant as a marker of type II pneumocyte (18). We double-stained slides from adjacent cuts with anti-influenza A and anti-surfactant monoclonal antibodies and showed that all influenza virus antigen–positive cells with nuclear staining showed intracytoplasmic staining of surfactant (Figure 3). Slides stained only with antibodies to surfactant showed intracytoplasmic, not intranuclear, staining. This finding confirmed that viral antigen–positive cells were type II pneumocytes. Although viral mRNA was present in the intestines, viral antigen was not detected in 4 blocks of tissue from the small and large intestines. In accordance with the absence of viral mRNA in other organs, viral antigen was not detected in those tissues. We also tested 2 blocks of tissue from the trachea. We did not detect any positive staining in columnar epithelium, which is the usual target for influenza virus infection in humans (19), which suggests that the virus targeted primarily lung tissue and not airway epithelium. Similarly, we did not find viral antigen in bronchiolar epithelium in the lung sections. Columnar epithelium in both the trachea and bronchiole was intact, thus providing adequate columnar epithelial cells for evaluation. The lack of pathologic changes is consistent with the absence of viral infection in these tissues.

The high pathogenicity of the H5N1 avian influenza virus has been proposed to be caused by induction of proinflammatory cytokines (20). Cytokine dysregulation could be the major cause of tissue damage in humans, especially in organs in which productive infection does not take place and cell damage cannot be accounted for by cytolytic viral infection. To investigate this aspect of viral pathogenesis, we tested for the presence of cytokine mRNA in tissues from various organs. We detected TNF-α mRNA in lung tissue, but not in other organs (intestines, stomach, spleen, brain, bone marrow, kidneys, liver, and pancreas) of this patient, or in lung tissue of patients who died of other causes (Figure 2C). We did not find any increase in levels of IFN-α, IFN-γ, and IL-6 mRNA in organs of this patient when compared with control tissues from healthy persons.

In accordance with previous reports showing the increased levels of serum cytokines, we found high levels

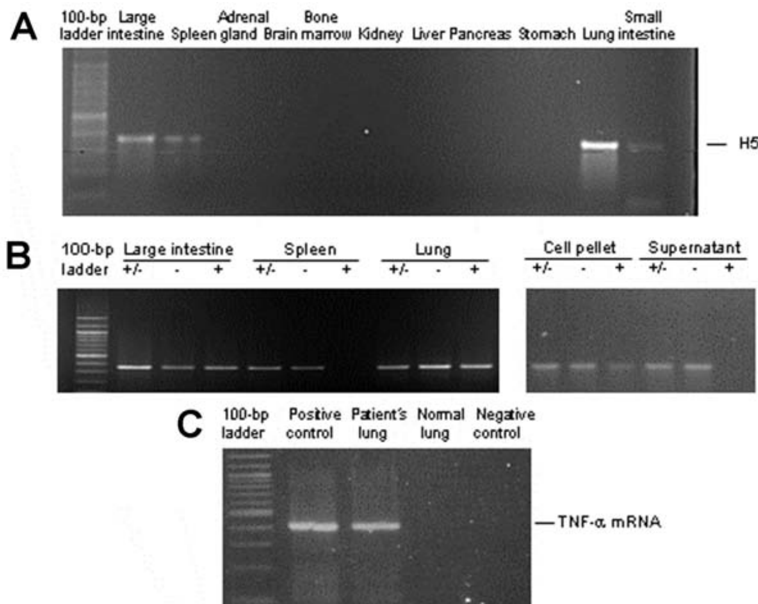

Figure 2. A) Detection of H5 influenza viral RNA in lungs, intestines, and spleen by reverse transcription-polymerase chain reaction (RT-PCR). B) Strand-specific RT-PCR detected positive-stranded viral RNA only in lungs and intestines but not in spleen. +/–, total RNA; –, negative-stranded RNA; +, positive-stranded RNA. RT-PCR products of an infected cell culture pellet and supernatant are shown as a control for proper amplification of the specific strands (lower panel). C) Tumor necrosis factor-α (TNF-α) mRNA was detected by RT-PCR only in lung tissue of the patient but not in lung tissue from a healthy control.

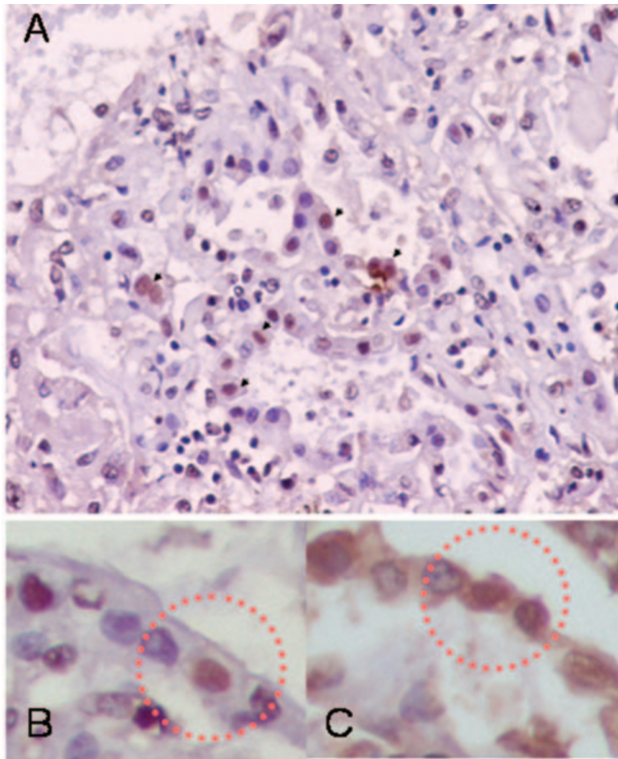

Figure 3. Immunohistochemical analysis showing influenza A antigen-specific staining in nuclei of cells lining the alveoli (A). To identify the cell type, slides from consecutive sections were stained with anti-influenza A antibody (B) and double-stained with anti-influenza A and antisurfactant antibodies (C). The sections were mapped, and the same area in each section was examined. Viral antigen-positive cells were stained both intranuclearly with anti-influenza antibody and intracytoplasmically with antisurfactant antibody, indicating that the viral antigen-positive cells were type II pneumocytes. Viral antigen-positive cell are marked by circles (magnification x400).

of interferon-induced protein 10 in serum samples collected on day 5 (37,000 pg/mL) and day 10 (4,300 pg/mL) of illness. These levels are comparable to those reported in H5N1-infected cases (10). However, we could not detect any significant levels of TNF-α and IFN-γ in these samples.

Discussion

Detailed autopsy data on patients with H5N1 disease are limited, and our data provide an insight into the pathogenesis of H5N1 virus in humans. We provide evidence that H5N1 viral replication is not confined to the respiratory tract but may also occur in the gastrointestinal tract. However, a fecal sample was not available for detection of virus. Although viral RNA was detected in the spleen, no evidence of viral replication was seen in this organ. The patient was treated with an antiviral agent for 2 days before death, which could have lowered the level of viral replication in the examined tissues. However, we still found viral mRNA in lungs and intestines, indicating that the viral replication was still ongoing. Viral replication in lungs and intestines was greater than in other sites. Our data agree with previous reports of human cases and cases in experimentally infected macaques, which also suggest that H5N1 influenza virus replication takes place predominantly in the lungs (10,11,21). We also show that type II pneumocytes, not columnar tracheal epithelial cells, are the major site of H5N1 viral replication in humans. Type II pneumocytes are surfactant-producing, alveolar epithelial cells and progenitor cells of both type I and type II cells. This cell type has been shown to contain sialic acid in newborn human lung (22). Whether the availability of the receptor alone determined the site of H5N1 infection needs further investigation.

Infection of the gastrointestinal tract by avian influenza virus, including H5N1, is common in avian species (23,24). However, involvement of the gastrointestinal tract in H1 and H3 influenza infection is rare in humans (25). A patient with H5N1 influenza virus infection was reported to have diarrhea as the initial symptom, which raises the question of whether the gastrointestinal tract may is another site of viral replication and shedding, similar to its function in avian species (26). In another recent report of a patient with a fatal H5N1 infection and severe diarrhea and encephalitis in Vietnam, the virus was found in a rectal swab (27). Our data confirm that H5N1 influenza virus replication can occur in the gastrointestinal tract even in the absence of diarrhea. However, we do not know the extent of viral shedding in stool in this patient. The absence of pathologic changes in the intestine, despite the viral replication, is intriguing.

The absence of viral antigen in the trachea indicated that the upper airway is probably not an active site of the viral replication. This finding is in marked contrast to the circumstances with human influenza, in which the upper respiratory tract and the tracheal and bronchial epithelium are primarily targeted (19). The predilection of H5N1 influenza virus for the lower airways may explain why detecting virus in upper airway specimens for diagnosis of H5N1 infection in humans is difficult (1). This finding also implies that specimens from the lower respiratory tract, such as sputum or bronchoalveolar lavage, would have a higher sensitivity for viral detection than an upper respiratory specimen, such as nasopharyngeal aspirates or throat swab specimens. Our data showing the absence of viral antigen in columnar epithelial cells contrast with a recently published report that H5N1 viral replication took place selectively in ciliated bronchial epithelial cells in an in vitro culture model (28). Whether this result was due to properties of specific viral strains or a difference attributable to the in vitro model needs further clarification.

In contrast to previous reports (10,11), we did not find prominent hemophagocytosis in any of the organs. The presence of hemophagocytosis in these reports supports the cytokine dysregulation model of pathogenesis. Whether the young age of our patient or prior treatment with immunosuppressive corticosteroids affected this manifestation in this patient is unclear.

TNF-α mRNA was detectable in the lungs but not in other tissues. This finding is in agreement with previous observations that H5N1 viruses isolated from human disease hyperinduce production of cytokines, most prominently TNF-α, in cultured human macrophages in vitro (20,29). The simultaneous presence of viral mRNA and cytokine mRNA in the same organ suggests a direct induction of cytokine in productively infected cells. In accordance with this finding, we also found that the viral isolate from this patient induced a high level of TNF-α production from primary human macrophages, which is comparable to the previously described strains (M. Peiris, unpub. data). However, we could not rule out the possibility that the superimposed fungal infection might have played a role in the induction of TNF-α in this patient. The hemagglutinin of the 1918 pandemic H1N1 influenza virus also appears to hyperinduce production of cytokines and chemokines in a mouse model of disease (30).

In conclusion, we have documented that H5N1 disease in humans is one in which viral replication is restricted to the respiratory and gastrointestinal tracts. The multiorgan dysfunction observed in human H5N1 disease, despite the apparent confinement of infection to the lungs, has remained an enigma. The hypothesis that cytokine dysregulation may contribute to the pathogenesis of severe H5N1 disease (20) remains a possibility. An understanding of the pathogenesis of human H5N1 disease is important in preparing for a pandemic.

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Dr. Uiprasertkul is a pathologist at the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok. His primary research interest is the pathogenesis of viral diseases.

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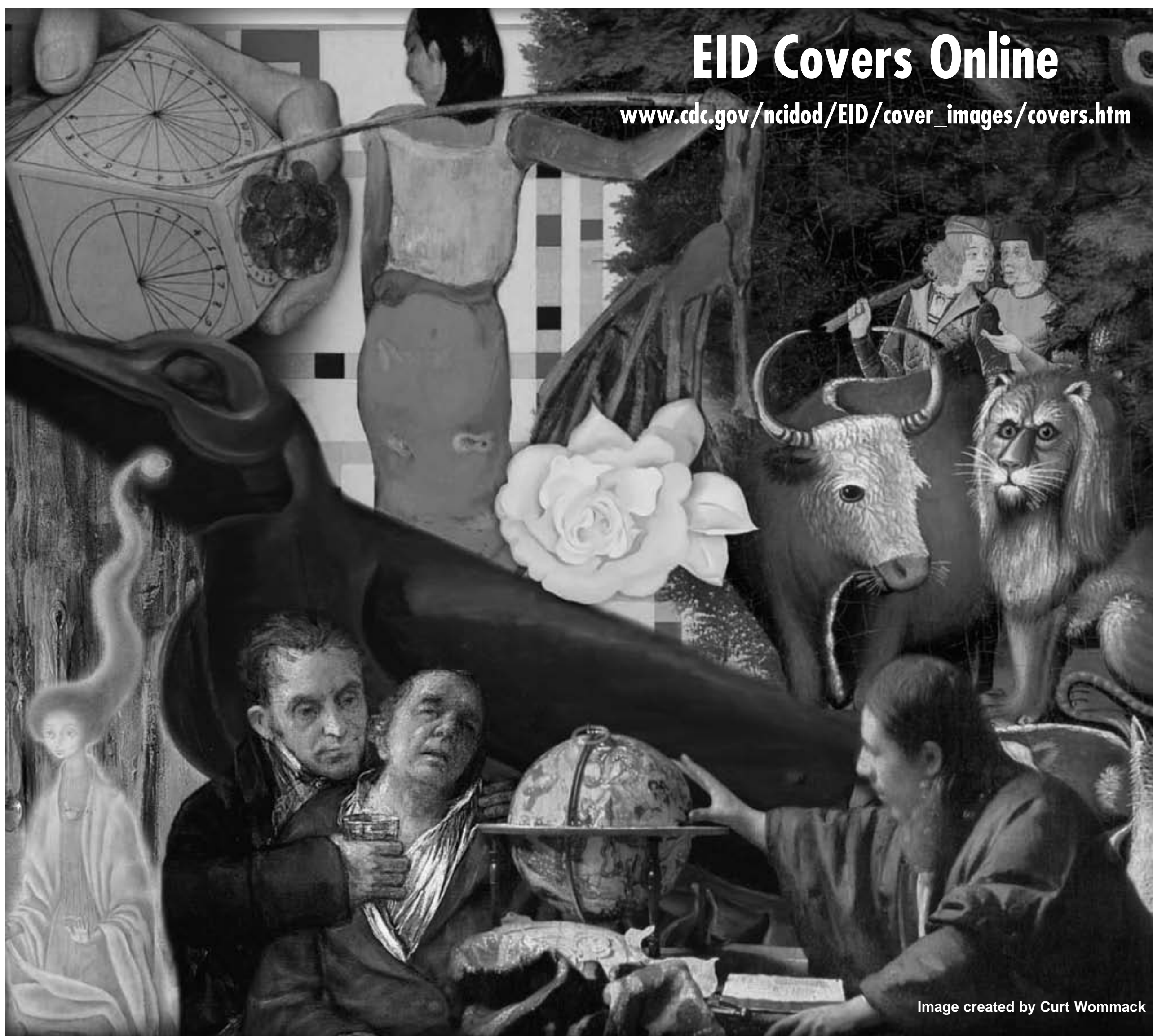
Address for correspondence: Prasert Auewarakul, Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, 2 Prannok Rd, Bangkok 10700, Thailand; fax: 66-2-418-4148; email: sipaw@mahidol.ac.th



Nipah Virus in Lyle's Flying Foxes, Cambodia

Jean-Marc Reynes,* Dorian Counor,* Sivuth Ong,* Caroline Faure,† Vansay Seng,* Sophie Molia,* Joe Walston,‡ Marie Claude Georges-Courbot,† Vincent Deubel,† and Jean-Louis Sarthou*

We conducted a survey in Cambodia in 2000 on henipavirus infection among several bat species, including flying foxes, and persons exposed to these animals. Among 1,072 bat serum samples tested by enzyme-linked immunosorbent assay, antibodies reactive to Nipah virus (NiV) antigen were detected only in *Pteropus lylei* species; *Cynopterus sphinx*, *Hipposideros larvatus*, *Scotophilus kuhlii*, *Chaerephon plicata*, *Taphozous melanopogon*, and *T. theobaldi* species were negative. Seroneutralization applied on a subset of 156 serum samples confirmed these results. None of the 8 human serum samples was NiV seropositive with the seroneutralization test. One virus isolate exhibiting cytopathic effect with syncytia was obtained from 769 urine samples collected at roosts of *P. lylei* specimens. Partial molecular characterization of this isolate demonstrated that it was closely related to NiV. These results strengthen the hypothesis that flying foxes could be the natural host of NiV. Surveillance of human cases should be implemented.

The new genus *Henipavirus* contains 2 species, Hendra virus (HeV) and Nipah virus (NiV), in the subfamily of the *Paramyxovirinae*, which also includes *Respirovirus*, *Rubulavirus*, *Morbillivirus*, and *Avulavirus* genera (1). HeV and NiV have emerged within the last 10 years and have been shown to be highly pathogenic in animals and humans. HeV was identified in 1994 in Brisbane during an outbreak of acute respiratory syndrome in 21 horses, of which 14 died. Two humans were also affected with the same syndrome, and 1 died (2,3). A second outbreak that occurred 1 year later ≈1,000 km north of Brisbane caused the death of 2 horses and their owner. Severe encephalitis followed by mild meningitis affected the owner 1 year after his exposure (4). NiV was discovered in Malaysia during a major outbreak of acute respiratory syndrome in pigs in 1998; it caused severe acute encephalitis among >283 pig farmers (with 109 deaths) and among 35 abattoir workers in Singapore (5,6). In 2001, 2003, and 2004, this virus emerged in Bangladesh; 4 outbreaks were reported with high death rates (32%–75%) (7). Person-to-person transmission was suspected during the last outbreak (8), which raised concerns about human infection from an epizootic or deliberate release of this highly virulent virus. Serologic and virologic investigations suggested that these 2 viruses shared *Pteropus* bats or flying foxes as natural host reservoirs (9–12). Furthermore, 2 other paramyxoviruses belonging to the genus *Rubulavirus* have been associated with flying foxes: Menangle virus, isolated in 1997 from pigs in Australia (13), and Tioman virus, isolated in 1999 from fruit bats in Malaysia (14).

Distribution of the henipaviruses is restricted so far to Australia, Malaysia, and Bangladesh. The distribution of flying foxes (≈58 species), considered as natural hosts of these viruses, extends from the east coast of Africa across south and Southeast Asia, east to the Philippines and Pacific islands, and south to Australia (15). It could be conjectured that henipavirus or similar viruses occur in flying foxes elsewhere and could emerge as a human pathogen. Three species belonging to the genus *Pteropus* (*Pteropus lylei*, *P. hypomelanus,* and *P. vampyrus*) have been identified in Cambodia (15), and antibodies to NiV-like virus have recently been detected in Lyle's Flying Fox (*P. lylei*) (16).

We conducted a survey in Cambodia, initiated in 2000, on henipavirus infection among several bat species, especially flying foxes, and persons exposed to these animals. Here, we confirm the presence of NiV antibodies and report NiV isolation and characterization from Lyle's bats in Cambodia.

*Institut Pasteur du Cambodge, Phnom Penh, Cambodia; †Institut Pasteur, Lyon, France; and ‡Wildlife Conservation Society, Phnom Penh, Cambodia

Materials and Methods

Sample Collection

A total of 1,303 bats were sampled from 35 locations in 9 Cambodian provinces, from September 2000 to May 2001. Of these, 467 came from restaurants in Phnom Penh and belonged to the species *P. lylei*. Blood samples were collected at the restaurants when the animals were killed for meat. The other 836 animals were captured in 9 provinces at roosts by hand and with hand nets, or along flyways by night with mist nets or harp traps. Anesthetized captured animals were euthanized by cardiac blood puncture. These animals belonged to 16 species representing 6 of the 7 bat families known from Cambodia (17). Serum samples from 1,072 animals were taken for serologic investigation.

Under 6 roosts of flying foxes (where individual bats from the 2 species *P. lylei* and *P. vampyrus* could live together), urine samples were collected from June 2003 to August 2004 on plastic sheets, following a published procedure (18). The roosts, of 400 to 600 flying foxes each, were located in Phnom Penh, Battambang, Kampong Cham, Kandal, Prey Veng, and Siem Reap Provinces (Figure 1). The roosts were visited 4 times, when the animals were present (the roosts are deserted from December to May). No roost of Variable Flying Foxes (*P. hypomelanus*) could be located. A total of 769 urine samples stored at –80°C were available for virologic investigation.

Serum samples were obtained in January 2001 from 8 persons who gave their oral consent and who were exposed to NiV-seropositive Lyle's Flying Fox bats. Four men worked in restaurants where bats are eaten and handled the animals; 4 women worked at the same place and slaughtered and cooked the bats.

Serologic Tests

Bat sera were first screened for antibodies against NiV by enzyme-linked immunosorbent assay (ELISA), as described (19). Antigens were prepared in the biosafety level Laboratory Jean Merieux in Lyon from Vero E6 cells infected with NiV and inactivated by γ irradiation as described (20). Sera were diluted 1:100. Peroxidase-labeled protein A/G (Pierce, Rockford, IL, USA) was used as conjugate. Three negative control serum samples and 1 positive control sample were included in each run.

Seroneutralization tests were carried out under biosafety level 4 conditions. Serum samples were heated for 30 min at 56°C and then were titrated with 3 dilutions (1:10; 1:20, and 1:40) in a 96-well microtiter plate (4 wells per dilution). Equal volume of NiV (100 50% tissue culture infective dose [$TCID_{50}$] in 50 µL) was added to all sera, and the plate was incubated for 1 h at 37°C. Vero E6 cells (5×10^4 in 100 µL) were added to all wells, and the plates were incubated at 37°C for 4 days in a CO_2 chamber at 37°C. Characteristic NiV cytopathic effect (CPE) showing large syncytia was observed under a microscope in each well. The number of virus-positive wells was confirmed after fixation in 10% formaldehyde for 1 h and amidoschwartz staining for 30 min. Positive (anti-NiV serum obtained from a convalescent-phase specimen) and negative sera were included for controls in each plate. Toxicity of the sera for Vero cells was observed on uninfected cells in the presence of 1:10 serum dilution. The neutralization titer of each sample was defined as the last dilution in which at least half of the monolayer was intact ($TCID_{50}$).

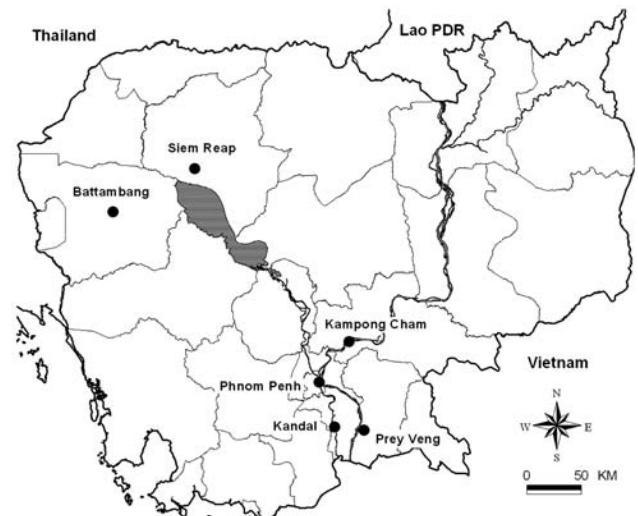


Figure 1. Bat urine sampling site. Cambodia, June 2003–July 2004.

Virus Isolation and Identification

Subconfluent Vero E6 cells (ATCC CRL-1586) in flasks of 25 cm^2 with filter caps were inoculated with 500 µL of viral transport medium containing the urine-impregnated cotton swab. The cell cultures were placed in a CO_2 incubator at 37°C and examined daily for CPE such as formation of multinucleated giant cells. If a CPE was observed, supernatants and cells were collected separately and frozen at –80°C.

RNA was extracted from supernatants by using QIAMP viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's procedure. Identification of paramyxoviruses or NiV were performed with reverse transcriptase–polymerase chain reaction (RT-PCR) with specific primers for the phosphoprotein (P) gene of *Paramyxoviridae* or specific primers for the nucleoprotein (N) gene of NiV (21).

Molecular Characterization

To amplify and sequence the coding domains of the N

and glycoprotein (G) genes, primers were designed by using Primer3 software (22), according to the published nucleotide sequences of NiV, with RNA extracted from supernatants of positive isolates. Reverse transcription and amplification with these primers were conducted. The amplified products were observed after electrophoresis on a 1% agarose gel with ethidium bromide staining; then purified, amplified products were sequenced with the dye termination cycle sequencing technique (Genome Express Company, Meylan, France). Sequences sent by the company were verified and aligned by using ClustalW program version 1.83 (23). Phylogenetic analysis was conducted with PHYLIP sequence analysis package (version 3.6 alpha 3) (24).

Results

Serologic Testing

Results of ELISAs for antibodies reactive with NiV antigens in 1,072 bats serum samples are shown in the Table. A positive signal was identified only in *P. lylei*. Fifty (10.9%) of the 458 tested samples from this species were positive.

Forty-three of the 50 ELISA-positive bat serum samples were available for confirmation by serum neutralization test. One produced cell toxicity, 1 was negative (titer <1:10), and 41 were positive. Neutralization at dilutions of 1:10, 1:20, and ≥1:40 was found in 1, 11, and 29 serum specimens, respectively. A subset (n = 156) of the 1,022 ELISA-negative serum specimens were tested by seroneutralization; 5 produced cell toxicity. Six among 43 ELISA-negative serum samples from *P. lylei* were positive (4 with a titer = 1:10 and 2 with a titer = 1:20). The 108 ELISA-negative serum samples from other captured species, *Cynopterus sphinx* (n = 15), *Hipposideros larvatus* (n = 15), *S. kuhlii* (n = 15), *Chaerephon plicata* (n = 27), *Taphozous melanopogon* (n = 12), and *T. theobaldi* (n = 24), were negative. None of the human sera were NiV seropositive with the seroneutralization test.

Virus Isolation and Molecular Characterization

Attempts to isolate viruses producing a CPE were positive for 2 samples among the 769 urine samples. These 2 samples, CSUR381 and CSUR382, were collected consecutively on the same morning (April 23, 2004) at the same roost of *P. lylei* specimens, located in a pagoda in the village of Bay Damran in Battambang Province. CPE showing syncytia was detected 6 days after injection into Vero E6 cells.

RT-PCR performed on RNA extracted from infected cell supernatants using specific primers for the P gene of *Paramyxoviridae*, as well as specific primers for the N gene of NiV, produced PCR products of the expected size

Table. Seum samples from 1,072 bats, Cambodia, reactive by enzyme-linked immunosorbent assay to Nipah virus, September 2000–May 2001

Species names	Negative	Positive
Frugivorous		
Cynopterus brachyotis	1	0
Cynopterus sphinx	68	0
Macroglossus sobrinus	1	0
Pteropus lylei	408	50
Roussetus leschenaulti	15	0
Insectivorous		0
Chaerephon plicata	153	0
Hipposideros armiger	1	0
H. larvatus	81	0
H. pomona	2	0
Murina cyclotis	1	0
Rhinolophus acuminatus	2	0
R. luctus	1	0
Scotophilus kuhlii	98	0
Taphozous melanopogon	69	0
T. theobaldi	121	0
Total	1,022	50

(138 bp and 228 bp, respectively). When the 2 partial N region products from the 2 Cambodian strains were sequenced, 2 identical nucleotide sequences were found that shared 97.4% homology to that of the NiV strain isolated in Malaysia, a finding that suggests that the Cambodian isolates belonged to the NiV species.

Further molecular characterization was achieved when the N and G genes of CSUR381 and CSUR382 isolates were amplified and sequenced. The nucleotide sequences of the 2 isolates were identical. The N sequence of CSUR381 was compared with the N sequences of the Malaysian NiV strains available in November 2004 in GenBank (AF212302, NC-0027281 derived from AF212303, AY029768, and AY029767 from human isolates; AJ564621, AJ564622, AJ564623, and AJ627196 from pig isolates; and AF376747 from a *P. hypomelanus* isolate). We observed that the Cambodian N nucleotide sequence shared 98% identity with the Malaysian AF21232 N sequence (32 nucleotides among 1,599 were divergent). The identity was 98.7% (525/532) for the N amino acid sequences (see online Appendix Figure 1, available at http://www.cdc.gov/ncidod/EID/vol11no07/ 04-1350-appG1.htm). All the changes of the 532 amino acid (aa) N protein occurred at the carboxyl terminus, with the following mutations: I429V, G432E, N457D, I502T, E511G, L518P, and A521T. The G sequence of the Cambodian CSUR381 strain was also compared to the available G sequences of the Malaysian NiV isolates. The percentage of nucleotide homology was 98.2% (32 nucleotide changes among 1,809), and the percentage of amino acid homology was 98.5% (593/602). Amino acid changes were N5S, V24I, R248K, G327D, I408V, V426I, L470Q, N478S, and N481D (see online Appendix Figure 2, available at

http://www.cdc.gov/ncidod/EID/vol11no07/ 04-1350-appG2.htm). These results confirmed that the Cambodian isolates are closely related to the other NiV isolates.

Phylogenetic analysis using parsimony method (24) and different N sequences of viruses of the *Paramyxovirinae* subfamily confirmed that the Cambodian isolates and the Malaysian NiV isolates were significantly similar and that the Cambodian strains probably belong to the NiV species (Figure 2). Analyses performed by using other methods (neighbor joining and maximum likelihood) or using the G sequences reached the same conclusion (data not shown). The N and G nucleotide sequences of the isolate CSUR381 were deposited in GenBank (accession nos. AY858110 and AY858111).

Discussion

Our serologic study confirms the presence in Cambodia of antibodies to a NiV-like virus among Lyle's Flying Foxes (*P. lylei*), as reported by Olson et al. in 2002 (16). We could not detect antibodies against NiV among bats belonging to other genera, including insectivorous bats. Some Malaysian specimens of 2 frugivorous species (*Cynopterus brachyotis* and *Eonycteris spelaea*) and 1 insectivorous species (*S. kuhlii*) have been found carrying neutralizing antibodies to NiV in Malaysia. These specimens were collected soon after an outbreak, when the virus was expected to circulate with high prevalence (10). Data on bat infection at sites of outbreaks in Bangladesh are limited, although antibodies against NiV have been detected in the Indian Flying Fox, *P. giganteus*, a species possibly conspecific with *P. vampyrus* (7). Although our sample is not representative of the bat population in Cambodia, our results strengthen the hypothesis that flying foxes could be the natural host of NiV. The results of both ELISA and neutralization tests used on this convenient panel of sera gave a relative sensitivity of 87% (41/47) and specificity of 99% (145/146). These findings are in accordance with the ELISA performance observed in other studies (25).

This description of NiV is the first in Cambodia. NiV has been isolated in 2 other countries. The NiV isolate obtained from *P. lylei* is the first isolate obtained from a *Pteropus* species different from *P. hypomelanus*, the first NiV-infected species in Malaysia. Investigation in Bangladesh, where the NiV outbreak occurred, did not detect any virus strains among bats; antibodies to Nipah-like virus were detected among specimens belonging to the species *P. giganteus* (7). Our results indicate that further henipavirus-related infection can be expected within the area of distribution of flying foxes and that NiV could emerge within this wide area.

Identification of the isolate was performed by molecular characterization on the N and G coding domains located at the 2 ends of structural proteins genes. We observed a higher amino acid diversity at the carboxyl terminus of the N protein between the Malaysian and Cambodian NiV isolates. The N central domain (from aa 171 to aa 383) of the members of the *Paramyxovirinae* family is the most conserved region, which seems to be involved in its inter-

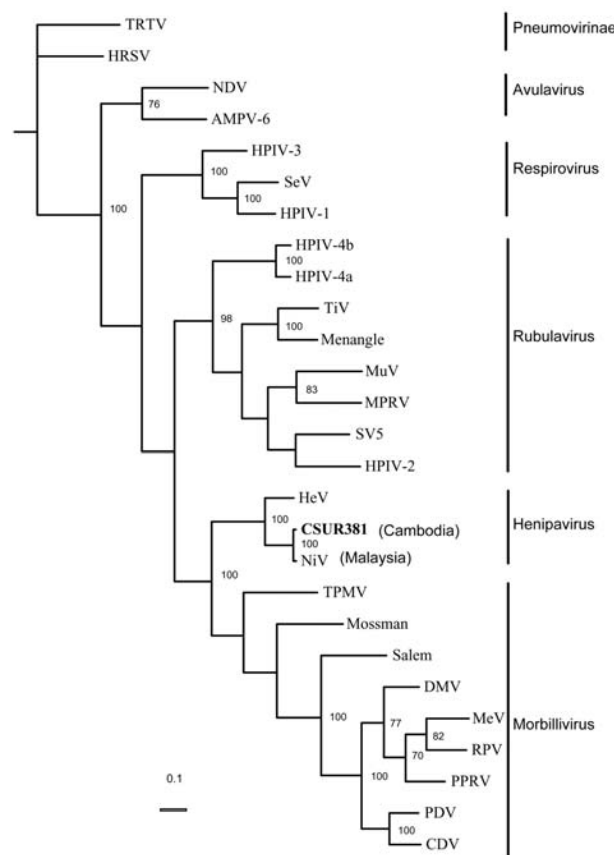


Figure 2. Phylogenetic analysis of the 1,599 nucleotides of the N gene coding domain sequence from the Nipah virus Cambodian isolate, members of the subfamily *Paramyxovirinae*, and 2 species of the subfamily *Pneumovirinae* used as outgroups. GenBank accessions numbers used are as follows: APMV-6: Avian paramyxovirus 6, AY029299; CDV: Canine distemper virus, AF014953; DMV, Dolphin distemper virus, X75961; HeV: Hendra virus, AF017149; HPIV-1: Human parainfluenza virus 1, D011070; HPIV-2: Human parainfluenza virus 2, M55320; HPIV-3: Human parainfluenza virus 3, D10025; HPIV4a: Parainfluenza virus type 4A, M32982; HPIV4b: Parainfluenza virus type 4B, M32983; HRSV: Human respiratory syncytial virus, X00001; MeV: Measles virus, K01711; MPRV: Mapuera virus, X85128; Menangle: Menangle virus, AF326114; Mossman: Mossman virus, AY286409; MuV: Mumps virus, D86172; NDV: Newcastle disease virus, AF064091; NiV: Nipah virus, AF212302; PDV: Phocid distemper virus, X75717; PPRV, Peste des Petits ruminants virus, X74443; RPV: Rinder pest virus, X68311; Salem: Salem virus, AF237881; SeV: Sendai virus, X00087; SV5: Simian virus 5, M81442; TiV: Tioman virus, AF298895; TPMV: Tupaia paramyxovirus, AF079780; TRTV: Turkey Rhinotracheitis virus, AY640317. Significant bootstrap values (≥70%) are indicated. The phylogram was generated by parsimony method and analyzing 100 bootstrap replicates.

actions with other functional proteins, such as the phosphoprotein (P) and the polymerase (L). Moreover the N proteins of paramyxoviruses possess 3 highly conserved regions (26). The first region (QXW(I,V)XXXK(A,C)XT, X representing any amino acid) is located between aa 171 and aa 181, the second region (FXXT(I,L)(R,K)u (G,A)(L,I,V)XT, where u represents an aromatic amino acid) from aa 267 to aa 277, and the third region (FXXXXYPXXuSuAMG) from aa 322 to aa 336. All 3 regions were conserved in the sequence of the Cambodian NiV strain. Conversely, the C-terminal part of N (from aa 384 to aa 532) overlaps antigenic epitopes and is the most variable domain (26). However, no change occurred in the 29 aa C terminal region (aa 468–496) involved in the binding to the P protein (27). Lastly, the N481D change observed in the Cambodian NiV G protein induced the disappearance of 1 of the 8 N-linked glycosylation sites of the protein (28).

Only 2 NiV isolates were obtained from 6 roosts in 6 provinces that were investigated. This finding is not unusual with these pathogens and bats: no NiV strain was isolated from Variable Flying Foxes (*P. hypomelanus*) and Large Flying Foxes (*P. vampyrus*) during the 1999 Malaysian outbreak (11). Two NiV isolates were obtained in the postoutbreak period (August 1999–June 2000) from 263 urine samples of Variable Flying Fox bats (12) and, to our knowledge, no isolate from bats in Bangladesh has been reported. Furthermore, only 3 isolates of HeV were obtained from 652 bats tissue samples collected around the epidemic sites in Australia (9). The presence of antibodies in roosts from Kompong Cham Province suggests that the virus could be widely present in the country. However, the low rate of NiV recovery from *P. lylei*, when compared to the high number of NiV antibody–positive bats (10.9%), suggests that the virus may not be sustained lifelong in animals or that it may remain at low titers and occasionally emerges and is released in biologic fluids. The 8 persons exposed to Lyle's Flying Fox bats, some of which were NiV-seropositive, were NiV-seronegative. Thus, the animals may not have been carrying the virus during their captivity.

This is the first time an NiV has been isolated in a country where no outbreak has been reported. This situation requires strategies to manage this pathogen and to prevent an outbreak. Recommendations should be made to the population in areas where flying foxes' roosts are identified to minimize exposure to them. A national hospital-based surveillance of acute encephalitis should be implemented; in the upcoming months, such surveillance will be instituted in the area where the virus was isolated. Surveillance of respiratory syndrome among pigs has to be estimated, as intensive pig farming is not the rule in the area. The potential of the Cambodian NiV isolate to cause fatal encephalitis in hamsters will also be investigated (29). Lastly, ecologic studies, including the dynamics of flying fox populations and their relationships with NiV, should be considered for a better understanding of its transmission and maintenance among these populations.

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Dr. Reynes is a veterinarian and a medical virologist. He is chief of the virology unit at the Institut Pasteur du Cambodge. His research interests include arboviruses, HIV, rabies, and emerging infectious diseases.

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Address for correspondence: Jean-Marc Reynes, Institut Pasteur de Madagascar, BP 1274, Ambatofotsikely, 101 Antananarivo, République de Madagascar; fax: 261-20-22-407-17; email: jmreynes@pasteur.mg

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Leptospirosis in Germany, 1962–2003

Andreas Jansen,* Irene Schöneberg,* Christina Frank,* Katharina Alpers,* Thomas Schneider,† and Klaus Stark*

Epidemiologic trends of human leptospirosis in Germany were investigated by analyzing national surveillance data from 1962 to 2003 and by conducting a questionnaire-based survey from 1997 to 2000. After a steady decrease of leptospirosis incidence from 1962 to 1997, surveillance data indicate an increase in disease incidence to 0.06 per 100,000 (1998–2003). Of 102 laboratory-confirmed cases in humans from 1997 to 2000, 30% were related to occupational exposures. Recreational exposures were reported in 30% (including traveling abroad in 16%), whereas residential exposure accounted for 37% of the cases. Direct contact with animals, mostly rats and dogs, was observed in 31% of the cases. We conclude that recent changes in transmission patterns of leptospirosis, partially caused by an expanding rat population and the resurgence of canine leptospirosis, may facilitate the spread of the disease in temperate countries like Germany. Preventive measures should be adapted to the changing epidemiology of leptospirosis.

Leptospirosis is a zoonotic disease of global importance, caused by spirochetes of the genus *Leptospira*. Based on antigenic relatedness, human pathogenic *Leptospira interrogans* strains have been differentiated into >200 serovars, which are classified into 24 serogroups (1). A variety of wild and domestic animals form the natural reservoir for pathogenic leptospires. Some serovars are associated with specific hosts, like *L. interrogans* serovar Icterohaemorrhagiae, which is primarily harbored by rats. Transmission to humans results from exposure to the urine of infected animals either by direct contact or through contaminated soil or water. Leptospirosis has recently been classified as a reemerging infectious disease, particularly in tropical and subtropical regions (2,3). Though the infection is considered rare in developed countries, low but persisting rates of autochthonous illness and death exist. Due to its nonspecific clinical features, a lack of awareness among physicians, and difficulties in isolating the organism and serologic testing, the incidence of leptospirosis is likely underestimated (4). Recent seroepidemiologic studies suggest that the incidence of leptospirosis in urban centers of some industrialized countries is remarkably high, with seroprevalence of leptospiral antibodies reaching levels ≥30% in selected urban populations (5–9). In Germany, the documented history of leptospirosis began in 1886 when Adolf Weil first described the severe form of the disease (10). Large agriculture-associated epidemics with several thousands of infected persons occurred from the 1920s to the 1960s (11). Although human leptospirosis is endemic in most industrialized countries worldwide (12), few systematic large-scale reports provide detailed information on the epidemiologic characteristics of the disease in temperate regions, including data on the relationship between humans and potential reservoir hosts and urban versus rural settings. However, this information is essential to implement appropriate control measures. The objective of this study is to describe current trends of laboratory-confirmed human leptospirosis in Germany, with special focus on modes of transmission, implicated reservoir hosts, prevalent serovars, and regional distribution of the infection.

Methods

Leptospirosis was a reportable disease in the former German Democratic Republic after 1958. In the former Federal Republic of Germany, cases of leptospirosis were reportable under the Federal Communicable Disease Act since 1962. After the reunification in 1990, the 2 reporting systems were combined. Hospitals and outpatient facilities reported cases of leptospirosis to the local health departments and through state health authorities to the Robert

*Robert Koch Institute, Berlin, Germany; and †Charité, Berlin, Germany

Koch Institute. In 2001, an improved surveillance system for mandatory case reporting of infectious diseases was implemented in Germany. Under the Infectious Disease Control Act, German laboratories notify local public health authorities of positive test results for leptospirosis. Local authorities then obtain additional information on the patient and electronically transfer the data through state authorities to the central database at the Robert Koch Institute. Additionally, official statistical data on deaths due to leptospirosis that were documented on death certificates (1962–2002) were obtained from the Federal Office of Statistics (http://www.gbe-bund.de).

From 1997 to 2000, data on demographics (age, sex, and residence), onset of symptoms, country in which infection was contracted, possible exposure risks, infecting serovars, mortality, and causes of death were evaluated by standardized questionnaires sent to local health departments for every reported case of leptospirosis. From 2001 to 2003, data on demographics, onset of symptoms, and country in which the infection was contracted were transmitted to the Robert Koch Institute as required by the newly implemented Infectious Disease Control Act. A case definition, demanding both clinical signs of leptospirosis (at a minimum, acute febrile illness) and laboratory confirmation (positive culture, polymerase chain reaction, seroconversion, or a single significant antibody titer as demonstrated by enzyme-linked immunosorbent assay, complement fixation testing, or microscopic agglutination testing), was applied to all reported infections from 1997 to 2003 (13). Identification of the presumptive infecting leptospiral serovar was performed either by culture (7 cases) or microscopic agglutination testing (38 cases) in laboratories with personnel who were experienced in these types of procedures. In 7 cases, a serovar-specific complement fixation test was conducted. All cases with documented cross-reactivity between different leptospiral serovars were excluded from the analysis.

To assess temporal trends, mean annual incidences were calculated for 6-year intervals from 1962 to 2003, referenced to the population of year 4 of each interval. Incidences for each of the 16 German states from 1997 to 2003 were calculated similarly. Statistical tests for trend were performed with the extension of the Mantel-Haenszel chi-square test. Data were analyzed with EpiInfo, version 6.04d (Centers for Disease Control and Prevention, Atlanta, GA, USA). The Mann-Whitney test was used to compare quantitative variables. A p value of <0.05 was considered to be significant.

Results

From 1962 to 2003, a total of 2,694 cases of leptospirosis were reported. During this 41-year period, the number of human leptospirosis cases generally declined, with a maximum of 147 cases in 1974 and a minimum of 25 cases in both 1991 and 1997 (median 59 cases) (Figure 1). The mean annual incidence decreased from 0.11 per 100,000 population from 1962 to1967 to the lowest observed incidence of 0.04 per 100,000 population ($p<0.001$) from 1992 to 1997. From the period 1992–1997 to the period 1998–2003, incidence increased to 0.06 per 100,000 population ($p = 0.001$). There were 234 deaths caused by leptospirosis from 1962 to 2002 (overall case-fatality ratio 9%). The highest fatality ratio was 12% in the period 1968–1973, while the lowest fatality ratio was 5% in the period 1998–2002. Five minor outbreaks of leptospirosis were reported: 5 cases in 1963, 14 cases in 1966, 3 cases in 1996, 2 cases in 1999, and 2 cases in 2002.

From 1997 to 2003, a total of 317 cases of leptospirosis were reported. Of these, 269 (85%) fulfilled the case definition and were included in the analysis. Information on age, sex, and area of residence was obtained for all 269 case-patients. Seventy-eight percent were men; 80% were 30–69 years of age (median age 45 years, range 1 month to 80 years) (Figure 2). Age-specific incidence was highest for persons 60–69 years of age, with a mean annual incidence of 0.1 per 100,000, and lowest incidence for children <10 years of age with 0.004 per 100,000 (Figure 2). Incidence was highest in the northeastern states of Germany (Mecklenburg-Western Pomerania with 0.12 per 100,000 population, followed by Brandenburg with 0.09 per 100,000 population) (Figure 3).

Presumed date and country in which the infection was contracted were reported for 248 cases (92%). In 39 cases (16%), the infection was likely contracted outside Germany. Of these, 13 patients named destinations inside Europe (4 cases from France, 2 cases each from Greece, Poland, and Hungary, and 1 case each from Norway, Croatia, and Bulgaria). Non-European countries were named in 26 cases, including 3 cases each from the Dominican Republic, Mexico, and Thailand, 2 cases each from Cuba and Argentina, and 1 case each from Vietnam,

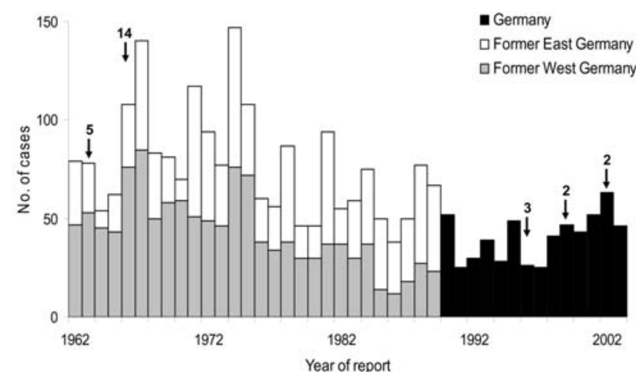

Figure 1. Number of reported leptospirosis cases in Germany, 1962–2003. Arrows indicate outbreaks and number of cases affected.

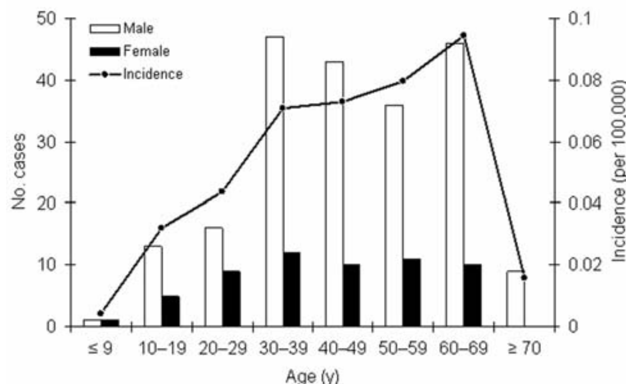

Figure 2. Leptospirosis cases 1997–2003, distribution by age and sex (N = 269).

Jamaica, Australia, Argentina, Bahamas, Costa Rica, India, Indonesia, Japan, Venezuela, Kenya, Turkey, and the Russian Federation. In most cases, onset of disease was August–November (61% in total, 62% of autochthonous cases, and 54% of imported cases) (Figure 4).

From 1997 to 2000, local health authorities returned 140 completed questionnaires for 156 reported case-patients (response rate of 90%). Of these, 14 (10%) did not fulfill the case definition and were excluded from analysis. Information on type of exposure was available for 102 (81%) of the remaining 126 cases. Occupational and recreational exposure risks were both present in 30% of the patients. Accidental exposures (falls into water, animal bites) were documented in 3%, while 37% of the exposures occurred through residential activities (Table). Traveling abroad was the single most frequent documented exposure risk (16%). Among patients with a known travel history, exposure to fresh water during travel was reported in 6 (38%) of 16 cases.

A similar distribution of exposure types was found in all age groups, except in persons $\geq$60 years of age, where residential exposure risks (60%) clearly dominated over recreational (16%) and occupational exposures (24%). Contact with domestic animals was reported in 12 (12%) of 102 cases, including dogs (7 cases), rabbits (2 cases), a mouse, a pet rat, and a horse (1 case each). Other animal exposures were reported in 20 cases (20%), including the following animals: rats (13 cases), cattle (3 cases), mice (2 cases), a pig, and a sheep (1 case).

Of the 102 patients with reported exposure risks, 24 (24%) lived in urban areas with 12 of those likely being infected in the cities, and 12 likely being infected during excursions or holidays outside the cities. Genuine urban cases were mainly related to residential exposures (gardening and associated activities in 6 cases) and owning dogs (4 cases). Accidental falls into urban waterways were reported in 2 cases.

A definite serovar identification was recorded in 52 (41%) of the 126 cases, while cross-reactivity between several serovars was observed in 22 cases (18%). In 52 cases (41%), no serovar differentiation was available. Serovars in the Icterohaemorrhagiae serogroup were the most common, accounting for 32 (62%) of the 52 identified isolates, followed by *L. interrogans* serovars Canicola and Grippotyphosa, each accounting for 5 (9%) of the identified isolates. Two cases (4%) with *L. interrogans* serovar Bataviae and 3 cases (6%) with serovar Pomona were identified, while serovars Sejroe, Hardjo, Hebdomadis, Bratislava, and Australis were found in single cases.

Of the 126 reported patients from 1997 to 2000, 10 patients died (90% men, case fatality 8%). The median age was 44 years of age for survivors and 60 years of age for nonsurvivors (p<0.03). Causes of death were multiple organ failure (including the triad of fever, renal failure, and jaundice) in 6 cases and massive pulmonary hemorrhage, intracerebral hemorrhage, cardiopulmonary failure, and acute respiratory distress syndrome in single cases. One of the nonsurvivors lived in an urban area and contracted the disease from an accidental fall into a waterway in

Figure 3. Regional distribution of leptospirosis in Germany, 1997–2003. Incidence per 100,000 population.

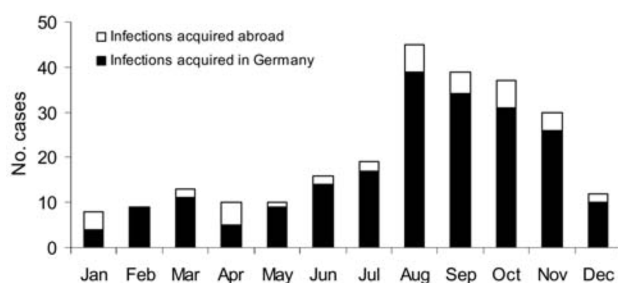

Figure 4. Seasonal trends of leptospirosis, 1997–2003 (N = 248).

Hamburg. Further exposure risks in the fatal cases included traveling abroad (3 patients), farming (2 patients), contact to a dog (1 patient), working in an abattoir (1 patient), mud or water around home (1 patient), and sewage work (1 patient). Serovars were identified in 3 of these cases (Bataviae, Icterohaemorrhagiae, and Canicola).

Discussion

After a steady decrease in the incidence of leptospirosis in Germany from 1962 to 1997, national surveillance data suggest an increased frequency of the disease in recent years. Though part of the increase in case reports since 2001 may reflect the acceptance of the improved surveillance system in Germany among laboratories that identify leptospirosis, the increase was already observable in the preceding surveillance system and likely reflects a true rise in cases. Presumably, the number of reported cases from Germany represents the tip of the iceberg, since less severe and nonspecific clinical manifestations of leptospirosis frequently go unrecognized, and several studies indicate that subclinical infection is common worldwide (14,15). As to the documented history of leptospirosis in Germany, this possible reemergence appears to be associated with distinct changes in the epidemiologic characteristics of the disease that affect routes of transmission, host animals, prevalent serovars, population at risk, and regional distribution.

In Germany, leptospirosis historically has been associated with agricultural exposure risks and mainly restricted to rural environments (11). *L. interrogans* serovar Grippotyphosa represented the most common infecting serovar, usually transmitted by field voles (*Microtus apodemus*) and European hamsters (*Cricetus cricetus*) as reservoir animals (16). Along with ecologic and structural changes, including mechanization of agriculture and improvements in sanitation, the relevance of leptospirosis in the rural economy diminished in the 1960s (17). A similar reduction in disease activity was reported from other industrialized countries, where leptospirosis has traditionally been a common occupational disease among agricultural workers (18,19). At present, some of the traditional occupations described as risks for leptospirosis (e.g., livestock farmers and sewage and abattoir workers) are still found in Germany. However, traveling abroad is the single most important exposure risk identified in the recent study. This finding is consistent with a general trend observed in several other countries, especially among the growing numbers of participants in adventure travel and water sports in exotic locations (20,21). Along with expanding long distance travel, the increase of leptospirosis as an emerging disease in tropical areas will necessarily be reflected in travelers returning from these regions.

However, the risk for exposure due to traveling is not restricted to the tropics, as 33% of the patients with a travel history contracted the infection while visiting European countries. France was unexpectedly the most common reported travel destination in this study. With an incidence of 0.44 per 100,000 population in 2000 (22), France has one of the highest reported incidences of leptospirosis in Western Europe. Leisure activities associated with exposure to fresh water (in particular, canoeing) were identified as a risk factor for the disease, and travelers to France with an interest in water sports should be aware of this association (23).

Table. Type of exposure for 102 confirmed cases of leptospirosis in Germany, 1997–2000

Type of exposure	No. cases (%)
Occupational	31 (30)
Park ranger	2
Sewer worker	6
Veterinarian	1
Zoo director	1
Abattoir worker	3
Miner	1
Military	1
Waste management	1
Construction work	3
Farmer with livestock	5
Farmer without livestock	7
Recreational	31 (30)
Fishing	5
Swimming	6
Camping	1
Traveling	16
Canoeing	3
Accidental	3 (3)
Fall in water	2
Bite	1
Residential	37 (37)
Keeping animals as pets	11
Rats/mice around home	4
Mud/water around home	5
Working on private canal, ditch, pond	9
Living on farm	2
Gardening or yard work	6
Total	102 (100)

In at least 12% of the reported cases, leptospirosis was contracted in urban areas and was primarily related to residential activities, like gardening and working on private ponds. Though urban leptospirosis has been established as a threat for certain occupational groups in Germany (17), residential exposures represent a new category of urban risk factors. The emergence of sporadic urban leptospirosis has recently been reported from several other industrialized countries, including the United States and Israel (9,18,24), and our data support the view of leptospirosis as an underrecognized urban health problem in industrialized countries (9). Since leptospirosis is still perceived as a zoonotic disease mainly restricted to occupational exposures and rural areas, clinicians may fail to consider the disease in urban settings (9).

In both urban and rural environments, we found domestic animals in general, and dogs in particular, implicated as putative reservoir hosts. The growing importance of canine leptospirosis in Germany is underlined by a seroepidemiologic study conducted from 1999 to 2002. Of 3,671 canine serum samples, 29.8% showed high antibody titers against several leptospiral serovars not covered by customary vaccines, including *L. interrogans* serovars Bratislava in 4.8% and Saxkoebing in 3.2% (25). In the United States and Canada, a rising prevalence of the disease in dogs has been observed since the early 1990s (26,27), and canine leptospirosis has recently been recognized as a reemerging zoonosis (28). Aside from the possible transmission of leptospirosis through direct contact with urine of infected pets, dogs and other domestic animals kept in gardens may also increase the likelihood of concomitant rodent infestation, as rodents are attracted by food and shelter because of the presence of domestic animals (29). The chance of rat infestations in such habitats is likely to rise, corresponding to a general increase of rat populations in both urban and rural environments noted in Germany (German Federal Environmental Agency, unpub. data).

In addition to dogs and rodents, a variety of domestic and wild animals were identified as potential reservoirs for leptospires in Germany. A nationwide seroepidemiologic study of almost 31,000 different animals showed the prevalence for *Leptospira* species was 14.4% in sheep and 4.5% in horses (30). In cattle, seroprevalence for *L. interrogans* serovar Hardjo was 10.3% and for *L. interrogans* serovar Saxkoebing was 11.3% (31). Leptospiral antibodies were also detected in foxes (2% of 1,253 animals) and wild boars (24% of 245 animals) (32,33), thus demonstrating the extensive distribution of *Leptospira* species in the German fauna and, consequently, the necessity for epidemiologic studies in defined occupational groups with close contact to these animals.

As to the distribution of infecting serovars, we found that *L. interrogans* serovar Icterohaemorrhagiae had replaced *L. interrogans* serovar Grippotyphosa as the most prevalent serovar in Germany. Although serologic identification of specific serovars by using microscopic agglutination testing can only give a broad idea of the common serovars in a certain population and has to be interpreted cautiously (34), our data indicate a principal shift from agriculture-related serovars to those primarily related to nonoccupational modes of transmission, with rats as their principal vector. This change has also been observed in other European countries (19,35). Underlying changes in transmission modes may also explain the shift to higher ages of infection that we observed when compared to ages noted in other studies. In several countries with predominant recreational or occupational modes of transmission, leptospirosis mainly affects persons 20–50 years of age (35–37). Although many patients in Germany are in this age group, the highest age-specific incidence was found in persons 60–69 years of age. This shift may be explained by the observed predominance of residential exposure risks, which are largely independent of physical fitness or ability to work. The seasonal distribution of leptospirosis in Germany was found to be similar when compared to the past and to recent studies from other temperate countries (19,35), which likely reflects the sensitivity of the bacteria to climatic variation and the seasonality of transmission patterns, irrespective of the predominant serovars.

Leptospirosis was traditionally most common in German states with a pronounced rural economy, including Bavaria, Schleswig-Holstein, Lower Saxony, and Saxony (11). From 1997 to 2003, however, the highest incidence of leptospirosis was found in the eastern states of Germany, particularly in Mecklenburg-Western Pomerania. The corresponding region of the former German Democratic Republic showed a high number of human leptospirosis caused by *L. interrogans* serovar Icterohaemorrhagiae already occurring in the 1950s. It has been suggested that this high incidence was correlated with large populations of Norway rats, which were attracted by the fishing industries (38). In addition, Mecklenburg-Western Pomerania has the highest proportion of surface fresh water among the German states (excluding the city states) and is particularly rich in lakes, canals, and branched rivers, which are frequently used for recreational activities by local persons and persons from nearby cities on weekend trips. Although this region was barely affected by agriculture-related leptospirosis in the past, it apparently provides favorable conditions for rat infestations and consequent transmission of *L. interrogans* serovar Icterohaemorrhagiae to humans by means of freshwater exposure.

Consistent with the literature, we found that nonsurvivors were significantly older than survivors (39). With respect to the increased proportion of elderly persons

affected by the disease in Germany, this finding is of major importance, since it suggests a possible increase in fatal courses of leptospirosis in the future. Finally, we documented the first fatal case of leptospirosis due to massive pulmonary hemorrhage observed in Germany. Pulmonary hemorrhage has increasingly been recognized as a grave manifestation of leptospirosis in several regions of Asia and South and Central America, but it has rarely been observed in central Europe (1). Since severe pulmonary involvement with leptospirosis is actually underrecognized in tropical regions of high endemicity (40), clinicians in industrialized countries are even more likely not to consider or recognize pulmonary complications in leptospirosis. Although the high case-fatality ratio demonstrated in our study may be biased toward including only the most severely ill or hospitalized patients, insufficient knowledge of the protean clinical manifestations of leptospirosis among clinicians clearly facilitates misdiagnosis and leads to delays in antimicrobial drug therapy of this potentially treatable infection.

Conclusion

In conclusion, this study suggests that the conditions for the transmission of leptospirosis to humans have become more favorable in recent years. Increasing international travel as well as the rising popularity of recreational and residential activities linked to freshwater exposures may explain some of the observed increase. In addition, the expansion of rat populations and the resurgence of leptospirosis in dogs potentially promote establishing an endemic substrate for the spread of the disease, especially in urban regions. Since these changes in transmission biology appear not to be restricted to Germany, leptospirosis should at least be an issue of increased concern in other industrialized countries. Reliable baseline information as provided by continuing surveillance and case reporting are indispensable for implementing appropriate preventive measures. In Germany, efforts should focus on rodent control and region-specific travel advice. In addition, developing and using canine vaccines that cover the prevalent serovars should be encouraged.

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Dr. Jansen is a physician and infectious disease epidemiologist at the Robert Koch Institute, Berlin, Germany. His research interests include zoonoses, enteric infections, and tropical infections.

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Address for correspondence: Andreas Jansen, Robert Koch Institute, Department of Infectious Diseases Epidemiology, Seestrasse 10, D-13353 Berlin, Germany; fax: 49-30-4547-3533; email: JansenA@rki.de

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Survey of Tickborne Infections in Denmark

Sigurdur Skarphédinsson,* Per M. Jensen,† and Kåre Kristiansen‡

We conducted a study of the distribution and prevalence of tickborne infections in Denmark by using roe deer as sentinels. Blood samples from 237 roe deer were collected during the 2002–2003 hunting season. Overall, 36.6% of deer were *Borrelia* seropositive, while 95.6% were *Anaplasma phagocytophilum* positive; all animals were negative for *Bartonella quintana* and *B. henselae* by indirect immunofluorescence assay. When a hemagglutination-inhibition test was used, 8.7% of deer were found positive for tickborne encephalitis (TBE)-complex virus. A total of 42.6% were found positive by polymerase chain reaction (PCR) for *A. phagocytophilum* with significant seasonal variation. All were PCR negative for *Rickettsia helvetica*. PCR and sequencing also showed a novel bacterium in roe deer previously only found in ticks. The study showed that the emerging pathogen *A. phagocytophilum* is widely distributed and that a marked shift has occurred in the distribution of TBE-complex virus in Denmark. This finding supports studies that predict alterations in distribution due to climatic changes.

A change in the distribution and frequency of vectorborne infections may be among the first signs of the effect of global climatic change on human health (1). Tickborne infections are the most frequent human vectorborne infections in Europe; the incidence of many of these diseases has been on the rise, and new infections have emerged. In Denmark, borreliosis is known to be endemic and widespread, while tickborne encephalitis (TBE) has been found only on the island of Bornholm. In recent years, human serosurveys have indicated that granulocytic ehrlichiosis caused by *Anaplasma phagocytophilum* is also found in Denmark (2); however, the distribution is unclear. Studies on *Ixodes ricinus* ticks have revealed the existence of other potential pathogens, among them *Rickettsia helvetica* and *Bartonella* spp. (3, S. Skarphédinsson et al., unpub. data). At the same time, increase in the incidence of TBE has been noted in neighboring countries like Germany, Poland, Lithuania, and Sweden (4). Changes in the distribution of TBE in Europe have been suggested to be related to climatic changes, and new foci have been predicted, some within Denmark (5). However, the role of climatic changes is unclear, and increased surveillance is needed to elucidate this in further detail.

*University of Southern Denmark, Odense, Denmark; †The Royal Veterinary and Agricultural University, Fredriksberg, Denmark; and ‡Medical Public Health Office, Rønne, Denmark

I. ricinus is the main vector of tickborne infections in Europe and the dominant tick in Denmark (>90%). Roe deer (*Capreolus capreolus*), an important host for *I. ricinus* ticks, have been used as sentinel animals to monitor tickborne infections in several studies, 2 of which have been performed in Denmark. In 1963, Freundt published a survey of TBE (6), and in 1994 Webster and Frandsen evaluated the seroprevalence of *Borrelia burgdorferi* in deer (7). In light of increasing tick density observed, as well as the finding of new pathogens in Denmark, reassessment is indicated (8). The aim of this study was to assess the seroprevalence and geographic distribution of TBE-complex virus, *Borrelia burgdorferi*, *A. phagocytophilum*, *Bartonella quintana*, and *Bartonella henselae* by using roe deer as natural sentinels; at the same time, we evaluated prevalence of infection with *A. phagocytophilum* and *R. helvetica* by using polymerase chain reaction (PCR).

Materials and Methods

Sample Collection and Serologic Testing

State forest rangers from the 25 Danish state forest districts were invited to participate during the regular hunting season of 2002 (May 15–July 15, 2002 [summer], and October 1, 2002–January 15, 2003 [fall]). They were asked to obtain blood samples from roe deer by cardiac puncture or from the thoracic cavity when dressing freshly killed animals in the field. For each animal, sex, age, and degree of tick infestation was also noted. Blood collection kits were distributed to all state forest districts by mail, and

blood samples were sent by mail to the laboratory. Because of limited amounts of material received from some districts, not all samples were available for all serologic tests.

B. burgdorferi serologic tests were performed by using indirect immunofluorescence assay (IFA). *B. burgdorferi* strain DK 6 was used as an antigen, and the conjugate was fluorescein isothiocyanate (FITC)-labeled rabbit anti-deer immunoglobulin (Ig) G (Kierkegaard & Perry Laboratories Inc, Guildford, UK). The cutoff point was 1:64.

B. henselae and *B. quintana* serologic tests were performed by using IFA. Slides coated with Vero cells infected with *B. henselae* and *B. quintana* (Focus Technologies, Cypress, CA, USA) were used as antigen, and the conjugate was FITC-labeled rabbit anti-deer IgG (Kierkegaard & Perry Laboratories Inc). The cutoff point was 1:64.

A. phagocytophilum serologic testing was performed by using IFA. Slides with HL-60 cells infected with a human isolate of *A. phagocytophilum* (Focus Technologies) were used as antigen, and FITC-labeled rabbit anti-deer IgG was used as conjugate. The cutoff point was 1:128. Slides were considered borderline positive when a definite-but-dim fluorescence equal to that observed for the positive control at its reference endpoint titer was found. Moderate-to-intense fluorescence of the morula was graded positive.

TBE-complex virus serologic testing was performed at the Institute of Virology, University of Vienna, Austria. Samples were first tested by using hemagglutination inhibition (HI) test. Positive samples were verified by using neutralization test.

DNA Extraction, PCR, and Sequencing

Total DNA was extracted from blood samples by using a QIAamp Blood kit (Qiagen, Albertslund, Denmark), according to manufacturer's instructions. Amplifications were performed in a Perkin Elmer GeneAmp PCR system 9600 (Perkin-Elmer Corp, Norwalk, CT, USA), and real-time PCR was performed in Bio-Rad iCycler iQ Quantitative thermal cycler (Bio-Rad, Herlev, Denmark). DNA amplification was done in a 25-µL reaction volume by using ReddyMix PCR Master Mix (ABgene, Epsom, United Kingdom), with 5 µL of sample DNA in each reaction. Cycling conditions included initial 3 min of denaturation at 96°C, followed by 39 cycles, each consisting of 15 s denaturation at 96°C, 15 s annealing at 58°C, and 15 s extension at 72°C. These 39 cycles were followed by an extension period of 3 min at 72°C. Real-time PCR was performed by using HotStartTaq Master Mix kit (Qiagen). Reaction volume was 25 µL with 5 µL sample DNA. Cycling conditions for *Anaplasma* real-time PCR included an initial activation of Taq polymerase at 95°C for 10 min, followed by 40 cycles, each consisting of 15 s denaturation at 95°C followed by 1 min annealing-extension at 60°C. *R. helvetica* real-time PCR included an initial activation of Taq polymerase at 95°C for 10 min followed by 45 cycles of 30 s denaturation at 95°C followed by 45 s annealing-extension at 52°C. Specimen processing, PCR setup, and amplification and detection procedures were all conducted in separate areas to minimize the potential for cross-contamination.

Anaplasma infection was detected with primers that specifically target the 16S rRNA gene of the *A. phagocytophilia* genogroup (9): forward primer 5′-GGTACCY-ACAGAAGAAGTCC and reverse primer 5′-TAGCAC-TCATCGTTTACAGC. PCR products were detected on 3% agarose gels stained with ethidium bromide. If samples were found positive, a second real-time PCR was performed. Primers specific for the *A. phagocytophilum msp*2 gene were used (10): ApMSP2f (5′-ATGGAAGGTAGT-GTTGGTTATGGTATT), ApMSP2r (5′-TTGGTCTTGA-AGCGCTCGTA), and a TaqMan probe ApMSP2p-HEX (5′-TGGTGCCAGGGTTGAGCTTGAGATTG). Primers were labeled at the 5′ and 3′ ends with hexachloro-6-carboxy-fluorescein (HEX) and Black Hole Quencher (BHQ), respectively.

R. helvetica infection was detected with primers that specifically target the 23S rRNA gene of *R. helvetica*: Rhf (5′-ATAGGGAGGAATTTGAAGGA) and Rhr (5′-GGTAATTTGTACGTCGATCC) and a TaqMan probe Rhpr-TR (5′-CGGAACACAGAACCGTAGCG). Primers were labeled at the 5′ and 3′ ends with Texas Red and BHQ, respectively. For quality control, negative and positive controls were included each time a PCR was performed.

PCR products used for DNA sequencing were purified by using GFX PCR DNA and Gel Band purification kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). For DNA sequencing reaction, ABI Prism Big Dye Terminator v3.0 kit was used (Perkin Elmer, Applied Biosystems Division). Removal of unincorporated dye terminators was performed by using DyeEx kit (Qiagen) Samples were run on ABI 373A sequencer (Perkin Elmer, Applied Biosystems Division). Sequences were compared with public domain database by using the Blast software. Sequences obtained are available in GenBank under accession nos. AY776165, AY776166, and AY776167.

Statistical Analysis

Data were analyzed by using STATA 8.2 (StataCorp LP, College Station, TX, USA). For analysis of seroepidemiologic results, Fisher exact test and the Mantel-Haenszel method were used. Values of $p<0.05$ were considered significant.

Results

A total of 237 blood samples from roe deer were collected from 22 of 25 state forest districts (Figure 1). Blood samples from 112 animals were collected in the summer

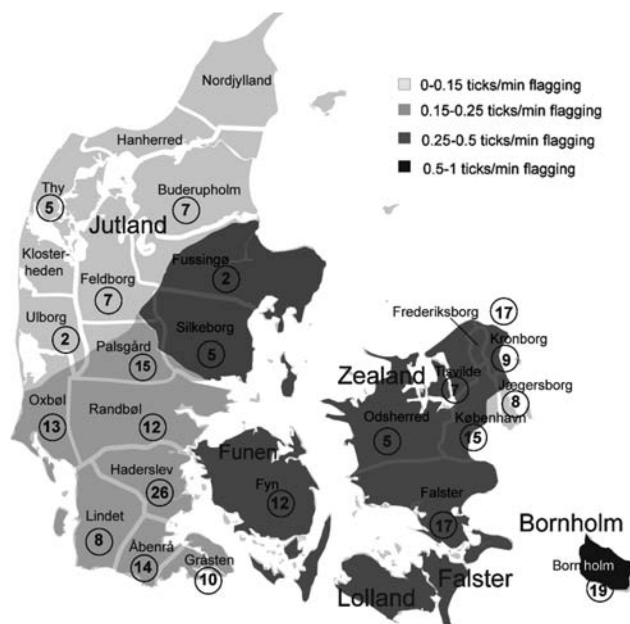

Figure 1. Geographic distribution of roe deer samples collected. The map shows the location of the 25 state forest districts in Denmark. Numbers in circles indicate number of samples collected in each district. Three districts, Klosterheden, Hanherred, and Nordjylland, did not submit samples. Also shown is the approximate density of *Ixodes ricinus* ticks in Denmark, redrawn from (11) as shaded areas. Flagging is the technique of collecting ticks by moving a piece of fabric mounted on a stick through the vegetation for a given period of time.

hunting season and from 125 animals in the fall hunting season. The mean age of roe deer was 2.9 years (median 2.5, range 0.5–10 years, Table 1); 60% were bucks, 36% were does, 4% were not defined. This skewed distribution is due to the fact that hunters are only allowed to hunt bucks during the first hunting period in the summer. Only 7% of the roe deer were heavily infested with ticks (defined as >100 engorged ticks/deer); 63% of these animals came from Zealand (p = 0.026).

B. burgdorferi and *Bartonella* spp.

Seroprevalence was assessed on 227 samples. Of these, 83 (36.6%) had positive results on *Borrelia* IFA, but of these 23 (10%) were borderline positive. Significant regional difference was found in *Borrelia* seropositivity when the mainland of Jutland was compared to the islands (Funen, Zealand, Lolland Falster, Bornholm; Figure 1), 27.1% versus 46.7% (p = 0.003). *Borrelia*-positive roe deer were found in 19 of 22 forest districts evaluated. Three districts in the northern part of Jutland were negative (Fussingø, Thy, and Ulborg; Figure 1), but only 9 blood samples came from these 3 districts. No significant differences in *Borrelia* antibody prevalence were found for sex, age, and season.

B. henselae and *B. quintana* seroprevalence was assessed on 227 samples. All samples were seronegative (95% confidence interval [CI] 0%–1.6%)

A. phagocytophilum

Seroprevalence was assessed on 227 samples. Of these, 217 (95.6%) were positive and 19 were borderline positive (8%). No significant difference in seroprevalence was seen for age, sex, season, or region. Among 237 samples tested by PCR for *A. phagocytophilum*, 101 (42.6%) were positive in both 16S rRNA and *msp*2 PCR analysis. Only samples positive for both genes (16S rRNA and *msp*2) were considered *A. phagocytophilum* positive. Four animals were PCR positive but had negative *Anaplasma* serologic results.

Marked seasonal difference was found with 70 (62.5%) positive roe deer during the summer hunting season and 31 (24.8%) positive animals during the fall hunting season (p<0.0001) (Figure 2). Fewer animals were PCR positive in Jutland (39.6%) than on the islands (47.6%), and when adjusted for seasonal difference in sample collection, the difference was significant (p<0.05). *A. phagocytophilum* PCR-positive samples came from all 22 state forest districts (Table 2). Twenty-one samples from Jutland, Funen, Zealand, Falster, and Bornholm that were PCR positive for *A. phagocytophilum* were sequenced (GenBank accession no. AY776165) and revealed 100% homologies with known *A. phagocytophilum* sequences. Sequence variation was only encountered in 1 sample from Oxbøl in Jutland (no. AY776166). No significant difference in sex or age for PCR-positive samples was found. Ten samples were found positive for *Anaplasma* genus on the primary PCR but

Table 1. Prevalence of *Borrelia*, *Anaplasma phagocytophilum*, and tickborne encephalitis (TBE)-complex virus by age group*

Age group	n	*Borrelia* positive (n = 227) (%)	*Anaplasma* positive (n = 227) (%)	*A. phagocytophilum* PCR positive (n = 237) (%)	TBE-complex virus positive (n = 229) (%)
Fawns (≤11 mo)	19	4/17 (23.5)	17/17 (100)	8 (42.1)	0
Yearlings (12–23 mo)	37	13/33 (39.4)	32/35 (91.4)	14 (37.8)	0
Young adults (24–35 mo)	56	13/52 (25.0)	49/51 (96.1)	26 (46.4)	7 (12.5)
Adults (≥36 mo)	100	42 (42.0)	94/99 (94.9)	47 (47.0)	9 (9.0)
Age unknown	25	11 (44.0)	25 (100)	6 (24.0)	4 (16.0)

*Because material received from some state forest districts was limited, not all samples were available for all serologic tests. Where not equal to n, denominator gives actual number of samples tested. PCR, polymerase chain reaction.

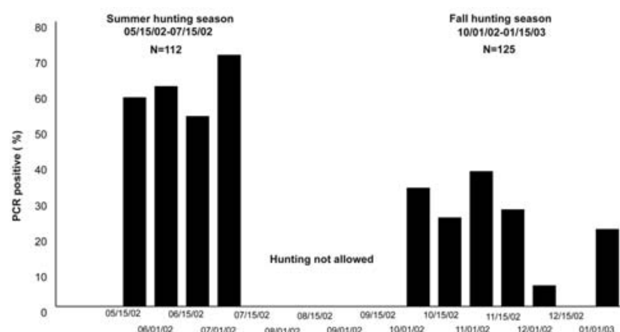

Figure 2. Seasonal variation in samples that were positive on polymerase chain reaction (PCR) for *Anaplasma phagocytophilum*.

negative on the more specific secondary PCR. Five of these samples were sequenced (no. AY776167); none was positive for *A. phagocytophilum*, but all had high homology (99%) with a sequence amplified from *I. ricinus* ticks collected from humans in Italy and previously deposited in GenBank as Rickettsiales bacterium it86 (no. AF525482.1).

R. helvetica and TBE-complex Virus

All 237 samples were tested by PCR for *R. helvetica*, but none were found positive (95% CI 0%–1.6%). Seroprevalence of TBE-complex virus was evaluated on 229 samples. Twenty samples (8.7%) were positive on the HI test. The verifying neutralization test was positive for 14 (6.1%) samples, but 4 could not be evaluated because of cell monolayer destruction, and 2 samples were not available for neutralization testing because of lack of material. Positive samples came from Bornholm (n = 6, 30%), Zealand and Falster (n = 3, 15%), and Jutland (n = 11, 55%) (Figure 3). Significant differences in seroprevalence could not be shown for sex or season, but TBE-complex virus–positive animals were significantly more common in the young adult–adult group than in the fawn–yearling group (p = 0.014).

Discussion

In the present study, roe deer were used as sentinels. They are widely distributed throughout Denmark and outnumber other large wild animals. They play a central role in the life cycle of *I. ricinus* by feeding large numbers of the tick at all 3 life stages (12). Density of *I. ricinus* is strongly related to the abundance of roe deer (13,14). Roe deer are considered incompetent as reservoirs of *Borrelia* spp. (15); however, their central role in the life cycle of the tick and the fact that they can respond immunologically to *Borrelia* infection renders them useful as sentinels for borreliosis (16). The finding of a *Borrelia* seroprevalence of 36.6% is lower than that found in the study by Webster and Frandsen (7) from 1994 (52%), but in the previous study all roe deer samples came from Zealand, and when this regional difference is taken into account the difference is not significant (50% vs. 52%, p = 0.773). The regional differences found correlate well with the known tick density in Denmark (Figure 1). The lack of seropositive roe deer from 3 districts in northern Jutland may, however, be due to the low sample size from these districts.

The role of ticks and roe deer in the transmission of *Bartonella* infections is still uncertain. Schouls et al. found that 60% of ticks collected from infested roe deer carried *Bartonella* spp. (17). *B. henselae* has also recently been found in ticks infesting humans in Italy (18), and *B. quintana* has been recovered from ticks in California (19). In 2001, McGill et al. found that 31% of elite orienteers (participants in the competitive sport of finding the fastest route between defined checkpoints with only a compass and map), a high-risk group for tick bites, were seropositive to *Bartonella elizabethae* (20). The recent finding of patients in the United States with *Borrelia burgdorferi*. and *B. henselae* co-infection also supports the idea that ticks play a role as a vector for *Bartonella* spp. (21). Our finding of no *B. henselae*– or *B. quintana*–seropositive roe deer does not support this idea. Whether roe deer are seropositive to other *Bartonella* spp., like *B. elizabethae*, remains to be evaluated, as well as the human pathogenic potential of *B. elizabethae*.

Roe deer are thought to be competent reservoirs of *A. phagocytophilum* (22), and a high seroprevalence has been found in previous studies in Europe. We found a seroprevalence of 95.6%, which is similar to findings from Norway (96%) and Slovenia (94%) (23,24). Larger variation has been found in the number of PCR-positive roe deer, from 12.5% in Czech Republic to 85.6% in Slovenia (24,25). Although differences in PCR protocols may to some extent explain this difference, the seasonal variation in the number of PCR-positive roe deer, as shown in this study, may also play a role (Figure 2). As the probability of roe deer being rickettsemic changes through the season, serology represents a better surveillance tool than PCR. The high proportion of roe deer that are bacteremic throughout the tick season and the importance of roe deer in the life cycle of *I. ricinus* may indicate that roe deer are the main reservoir of *A. phagocytophilum* in Europe.

Although regional variation was found in the number of PCR-positive animals, *A. phagocytophilum* is widespread in Denmark and seems correlated to tick and roe deer density. Limited variation was found among the 16S RNA sequences analyzed. Reliable differentiation of possible *A. phagocytophilum* strains in Denmark would be better accomplished by sequencing genes with higher variation than the conserved sequence of 16S RNA; among better candidates are the Ank-gene and the groESL operon.

Table 2. Regional distribution of *Borrelia*-, *Anaplasma phagocytophilum*–, and tickborne encephalitis (TBE)-complex virus–positive roe deer*

Region + state forest district	n	% *Borrelia* positive†	% *A. phagocytophilum* positive*	% *A. phagocytophilum* PCR positive	% TBE-complex virus HI-test positive
Jutland					
Åbenrå	14	21.4	100	21.4	14.3
Buderupholm	7	71.4	100	30.0	0
Feldborg	7	25.0 (n = 4)	80.0 (n = 5)	14.3	0
Fussingø	2	0	50.0	50.0	0
Gråsten	10	50.0	88.8 (n = 9)	50.0	10
Haderslev	26	16.0 (n = 25)	91.7 (n = 24)	61.5	16.0
Lindet	8	25.0	100	50.0	12.5
Oxbøl	13	46.2	100	53.8	0
Palsgård	15	9.1 (n = 11)	90.9 (n = 11)	20.0	9.1
Randbøl	12	16.7	100	8.3	8.3
Silkeborg	5	60.0	100	80.0	20.0
Thy	5	0	80.0	20.0	0
Ulborg	2	0	100	50.0	0
Funen					
Fyn	12	41.7	100	50.0	0
Zealand-Lolland-Falster					
Falster	17	46.7 (n = 15)	100 (n = 16)	41.2	6.3
Frederiksborg	17	52.9	88.2	52..9	5.8
Jægersborg	8	50.0	100	25.0	12.5
Kronborg	9	55.6	100	66.6	0
København	15	40.0	100	46.6	0
Odsherred	5	80.0	100	80.0	0
Tisvilde	7	42.9	85.7	14.3	0
Bornholm	19	36.8	100	46.2	31.6
Region unknown	2				

*PCR, polymerase chain reaction; HI, hemagglutination inhibition.
†Because the amount of material received from some state forest districts was limited, not all samples were available for *Borrelia* and *Anaplasma* serologic testing. Where not all samples were tested, n is indicated in parentheses.

Further characterization of European strains of *A. phagocytophilum* is needed; the finding of a high *A. phagocytophilum* prevalence in roe deer compared to the low number of human anaplasmosis cases reported in Europe may indicate the existence of strains less virulent or nonpathogenic to humans (26). The finding of sequences in roe deer blood that are related to sequences previously amplified only from ticks in Italy (It86-Belluno) is of interest and should be studied further (27). Whether this organism is pathogenic to roe deer or can cause human infection remains to be elucidated. The accuracy of diagnostic assays used is critical to any pathogen surveillance, and the potential for serologic cross-reaction is an important consideration. Recently *R. helvetica* has been found in ticks from Bornholm and Jutland, and a seroprevalence of 12.5% was described in high-risk groups in northern Jutland (3, S. Skarphédinsson et al., unpub. data). Even though it is not phylogenetically close to *A. phagocytophilum*, and even though serologic cross-reactivity has not been reported to date, it is the only other *Rickettsia* species reported in Denmark. We therefore looked for *R. helvetica* in roe deer, but found no PCR-positive deer. The explanation for this finding may be that roe deer are not competent as reservoirs or that the bacteremic phase is very short. Another possibility is that *R. helvetica* has a very focal distribution or is even disappearing from Denmark, as the seroprevalence study of Nielsen et al. showed a gradual decrease in seroprevalence from 29% in 1997 to 0% in 2000 (3).

Tickborne encephalitis was the first tickborne infection to be recognized in Denmark. During the years 1958–1962, E.A. Freundt did a survey for TBE-complex virus using human and animal sera from all parts of Denmark. He found, using both HI, neutralization, and complement fixation tests, that TBE-complex virus was present only on the island of Bornholm. He found an overall seroprevalence of 8.6% in Danish roe deer (the local seroprevalence on Bornholm was 83%) (6). Since then a very limited surveillance of TBE has since been carried out in Denmark. Recent increases in TBE cases in neighboring Sweden have been suggested to be related to climatic changes (28), as milder climate has been followed by a northern shift in the distribution limit of *I. ricinus* as well as a general increase in tick density (29). However, the variable patterns of changing TBE case numbers in Europe indicate that changing climate is not the sole causal factor.

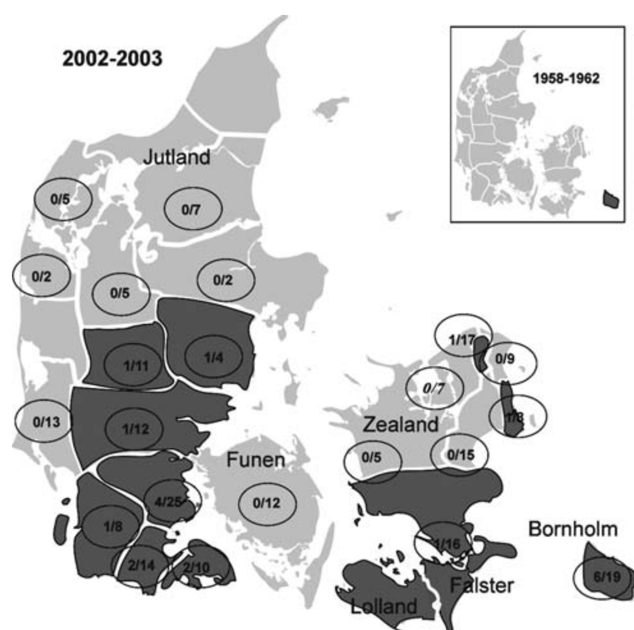

Figure 3. Distribution of tickborne encephalitis (TBE)-complex virus-positive state forest districts (dark shading) in Denmark, 2002–2003 vs. 1958–1962. Numerators indicate number of TBE-complex virus–positive roe deer; denominators indicate number of deer tested.

Changes in the densities of hosts for ticks and sociopolitical circumstances may play a role as well (30). TBE has also been suggested by the World Health Organization–European Centre for Environment and Health working group on the early implication of climatic change to be a priority infection for surveillance during climatic change (31) because of the fragile and temperature-dependent natural cycle of TBE virus. Using geographic information systems and remote sensing, Randolph et al. have predicted the present as well as future distribution of TBE in northern Europe with changing climate (32). These predictions seem to correlate well with the findings in our study of a change in the distribution of TBE-complex virus in Denmark. A strict correlation between TBE-complex virus–positive areas and tick and roe deer density was, on the other hand, not found.

However, recent studies on ticks in Bornholm have shown that not only the Western European subtype of TBE-complex virus is to be found. Louping ill virus, another flavivirus belonging to the TBE antigenic complex, is now also found in Bornholm (33). Further studies are needed to clarify the possible role of serologic cross-reactivity between these 2 closely related viruses. Although we found the same overall TBE seroprevalence now as in 1962, the local seroprevalence in Bornholm is significantly lower than before (31.6% vs. 83%, $p = 0.001$). Whether the emergence of Louping ill virus plays a part in this decrease is of interest. The fact that only roe deer 24 months of age or older were TBE-complex virus–seropositive may indicate that although TBE-complex virus has emerged in new areas in Denmark, the infection is still rare and focal in distribution.

Using roe deer as sentinels, we have shown that *A. phagocytophilum* is now widely distributed in Denmark and that roe deer may be the main reservoir. Also, while *Borrelia* prevalence has remained stable, the distribution of TBE-complex virus has changed, which supports the predicted effect of climatic change on vectorborne infections in northern Europe. In a shifting climate, continued long-term monitoring of tickborne infections is of importance. Healthcare providers should also be aware of the dynamic changes in distribution and prevalence of these infections when treating a patient with compatible illness, specifically after exposure to ticks.

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Dr. Skarphédinsson is a physician working at the Clinical Microbiology Research Unit, Institute of Clinical Research, University of Southern Denmark, and a board member of the Center for Health and Biodiversity, Denmark. His research interests include the epidemiology of zoonotic and vectorborne infections.

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Address for correspondence: Sigurdur Skarphédinsson, Department of Clinical Microbiology, Winsloewparken 21,2, DK-5000 Odense C, Denmark; fax: 45-6541-4785; email: siggi@inet.uni2.dk

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Risk Factors for Pediatric Invasive Group A Streptococcal Disease

Stephanie H. Factor,*† Orin S. Levine,* Lee H. Harrison,‡ Monica M. Farley,§ Allison McGeer,¶ Tami Skoff,* Carolyn Wright,* Benjamin Schwartz,* and Anne Schuchat*

Invasive group A *Streptococcus* (GAS) infections can be fatal and can occur in healthy children. A case-control study identified factors associated with pediatric disease. Case-patients were identified when *Streptococcus pyogenes* was isolated from a normally sterile site, and matched controls (≥2) were identified by using sequential-digit dialing. All participants were noninstitutionalized surveillance-area residents <18 years of age. Conditional regression identified factors associated with invasive disease: other children living in the home (odds ratio [OR] = 16.85, p = 0.0002) and new use of nonsteroidal antiinflammatory drugs (OR = 10.64, p = 0.005) were associated with increased risk. More rooms in the home (OR = 0.67, p = 0.03) and household member(s) with runny nose (OR = 0.09, p = 0.002) were associated with decreased risk. Among children, household-level characteristics that influence exposure to GAS most affect development of invasive disease.

Invasive group A *Streptococcus* (GAS) infections include sepsis, bacteremic pneumonia, and dramatic, rapidly progressive syndromes such as necrotizing fasciitis and streptococcal toxic shock syndrome (STSS). An estimated 9,100 cases and 1,350 deaths occur in the United States each year, many of these among previously healthy children (1).

Previous studies identified associations between individual-level risk factors and pediatric invasive GAS disease. Many hospital-based case series (2–13) and 2 population-based studies (14,15) have associated varicella-zoster virus (VZV) infection with an increased risk for invasive GAS disease. Use of nonsteroidal antiinflammatory drugs (NSAIDs) was also associated (9,12), but whether NSAID use predisposed to or increased the severity of GAS infection (16) or was a marker of disease severity (17) is unclear. These previous studies were limited by the completeness of the data available from medical and laboratory records.

Household-level risk factors appear to play a role in disease development. Studies conducted in the 1950s demonstrated that school-age children were most often responsible for introducing a GAS strain into a household (18). Household transmission of GAS infection facilitated a communitywide GAS outbreak in southeastern Minnesota (19). Household crowding, measured by the number of persons in the home, and exposure to children with a sore throat in the home have been associated with increased risk for invasive GAS in adults (20). Household exposures have not been studied concomitantly with individual-level risk factors in children.

We conducted a population-based, case-control study with parental interviews to concomitantly study and identify individual- and household-level risk factors for invasive pediatric GAS disease. Parents could provide more complete data than medical record review. Simultaneous study of both types of data could also assess the relative effects of individual and household characteristics.

Methods

Invasive GAS disease was defined as the isolation of *Streptococcus pyogenes* from a normally sterile site (including blood, cerebrospinal fluid, pleural fluid, peritoneal fluid, pericardial fluid, joint fluid, surgical specimens, bone, and scrotal fluid) in a noninstitutionalized resident, <18 years of age, in a surveillance area. Children who had GAS isolated from a sterile site >48 hours after hospital admission were presumed to have a nosocomial infection (21) and were excluded.

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †New York City Department of Health and Mental Hygiene, New York, New York, USA; ‡Johns Hopkins University, Baltimore, Maryland, USA; §Emory University School of Medicine and the VA Medical Center, Atlanta, Georgia, USA; and ¶Mount Sinai Hospital, Toronto, Ontario, Canada

Cases of invasive GAS disease were identified through active, laboratory-based surveillance in 3 areas: metropolitan Atlanta, Georgia, from July 1, 1997, to June 30, 1999; metropolitan Baltimore, Maryland, from July 1, 1997, through June 30, 1999; and the Toronto-Peel region, Ontario, Canada, from July 1, 1997, through December 31, 1997. The surveillance area population was estimated to include 9 million people (3.6 in metropolitan Atlanta, 2.4 in metropolitan Baltimore, and 3.0 in the Toronto-Peel region based on 1997 Bureau of Census estimates [22]). All acute-care hospitals and laboratories serving the residents of the surveillance area were contacted biweekly and audited semiannually to identify patients with invasive GAS disease. For each case of pediatric invasive GAS identified, the standardized Active Bacterial Core Surveillance case-report form was completed by reviewing the hospital medical record. This form is used for all organisms under surveillance and includes all laboratory and clinical data needed to fulfill the criteria for streptococcal toxic shock syndrome (STSS) (23) and other clinical syndromes.

The method used to reach case-patients and identify controls has been described elsewhere (20). Briefly, a "case algorithm" was used to contact persons infected with invasive GAS. For each case-patient identified, up to 15 separate telephone calls were made to contact the parent or guardian. To maximize the likelihood of contacting the patient, the telephone calls were made on 5 nonconsecutive days, including at least 1 weekend day, during each of 3 different time periods (8:00 a.m.–12:00 p.m., 12:01–5:00 p.m., 5:01–8:00 p.m.). Case-patients were eligible if their enrollment was complete within 3 months of onset of GAS disease. Family members of deceased case-patients were interviewed, and non–English-speaking patients were included if individual surveillance sites had the resources to communicate with the patients in their language.

A population-based sample of matched controls was selected through systematic, sequential-digit telephone dialing. Case-patients and controls were matched by age group, postal or zip code, and telephone exchange to control for age and socioeconomic status. Age groups were defined as 0–23 months, 24–59 months, and 5–17 years. Case-patients without telephones were excluded.

When experienced surveillance personnel reached the parent or guardian of a case-patient or control, they explained the purpose of the study, obtained informed consent, and administered a standardized questionnaire. The questionnaire included questions on demographics, socioeconomic status, age-specific activities like breastfeeding and preschool, medical history, and household characteristics. The household characteristics included physical space, number of persons in the household, persons who smoke, and symptoms in other persons in the past 2 weeks for controls or in the 2 weeks before invasive GAS disease among case-patients. Within the medical history section, we differentiated between "regular" NSAID use and "new" NSAID use. New NSAID use indicated that the case-patient had started using NSAIDs in the 2 weeks before illness was diagnosed or that a control participant had started using NSAIDs in the 2 weeks before the interview. The parent or guardian for case-patients and controls was allowed to self-define regular use of NSAIDS. This study was approved by the institutional review boards at the Centers for Disease Control and Prevention and at each surveillance site.

Odds ratios (ORs) for each potential risk factor were determined by using conditional logistic regression (Proc PHREG, SAS Version 6.12, SAS Institute Inc., Cary, NC, USA), controlling for sex and race. Variables with $p<0.20$ in individual analyses were included in multivariable analysis. Computer-assisted and manual forward, backward, and stepwise conditional logistic regression identified risk factors independently associated with invasive GAS disease. ORs with 95% confidence intervals (CIs) that did not include 1.00 and p values <0.05 were considered significant in multivariable analysis.

Results

Surveillance identified 72 episodes of invasive GAS disease among children <18 years of age. Eight had nosocomial infection and were ineligible. Of the 64 remaining, 38 were enrolled, 5 parents or guardians refused to participate, 3 were not reached after exhausting the telephone call algorithm, and 18 did not participate for other reasons, including a time lapse of >3 months after the illness, incomplete or incorrect contact information (i.e., wrong phone number, disconnected phone, no phone, homelessness), and difficulty communicating over the phone (i.e., poor communication skills, non–English-speaking parent or guardian). No statistical differences in race, sex, age, or death rate were seen between enrolled and nonenrolled patients.

The number of case-patients enrolled varied by area: 20 (53%) from Atlanta, 11 (29%) from Baltimore, and 7 (18%) from Toronto. Ten (26%) patients were 0–23 months of age, 7 (18%) were 24–59 months, and 21 (55%) were 5–18 years. Forty-seven percent were boys. Of enrolled patients, 24 (63%) were white, 13 (34%) were African American, and 1 (3%) did not specify ethnicity. Two patients (5%) died of the disease; both were diagnosed with primary bacteremia without focus. Primary bacteremia and cellulitis were the 2 most common diagnoses (Table 1). No cases of STSS were found.

Several factors were associated with invasive GAS disease (Table 2). When sex and race were controlled for in individual analysis, having a primary caretaker who

Table 1. Clinical syndromes of children with invasive group A streptococcal disease, Atlanta, Baltimore, and Toronto, 1997–1999*

Clinical syndrome	No. patients (%)
Primary bacteremia (without focus)	18 (47)
Cellulitis	6 (16)
Septic arthritis	4 (11)
Necrotizing fasciitis	1 (3)
Pneumonia	1 (3)
Otitis	1 (3)
Peritonitis	1 (3)
Abscess	1 (3)
Appendicitis	1 (3)

*Patients may appear in >1 category; data not available for all patients.

smokes, presence of ≥1 other children in the home, and new use of NSAIDs were associated with an increased risk for invasive GAS disease (p≤0.05); more rooms in the home, higher level of parental education, and a household member with a runny nose (rhinitis) in the past 2 weeks were associated with a decreased risk for invasive GAS disease (p≤0.05). By using multivariable conditional regression and controlling for sex and race, 4 risk factors were found to be independently associated with invasive GAS disease: having ≥1 other children in the home (OR = 16.85, p = 0.0002) and new use of NSAIDs (OR = 10.64, p = 0.005) were associated with an increased risk, and more rooms in the home (OR = 0.67, p = 0.03) and having a household member with a runny nose in the past 2 weeks (OR = 0.09, p = 0.002) were associated with a decreased risk.

VZV and HIV infection occurred only in case-patients (n = 3 and n = 1, respectively). Patients and controls were equally likely to be vaccinated against VZV (OR = 0.93, p = 0.88). Of the 3 case-patients with history of VZV infection, 1 reported new use of NSAIDs. Among participants 0–23 months of age, 12 (71%) of 17 controls were currently being or had ever been breastfed compared to 0 of 8 case-patients (OR = 0, p = 1.00).

Discussion

This study suggests that children bring GAS into the home and that crowding, measured by the number of rooms in the home, influences the development of invasive GAS disease. The protective association of rhinitis was unexpected, and the mechanism of protection is not obvious. Individual-level risk factors seem to play a less important role. Although NSAID use is associated with invasive disease, the measurements of new use and regular use are too crude to clearly identify their role as a risk factor.

The roles of children and crowding are expected and have been suggested by previous studies. Children are most likely to introduce GAS infection into the home (18), children spread GAS in the home (19), and children with sore throats are likely reservoirs of GAS for adults who develop invasive disease (20). Crowding, measured by number of people in the home, increases risk for acquiring disease among adults >45 years of age (20).

Although the association between rhinitis and invasive GAS infection may be spurious, data support a true relationship. Among persons with sore throats, those with rhinitis are less likely to have GAS pharyngitis than are those without rhinitis (24,25). This finding suggests that controls were less likely to be exposed to persons with GAS pharyngitis than were case-patients.

Although only significant in individual analysis in this study, cigarette smoke has been independently associated with other invasive bacterial infections in other studies. Increased risk for invasive meningococcal disease in children <18 years is associated with having a mother who smokes (26), and increased risk for invasive pneumococcal disease in immunocompetent, nonelderly adults is associated with both being a smoker and being exposed to other smokers (27). Larger numbers of case-patients may show an association.

A large difference was seen in the proportions of patients and controls who have been breastfed, although this difference was not significant. Breastfeeding may protect against invasive GAS disease as it does against other invasive bacterial diseases. Previous studies found current breastfeeding protective against invasive pneumococcal disease in children 2–11 months of age (28) and against invasive *Haemophilus influenzae* type B disease in children <6 months of age (29). Data suggest that in addition to containing protective antibodies against these organisms, breast milk can inhibit bacterial colonization independent of antibody concentration (30). Although HIV infection is a risk factor for invasive GAS infection in adults (20,31) and VZV infection is a risk factor for invasive GAS infection in children (14–15), this study had too few patients to comment on either.

This study has several limitations. The small size limits the statistical power to identify associations between individual- and household-level characteristics and invasive GAS disease. Some questions were only asked of subgroups, further decreasing power to detect associations. Using sequential-digit dialing and matching on zip or postal codes controlled for socioeconomic and community-level risk factors. These factors could therefore not be studied. This method may limit the generalizability of the findings; the study population included only persons with phones and, specifically, persons likely to answer their phones.

The associations in this study all suggest that development of pediatric invasive GAS disease is largely related to opportunities for exposure to GAS, as measured by exposure to children, other persons, and persons with GAS infections. Individual-level risk factors in children are less

Table 2. Individual and multivariable analysis for risk factors for invasive group A streptococcal disease among case-patients and controls*

Variable	Case-patients (N = 38) (%)	Controls (N = 78) (%)	Individual analysis OR (95% CI)	Individual analysis p value	Multivariable analysis OR (95% CI)	Multivariable analysis p value
No. persons in home (mean)	4.42	4.02	1.28 (.91–1.78)†	0.15		
Live in single-family home	23 (62)	59 (76)	0.49 (0.21–1.16)	0.11		
No. rooms in home (mean)	6.39	7.27	0.81 (0.66–0.99)‡	0.04	0.67 (0.51–0.88)‡	0.03
No. smokers in home						
0	21 (57)	53 (68)	1.43 (0.87–2.32)§	0.16		
1	9 (24)	16 (21)				
2	6 (16)	7 (9)				
3	1 (3)	2 (3)				
Primary caretaker is a smoker	11 (30)	13 (17)	2.71 (1.02–7.21)	.05		
≥1 other child <18 years living in the home	33 (87)	43 (56)	5.76 (1.95–16.96)	.002	16.85 (3.90–72.84)	0.0002
≥1 other person in the household with a cough	4 (11)	17 (22)	0.40 (0.12–1.32)	0.13		
≥1 other person in the household with a runny nose	4 (11)	26 (34)	0.25 (0.08–0.80)	0.02	0.09 (0.01–0.40)	0.002
HIV-positive	1 (3)	0	Undefined	0.99		
Eczema	8 (21)	16 (21)	0.99 (0.38–2.64)	0.99		
Varicella-zoster virus infection	3 (8)	0	Undefined	0.99		
Vaccinated with varicella-zoster virus vaccine	12 (46)	26 (47)	0.93 (0.36–2.40)	0.88		
New use of NSAIDs	9 (24)	7 (9)	3.15 (1.07–9.29)	0.04	10.64 (2.08–54.61)	0.005
Use of corticosteroids	1 (3)	2 (3)	0.93 (0.08–11.02)	0.95		
Parent/guardian education						
Some HS	4 (11)	3 (4)	0.69 (0.51–0.91)	0.01		
HS graduate or GED	12 (32)	13 (17)				
Technical school	2 (5)	5 (6)				
Some college	10 (26)	23 (29)				
College graduate	7 (18)	23 (29)				
Postgraduate study or professional	2 (5)	10 (13)				
Household income						
<$15,000	7 (21)	2 (3)	0.70 (0.48–1.01)	0.06		
$15,001–$30,000	8 (24)	11 (18)				
$30,001–$45,000	4 (12)	13 (22)				
$45,001–$60,000	6 (18)	17 (28)				
≥$60,001	9 (26)	17 (28)				
Ever breastfed¶	0	12 (71)	0 (Undefined)	1.00		

*Data were not available for all variables for all case-patients and controls. Analyses controlled for race and sex. OR, odds ratio; CI, confidence interval; NSAID, nonsteroidal antiinflammatory drug; HS, high school; GED, general equivalency diploma.
†Increase in risk with each additional person living in home. Persons living in the home were evaluated as a continuous variable in conditional logistic regression.
‡Decrease in risk with each additional room in home. Rooms were evaluated as a continuous variable in conditional logistic regression.
§Increase in risk with each additional smoker in home. Smokers in the home were evaluated as a continuous variable in conditional logistic regression.
¶Question asked only of children <2 years of age; 8 case-patients and 17 controls were <2 years. Because none of the case-patients were breastfed, the calculated OR = 0, and the confidence interval is not defined.

important. Breastfeeding young children and nonsmoking by their household contacts may be preventive and should be encouraged.

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Dr. Factor is a CDC medical epidemiologist in the Bioterrorism Preparedness Response Program assigned to the New York City Department of Health and Mental Hygiene to develop bioterrorism and emergency response plans for New York City. In addition to emergency preparedness research, she is interested in respiratory and bloodborne diseases.

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Address for correspondence: Stephanie H. Factor, Bureau of Emergency Management, New York City Department of Health and Mental Hygiene, 125 Worth St, New York, NY 10013, USA; fax: 212-788-4734; email: sfactor@health.nyc.gov

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Emergency Department Response to SARS, Taiwan

Wei-Kung Chen,* Hong-Dar Isaac Wu,* Cheng-Chieh Lin,† and Yi-Chang Cheng†

How emergency departments of different levels and types cope with a large-scale contagious infectious disease is unclear. We retrospectively analyzed the response of 100 emergency departments regarding use of personal protective equipment (PPE) and implementation of infection control measures (ICMs) during the severe acute respiratory syndrome outbreak in Taiwan. Emergency department workers in large hospitals were more severely affected by the epidemic. Large hospitals or public hospitals were more likely to use respirators. Small hospitals implemented more restrictive ICMs. Most emergency departments provided PPE (80%) and implemented ICMs (66%) at late stages of the outbreak. Instructions to use PPE or ICMs more frequently originated by emergency department administrators. The difficulty of implementing ICMs was significantly negatively correlated with their effectiveness. Because ability to prepare for and respond to emerging infectious diseases varies among hospitals, grouping infectious patients in a centralized location in an early stage of infection may reduce the extent of epidemics.

Severe acute respiratory syndrome (SARS) is a newly emerging infectious disease in humans. The initial outbreak, which occurred in November 2002 in China, marked the beginning of a pandemic that spread rapidly around the globe, resulting in >8,000 reported cases (1). Taiwan was the third region to be affected by the outbreak because of its frequent contact with China and Hong Kong. The first case in Taiwan was reported on February 1, 2003, and a total of 671 probable cases had been reported by June 15 (2). The turning point of the SARS outbreak in Taiwan occurred when a healthcare laundry worker with atypical SARS symptoms visited the emergency department of hospital A (located at Taipei City) 3 times (on April 12, 14, and 15) and was admitted to an ordinary ward without quarantine (3). The outbreak at hospital A occurred on April 22, then spread from hospital A to other hospitals. Before the hospital A outbreak, most hospitals did not anticipate the extent of the SARS epidemic.

Many healthcare workers (HCWs) were infected during the SARS epidemic (3–8). Protecting HCWs from contamination was the first priority of infection control measures (ICMs) in hospitals. In response to the growing epidemic, most hospitals followed the recommendations of the Department of Health (DOH), which were similar to those of the Centers for Disease Control and Prevention and the World Health Organization (9–11). However, in some hospitals, additional measures were also taken to prevent a hospital outbreak when hospital managers believed that the existing recommendations were ineffective or insufficient. During the SARS outbreak, the emergency department played a vital role in infection control because many patients with fever sought medical attention in an emergency department. Facing a new disease, emergency department personnel were unable to make decisions regarding timing of personal protective equipment (PPE) usage and classification of infectious disease because the means of transmission were unclear and early identification was difficult (unclear clinical symptoms and lack of a laboratory test).

Previous studies have demonstrated that effectively implementing ICMs can control and prevent an outbreak (12,13). However, the problem is not just a question of control in individual hospitals. The control measures must be coordinated throughout the healthcare system, and these measures must be implemented in the initial stage, not just in the late stage. Whether emergency departments had the ability to make adequate preparations or implement all the necessary ICMs was unclear. Because policymakers lacked adequate information about the capacity and ability of hospital or emergency departments to implement such measures, no decisions were made in the early stages of the outbreak about whether to divert or group persons with suspected or probable cases of SARS into a centralized

*China Medical University Hospital, Taichung, Taiwan; and †China Medical University, Taichung, Taiwan

section in emergency departments. The purpose of this hospital-based study was to collect data from hospitals of various levels and analyze the ability of the hospitals' emergency departments to cope with the SARS outbreak. These data may be used to improve the effectiveness of existing emergency protocols before the reemergence of SARS, influenza, or other infectious disease. Hence, policymakers, as well as administrators of hospitals and emergency departments, will be able to make more effective decisions in the early stage of an infectious disease.

Materials and Methods

Taiwan had 468 hospitals in 2003, 393 (83.9%) private and 75 (16.1%) public. Data were collected from emergency departments in which the number of patients exceeded 500 per month. Questionnaires were sent to chiefs of staff at emergency departments in late June 2003. The questionnaire was designed by emergency department experts after panel discussions. The data collected included the following: accreditation of the hospitals, the average monthly volume of emergency department patients from March to May 2003, the effects of the SARS epidemic on emergency department workers, types of PPE supplied, kinds of ICMs implemented, as well as timing and origin of instruction to use PPE and ICMs during the SARS epidemic.

Before data analyses, hospitals were classified into 3 levels, medical center (level A), regional hospital (level B), or local hospital (level C). In general, the number of hospital beds at these hospitals, based on reference data, was >500 in level A, 200–500 in level B, and <200 in level C. The average monthly volume of emergency department patients was an average of the monthly emergency department volumes during the 3-month period. The hospitals were grouped first by level, and then the average was calculated. The average of the averages for each hospital was then determined. Emergency department workers were classified as physicians, nursing staff, or paramedics. The effects on emergency department staff were measured according to a 4-point scale, as follows: 1) had fever and needed to stay at home, 2) quarantined at home with fever or no fever, 3) quarantined at hospital, and 4) probably had SARS.

The basic PPE recommended by the DOH included head and shoe covers, goggles, face shield, gloves, apron, disposable gown, surgical mask, and N95 respiratory mask. Hand hygiene was excluded as a protection measure because accurate assessment was difficult. The high-level protective respirators were defined as P100/ N100/ FFP3 (approved by the National Institute for Occupational Safety and Health [NIOSH]) and powered air-purifying respirators with full-body isolation suit. ICMs included the guidelines from the DOH (defined as basic ICMs) as well as additional measures, such as having a fever triage ward or referral to a SARS screening team, implemented by emergency departments during the SARS outbreak. The timing of complete PPE implementation or having ICMs completely in place was classified into 2 stages: 1) early stage, from March to late April, 2) late stage, from late April to mid-June. The order to use PPE or ICMs came from 3 sources: 1) emergency department workers themselves, 2) emergency department administrators, and 3) hospital administrators. The difficulty of implementing or instituting ICMs was rated on a scale from 1 (mildly difficult) to 5 (very difficult). The effectiveness of implementing ICMs was rated on a scale from 1 (less effective) to 5 (very effective). All ratings were based on self-assessments of hospital staff.

One-way analysis of variance (ANOVA) was used to test the differences in the average monthly volumes of patients among different levels and types of emergency departments. The associations between categorical variables were analyzed by chi-square and Fisher exact test. The rating scale of difficulty and effectiveness of ICMs was represented by median and interquartile range (IQR). The correlation between difficulty and effectiveness was analyzed by Spearman rank correlation; p values <0.05 were considered significant.

Results

A total of 213 emergency departments were initially included in this study; 152 (71.4%) were private hospitals and 61 (28.6%) were public hospitals. One hundred emergency departments responded to the questionnaire (respondent rate = 46.9%). Among these, 15 emergency departments were medical centers (respondent rate = 65.2%, including 6 public and 9 private emergency departments), 28 emergency departments were regional hospitals (respondent rate = 38.9%, including 10 public and 18 private), and 57 emergency departments (respondent rate = 44.9%, including 14 public and 43 private) were local hospitals. The overall response rate was 46.0% in public hospitals and 49.1% in private hospitals.

The emergency department volumes and assessment of the effects of the SARS outbreak on emergency department workers are shown in Table 1. From March to May 2003, the average monthly volume of emergency department patients in level A hospitals was 6,200 (range 3,429–11,080) and 3,828 (range 1,864–5,770) in level B hospitals, both of which were significantly larger than the average number of patients in level C hospitals (average 2,246, range 729–3,236) (p = 0.001). No significant differences in emergency department volume were found between public (average 2,642, range 1,364–6,258) and private hospitals (average 3,398, range 729–11,080). The most frequent effect of the SARS outbreak on emergency

Table 1. Effects of SARS epidemic on emergency departments*

	Hospital level				Hospital type		
No. EDs with ED staff affected as follows†	A (%), n = 15	B (%), n = 28	C (%), n = 57	p value	Public (%), n = 30	Private (%), n = 70	p value
Fever and needed to stay at home	7 (47)	8 (29)	5 (9)	0.002	7 (23)	13 (19)	0.585
Physician	6 (40)	1 (4)	0	0.000	2 (7)	5 (7)	0.932
Nursing staff	6 (40)	7 (25)	5 (9)	0.010	7 (23)	11 (16)	0.363
Paramedic	2 (13)	4 (14)	0	0.014	2 (7)	4 (6)	0.854
Quarantine at home	3 (20)	7 (25)	6 (11)	0.208	7 (23)	9 (13)	0.190
Physician	3 (20)	6(21)	4 (7)	0.122	7 (23)	6 (9)	0.044
Nursing staff	3 (20)	6 (21)	4 (7)	0.122	5 (17)	8 (11)	0.475
Paramedic	2 (13)	3 (11)	3 (5)	0.487	3 (10)	5 (7)	0.629
Quarantine in hospital	4 (27)	2 (7)	0	0.001	5 (17)	1 (1)	0.003
Physician	3 (20)	1 (4)	0	0.002	4 (13)	0	0.002
Nursing staff	3 (20)	2 (7)	0	0.006	4 (13)	1 (1)	0.012
Paramedic	3 (20)	1 (4)	0	0.002	3 (10)	1 (1)	0.045
Probable case-patients	3 (20)	3 (11)	0	0.007	4 (13)	2 (3)	0.043
Physician	1 (7)	0	1(2)	0.057	0	1 (1)	0.511
Nursing staff	3 (20)	2 (7)	0	0.006	3 (10)	2 (3)	0.133
Paramedic	3 (20)	2 (7)	0	0.006	3 (10)	2 (3)	0.133

*SARS, severe acute respiratory syndrome; ED, emergency department.
†Represents the number of EDs that responded "yes" to at least 1 ED staff member in a given category of possible SARS impact. The number in parentheses is the percentage of the total EDs in a particular hospital level or type.

department workers was "fever and needed to stay at home." Emergency department workers in level A and B hospitals had a higher probability of being affected during the SARS outbreak, regardless of job type. When the effects of the SARS outbreak on public and private hospitals were compared, significant differences were found between the type of quarantine at hospitals.

PPE supplied by emergency departments is shown in Table 2. The use of basic PPE did not differ significantly among emergency departments at different hospital levels. However, level A emergency departments used high grade PPE (P100/N100/FFP3 or powered air-purifying respirator) more often than emergency departments at level B and C hospitals. The implemented ICMs in different hospitals are shown in Table 3. Most of the hospitals were able to follow the guidelines of the DOH. However, in terms of additional ICMs, emergency departments of level C hospitals used more restrictive measures when transferring patients in and out. The use of ICMs in public and private hospital was significantly different in patients who were transferred out. The timing of PPE usage or implementation of ICMs is shown in Table 4. Eighty percent (80/100) of hospitals completely implemented use of PPE, and 66% (66/100) of hospitals implemented their ICMs at the late stage of the SARS outbreak. The instruction to use PPE originated from emergency department managers in 60% of level A, 46% of level B, and 23% of level C hospitals. The order to implement ICMs came from hospital managers in 33% of level A, 50% of level B, and 62% of level C hospitals.

Table 5 shows the rating scales and correlations of difficulty and effectiveness of ICMs in emergency departments.

Table 2. Supply of personal protection equipment (PPE) in emergency departments by hospital level and type

	Hospital level				Hospital type		
PPE	A (%), n = 15	B (%), n = 28	C (%), n = 57	p value	Public (%), n = 30	Private (%), n = 70	p value
Basic PPE							
Head or shoe covers	13 (87)	25 (89)	52 (91)	0.862	27 (90)	63 (90)	1.000
Goggles	12 (80)	21 (75)	46 (81)	0.529	27 (90)	52 (74)	0.182
Face shield	15 (100)	25 (89)	47 (82)	0.182	26(87)	61(87)	0.948
Gloves	14 (93)	24 (86)	55 (96)	0.187	27 (90)	66 (94)	0.425
Apron	11 (73)	22 (79)	42 (74)	0.876	22 (73)	53 (76)	0.805
Disposable gown	10 (67)	25 (89)	35 (61)	0.030	23 (77)	47 (67)	0.476
Surgical mask	10 (67)	17 (61)	38 (67)	0.855	21 (70)	44 (63)	0.493
N95 respiratory mask	12 (80)	23 (82)	56 (98)	0.014	26 (87)	65 (93)	0.322
High grade PPE							
P100/N100/FFP3	11 (73)	13 (46)	5 (9)	0.000	14 (47)	15 (21)	0.011
Powered air-purifying respirators	6 (40)	2 (7)	4 (7)	0.001	5 (17)	7 (12)	0.347

Table 3. Implemented infectious control measures in different hospitals by level and type*

Infection control measures (ICM)	Hospital level			p value	Hospital type		p value
	A (%), n = 15	B (%), n = 28	C (%), n = 57		Public (%), n = 30	Private (%), n = 70	
Basic ICM							
Entrance body temperature screen	15 (100)	28 (100)	57 (100)	-	30 (100)	70 (100)	-
Visitors restriction	14 (93)	24 (86)	55 (96)	0.193	30 (100)	63 (90)	0.347
Quarantine of fever patients outside of EDs	14 (93)	28 (100)	56 (98)	0.341	29 (97)	69 (99)	0.533
Quarantine fever patients in isolation room	15 (100)	24 (86)	46 (81)	0.158	26 (87)	59 (84)	0.760
Instituted fever screening station	15 (100)	28 (100)	55 (96)	0.847	30 (100)	68 (97)	0.373
Additional ICM							
Instituted fever triage ward	11 (73)	22 (79)	38 (67)	0.252	24 (80)	47 (67)	0.194
Restricted fever patient admission	13 (87)	21 (75)	52 (91)	0.128	26 (87)	60 (86)	0.900
Restricted patients transfer in	5 (33)	7 (25)	31 (54)	0.026	13 (43)	30 (43)	0.965
Suspected case-patients transfer out	4 (27)	9 (32)	43 (75)	0.000	12 (40)	44 (63)	0.048
SARS screening team	8 (53)	13 (87)	40 (70)	0.087	16 (53)	45 (64)	0.303
Closure of ED	3 (20)	5 (18)	15 (26)	0.654	9 (30)	14 (20)	0.276

*ED, emergency department; SARS, severe acute respiratory syndrome.

Of the basic ICMs, instituting isolation at emergency departments was thought to be the most difficult (median = 3 score). Additional ICMs were more difficult to implement than basic ICMs. The effectiveness of additional ICMs was also thought to be less than that of basic ICMs. Closing emergency departments was thought to be less effective. Overall, the effectiveness of ICMs decreased as difficulty of implementation increased. Significant negative correlations were seen between perceived difficulty and effectiveness of implemented ICMs, except for body temperature at admission, institution of a fever screening station, and closure of the emergency department.

Discussion

Impact of SARS on Emergency Departments in Larger Hospitals

In our study, emergency department workers in larger hospitals were more severely affected by the SARS outbreak than staff at smaller hospitals. Several possible reasons could explain this finding. First, as our data showed, emergency departments at larger hospitals tend to have more patients requiring emergency services than those at smaller emergency departments. Thus, overcrowding and more frequent contact with patients would increase the incidence of person-to-person transmission. Emergency department workers may have become infected even without contact with a hospitalized SARS patient (14). Second, most larger hospitals were located in an urban area. Persons living in urban areas may be more likely to travel overseas, which would increase their chances of contracting an infectious disease. A similar phenomenon was reported at hospitals in cities with a high population density, such as Beijing, Hong Kong, Singapore, and Toronto (14–19). Third, fever patients tended to visit larger hospitals in the belief that they would be able to see a specialist who could identify the fever source. Finally, emergency departments at larger hospitals

Table 4. Features of infectious control measures in different hospitals by level and type*

Response to the SARS outbreak	Hospital level			p value	Hospital type		p value
	A (%), n = 15	B (%), n = 28	C (%), n = 57		Public (%), n = 30	Private (%), n = 70	
Timing of PPE (complete preparedness)				0.132			0.082
Early stage	4 (27)	2 (7)	14 (25)		3 (10)	17 (24)	
Late stage	11 (73)	26 (93)	43 (75)		27 (90)	53 (76)	
Start of PPE use				0.015			0.006
From ED workers themselves	1 (7)	2 (8)	8 (14)		3 (10)	8 (11)	
From ED administrators	9 (60)	13 (46)	13 (23)		17 (57)	18 (26)	
From hospital administrators	5 (33)	13 (46)	36 (63)		10 (33)	44 (63)	
Timing of ICMs (complete set-up)				0.087			0.559
Early stage	3 (20)	8 (29)	24 (42)		10 (33)	24 (34)	
Late stage	12 (80)	20 (71)	33 (58)		20 (67)	46 (66)	
Order of infection measures				0.058			0.136
From ED workers themselves	1 (7)	1 (4)	7 (12)		3 (10)	7 (10)	
From ED administrators	9 (60)	13 (46)	15 (26)		16 (53)	21 (30)	
From hospital administrators	5 (33)	14 (50)	35 (62)		11 (37)	42 (60)	

*SARS, severe acute respiratory syndrome; PPE, personal protective equipment; ICM, infection control measures; ED, emergency department.

Table 5. Rating scale and correlation of difficulty and effectiveness of infection control measures (ICM) implemented*

Response to the SARS outbreak	Difficulty		Effectiveness		Correlation	p value
	Median	IQR	Median	IQR		
Basic ICM						
Entrance body temperature screen	1	1	5	1	–0.061	0.55
Visitors restriction	2	2	4	2	–0.309	0.002
Quarantine of fever patients outside of EDs	2	3	5	1	–0.283	0.005
Institution of isolation room at ED	3	3	4	1	–0.226	0.026
Institution of fever screening station	1	2	5	1	–0.128	0.214
Additional ICM						
Institution of fever triage ward	3	2	4	4	–0.210	0.042
Restriction of fever patient admission	3	2	4	2	–0.283	0.005
SARS screening team	4	2.25	4	3	–0.408	0.000
Restriction of transfer in	4	2	3	2	–0.269	0.010
Suspected cases transfer out	3	3	3	3	–0.210	0.040
Closure of ED	4	2	2	3	–0.153	0.140

*SARS, severe acute respiratory syndrome; IQR, interquartile range; ED, emergency department.

had more patients transferring in, so some patients with fever of unknown origin may have been transferred from lower level hospitals.

In Taiwan, emergency department volume does not always correlate well with either hospital bed number or with hospital location (such as urban versus. nonurban). This lack of correlation may explain, in part, the large variation in numbers of emergency department patients in level A hospitals. During the SARS outbreak, some patients were transferred to larger hospitals in both urban and semiurban areas. Therefore, preventing outbreaks at larger hospitals during an epidemic of an emerging disease is essential.

Use of High-Grade PPE and Additional ICMs

In our analysis, most of the emergency departments in Taiwan followed the guidelines for basic PPE provided by the DOH. Some hospitals did not use these basic recommended PPE because they already had other PPE that performed the same function. However, some hospitals may have had an inadequate supply of PPE. In fact, a substantial problem for hospitals during the SARS epidemic was the cost of basic PPE, such as surgical masks and N95 respiratory masks, which increased costs markedly during this period. The quantity of PPE required by larger hospitals was very large, which placed a financial hardship on these hospitals, even though many of them did not encounter any SARS cases. Some hospitals were so anxious to acquire sufficient basic PPE that they even requested recycled PPE if it was available. The supply of higher grade respirators was greater at larger hospitals than at smaller hospitals. This finding may have been because hospital outbreaks were generally more common at larger hospitals and more patients who needed emergency resuscitation were transferred to larger hospitals. Transfers inevitably increased the risk for transmission to emergency workers (14).

In our analysis, most hospitals implemented basic ICMs during the SARS epidemic, but smaller emergency departments more frequently used more restrictive ICMs. Paradoxically, smaller hospital appeared to be more alert to the emerging disease, although the number of emergency department patients was lower than in larger emergency departments. This may have been because smaller hospitals were aware that they lacked the ability and capacity to treat SARS patients and therefore implemented additional ICMs to prevent an outbreak. Smaller hospitals were more likely than larger hospitals to restrict the patients from being transferred in than to transfer out suspected case-patients during the epidemic. Placing the suspected SARS patients in an isolation room was recommended. However, most hospitals, both public and private, found this a considerable challenge (20–22). Lack of isolation rooms became the key reason for transferring patients out and restricting the transfer of patients in. Because of the difficulty of isolating all suspected patients, implementing additional ICMs became the best strategy for most emergency departments. This strategy may have resulted in more patients with fever being transferred to a large hospital, thereby exposing these hospitals to a high risk of an outbreak. An inadequate number of isolation rooms will still be a problem in the next large-scale epidemic.

Use of PPE and ICMs in Late Stage of Epidemic

Because the effects of SARS on the healthcare system were unknown in early stages, most hospitals had no clearly defined response plan and were unsure when to implement ICMs. In our analysis, use of PPE or ICMs in the emergency department usually began at the outset of the epidemic (outbreak at hospital A). The attitude of most hospital administrators was to keep an eye on the situation, especially in private hospitals. Administrators were concerned that additional ICMs would decrease the volume of

services. This attitude was similar to that of health policymakers in the initial stage who were concerned that the SARS epidemic would have a devastating effect on the nation's economy and would cause widespread panic. Few hospitals actually prepared an infection response plan in the early stage that included the preparation of PPE and design of ICMs. Some hospitals did not even begin to consider how to implement these measures until they were directly facing the SARS epidemic.

PPE and ICMs were a financial hardship for private hospitals (>80% of hospitals in Taiwan). The reimbursement for private hospitals comes from the Bureau of National Health Insurance, depending on what services are provided. During the SARS epidemic, the overall volume of patients decreased, which affected the income of emergency departments and hospitals (23). In a future epidemic, without immediate government assistance at the crucial early stage, the effectiveness of hospitals' response will be reduced.

Implementing Additional ICMs

Implementing basic ICMs was easier and more effective than implementing additional ICMs. Most of the basic ICMs were directly ordered by the DOH, so hospitals were required to fully support the emergency departments. This fact may explain why these basic ICMs were rated low in difficulty to implement and thought to have high effectiveness. Whether additional ICMs protected smaller hospitals is unclear, but these measures did appear to decrease the risk of an outbreak in lower level hospitals. However, difficulty and effectiveness of ICMs had a significant negative correlation. This finding may have been because physicians were required to spend more time communicating with patients or with outside hospitals, which also had the effect of causing the number of complaints and disagreements between physicians and patients to rise. The effectiveness of additional ICMs could be increased by making their implementation less difficult. Some of these additional ICMs will place a great strain on the healthcare system and render it incapable of functioning normally. Early recognition and rapid initiation of infection control precautions are the most important strategies for controlling large-scale infectious disease outbreaks (24). If recognizing a new or large-scale contagious infectious disease in the early stage is not possible, implementing additional ICMs in hospitals, especially smaller hospitals, may be unavoidable because the first priority for hospital managers is to prevent a hospital outbreak. To avoid disrupting the healthcare system when additional ICMs are implemented, the DOH should do its utmost to provide full financial support and other assistance. If equal support from the DOH for all hospitals is not practical, a centralized system for suspected patients may be considered as a strategy to reduce the severity and extent of an epidemic. This strategy may decrease the high incidence of person-to-person transmission in larger hospitals and may enhance the ability of smaller hospitals to treat patients with suspected cases. Implementing a centralized system of quarantine is controversial, however, because ethical issues are involved (25).

The response rate of the present study was low, so results may have been affected by nonresponse bias. The nonresponding emergency departments may have had less effective systems in place when they responded to the SARS epidemic. Thus, the degree of variability among emergency departments may have been underestimated. In addition, little seasonal variation in emergency department volume occurs in Taiwan, and any seasonal variation in the 3-month period was likely to be negligible.

The findings of this study suggest that policymakers should understand the different abilities of hospitals to respond to an epidemic. In addition, support and control measures should be implemented more effectively and made immediately available to all hospitals, whether public or private. Understanding the ability and capacity of different hospitals to respond to a contagious disease will enable policymakers to design effective infection control measures to safeguard the health of the nation.

Dr. Chen is chief of staff of the emergency department of China Medical University Hospital, a medical center and teaching hospital in central Taiwan. His primary research interests include disaster response planning and emergency department management.

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Address for correspondence: Wei-Kung Chen, Department of Emergency Medicine, China Medical University Hospital, No 2, Yuh-Der Rd, Taichung City, 404, Taiwan, ROC; fax: 886-4-22031311; email: ercwk@www.cmuh.org.tw

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Human Metapneumovirus Genetic Variability, South Africa

Herbert P. Ludewick,* Yacine Abed,† Nadia van Niekerk,* Guy Boivin,† Keith P. Klugman,*‡ and Shabir A. Madhi*

The molecular epidemiology and genetic diversity of the human metapneumovirus (hMPV) were characterized for a 3-year period (2000–2002) from viruses that were identified in South Africa. Two major genetic groups (A and B) and 2 subgroups (1 and 2) of hMPV were identified, as well as 2–6 possible genotypes within the subgroups. A shift in the predominant group was documented in successive seasons. Whereas the F gene was relatively conserved between subgroups, a high degree of variation was observed in the extracellular domain of the G gene of the virus. The G protein identities between groups A and B were 45.1%–53.1% at the nucleotide level and 22.4%–27.6% at the amino acid level. These results provide evidence for the diversity of both surface glycoproteins of hMPV in Africa, which may be a prerequisite to understanding protective immunity against hMPV.

Human metapneumovirus (hMPV) is an important cause of acute respiratory tract infections worldwide in both children and adults (1–11). It causes annual epidemics during the winter-spring months in temperate regions. Taxonomically, hMPV belongs to the family *Paramyxoviridae*, subfamily *Pneumovirinae*, and is the only known human pathogen of the genus *Metapneumovirus* (1,12). Genetically, its closest relative is the avian pneumovirus type C (1,13,14); however, clinically, it resembles the respiratory syncytial virus (RSV) (15), a common respiratory pathogen classified in the family *Paramyxoviridae*, subfamily *Pneumovirinae*.

Genetic studies on hMPV have demonstrated the presence of 2 distinct hMPV groups and subgroups within these groups (2,4,7,10,14–18); more recently, evidence has been shown that multiple lineages may exist (19,20). Limited available data indicate that both groups can circulate in a single season with the possibility of the predominant group switching in successive seasons (2,4,17,21). Genetic variation in the hMPV attachment glycoprotein (G protein) indicates a high degree of nucleotide variation, which results in amino acid changes (17–19). This sequence variation within the hMPV G gene has been postulated to be due to immunologic pressure. Our study was designed to examine the extent of genetic variation and the circulation pattern of hMPV in a single South African community in 3 consecutive years (2000–2002) by sequence analysis of the 2 surface (F and G) glycoprotein genes from South African strains.

*University of the Witwatersrand, Johannesburg, South Africa; †Laval University, Quebec City, Quebec, Canada; and ‡Emory University, Atlanta, Georgia, USA

Materials and Methods

Specimens

Nasopharyngeal aspirates were obtained from children enrolled in a vaccine efficacy study that was conducted in Soweto, South Africa (22). The samples were obtained from children hospitalized for a lower respiratory tract infection in a 3-year period (2000–2002). Samples were stored at –70°C until processed for this study. Details regarding the cohort of children, procedure for collecting nasopharyngeal aspirate samples, and other viruses isolated from these samples have been published in part (22,23). The samples used in this study were from the entire year and not confined to samples obtained from the winter-spring months. Because of resource constraints, we sequenced a minimum of 30% of the hMPV-positive samples from each month; 92 (45%) of 206 hMPV-positive samples were sequenced for the F gene, and 61 (30%) of 206 were sequenced for the G gene. All samples that were sequenced for the G gene were sequenced for the F gene. Viral RNA was isolated from the stored frozen nasopharyngeal aspirate samples by using the QIAamp viral RNA kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. The study was approved by

the Committee for Research on Human Subjects at the University of the Witwatersrand, South Africa.

hMPV Detection of F Gene

A nested reverse transcription–polymerase chain reaction (RT-PCR) assay to amplify a fragment of the hMPV F gene was used to detect hMPV. RT-PCR was performed with the SUPERSCRIPT One-Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA) with primers 5′-ATGTCTTGGAAAGTGGTG-3′ (corresponding to nucleotide position 3052–3069 in the NL/1/00 genome accession no. AF371337) and 5′- CCATGTAAATTACGGAGCT-3′ (nucleotide position 3844–3862 in NL/1/00 in genome) under the following conditions: 50°C for 30 min; 94°C for 2 min; 94°C for 30 s, 45°C for 45 s, and 68°C for 1 min for 35 cycles; 68°C for 7 min.

The nested PCR was performed with primers 5′-TCATGTAGCACTATAACT-3′ (nucleotide position 3130–3149) and 5′-TCTTCTTACCATTGCAC-3′ (nucleotide position 3794–3810) under the following conditions: 94°C for 2 min; 94°C, 48°C, and 72°C for 1 min for 30 cycles; and 72° for 7 min. The PCR product was analyzed by electrophoresis on a 2% ethidium bromide–stained agarose gel.

hMPV Detection of G Gene

The G gene open reading frame (ORF) was amplified with the following primers: HMPVGunivF: 5′-GAGAACATTCGRRCRATAGAYATG-3′ (nucleotide position 6262–6285 of NL/1/00, GenBank accession no. AF371337) and HMPVGunivR: 5′-AGATAGACATTRACAGTGGATTCA-3′ (nucleotide position 7181–7204) under the following conditions: 50°C for 30 min; 95°C for 3 min; 94°C for 1 min, 59°C for 1 min, and 72°C for 2 min for 38 cycles; and 72°C for 7 min. The PCR product was analyzed on a 2% ethidium bromide–stained agarose gel. When necessary to increase the yield for sequencing, a nested PCR was performed with the same primer set.

Sequencing of hMPV F and G Genes

The PCR product generated for both F and G genes was purified with the QIAquick gel extraction kit (Qiagen, Inc.) and sequenced in both directions by using the nested primers for the F gene and the primers used for detecting the G gene. The PCR product was sequenced by using the BigDye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on the ABI 310 Genetic Analyzer (Applied Biosystems).

Phylogenetic Analysis

Nucleotide sequence alignments were generated with the ClustalX 1.81 software (24). Phylogenetic analysis was performed by using MEGA version (2.1) (25). Strains from the Netherlands, NL/1/00, NL/17/00, NL/1/99, and NL/1/94 (GenBank accession nos. AF371337, AY304360/AY296021, AY304361/AY296034, and AY304362/AY296060, respectively) and Canada, CAN97-83, hMPV13-00, CAN98-75, and hMPV33-01 (GenBank accession nos. AY485253/AY145296, AY485232, AY485245/ AY145289, and AY485242, respectively) were used as prototypes of the 2 groups and subgroups. The hMPV sequences for the F and G protein genes presented in this article have been deposited in GenBank under the accession numbers AY694693–AY694784 and AY848859–AY848919, respectively.

Results

A total of 2,802 samples collected in the 3-year study period were analyzed by RT-PCR for hMPV by amplification of the F gene protein. hMPV was identified in 206 (7.4%) samples in the 3-year period. One hundred one (9.6%) of the 1,057 samples from the year 2000, 82 (7.3%) of 1,128 samples from 2001, and 23 (3.7%) of 617 samples from year 2002 were positive for hMPV.

Molecular Epidemiology of hMPV

We examined the circulation pattern of hMPV during the 3 years by sequencing part of the hMPV F gene and performing phylogenetic analysis for 92 hMPV-positive samples. These samples, numbering 40, 34, and 18 from years 2000, 2001, and 2002 respectively, were distributed over all the months when hMPV was identified and accounted for ≈30%–100% of the samples from each month. Phylogenetic analysis indicated 2 major groups (A and B), each divided into 2 subgroups (1 and 2), causing a complex circulation pattern over the course of the study. Both groups A and B viruses cocirculated throughout the study period. Of the 92 hMPV-positive samples that were categorized, 56 (60.9%) of the viruses belonged to group A and 36 (39.1%) to group B.

During the 2000 epidemic, subgroups B2 and A2 cocirculated, with 72.5% of the circulating viruses belonging to subgroup B2. In 2001 subgroups A1, A2, and B2 cocirculated. Subgroup B2 virus significantly declined (4 [11.8%] of 34) in 2001 compared to 2000 (29 [72.5%] of 40), $p<0.0001$. Subgroup A1 emerged as the dominant strain, causing 67.7% of infections, compared to 20.5% observed for subgroup A2 and 11.8% for subgroup B2. Subgroup A1 also dominated in 2002, causing 83.3% of infections, and cocirculated with the emergent subgroup B1.

Sixty-one (66.3%) of the 92 hMPV-positive samples sequenced for the F gene were further sequenced for the G gene (Figure). Phylogenetic analysis of both the F and G gene nucleotide sequences also showed 2 major genetic groups (A and B) that could be further divided into 2 subgroups (1 and 2). The existence of these 2 major genetic

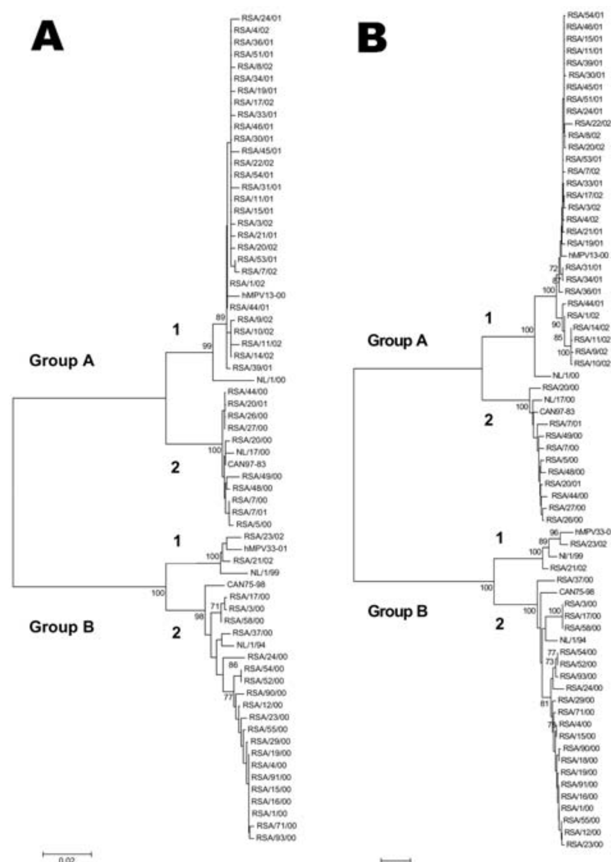

Figure. Neighbor-joining trees based on nucleotide sequences from A) the partial F gene and B) the G gene open reading frame from 61 South African human metapneumovirus (hMPV) isolates. The trees were computed with MEGA version 2.1 with the nucleotide Kimura 2-parameters. Bootstrap probabilities for 500 replicas are shown at the branch nodes. Only values of 70% to 100% are indicated. Isolates from South Africa are indicated by RSA, followed by the isolate number and year (e.g., RSA/18/02). The viruses from Canada (CAN97-83, hMPV13-00, CAN75-98, and hMPV33-01) and the Netherlands (NL/1/00, NL/17/00, NL/1/99, and NL/1/94) are prototypes from each subgroup.

groups was strongly supported by bootstrap values (100% of bootstrap replicas into 2 major groups and 99%–100% of bootstrap replicas into 2 minor subgroups).

From the topology of the trees, subgroup B2 was the most divergent. Although the South African hMPV clustered with both Canadian and Netherlands prototypes, the South African subgroup A1 virus clustered more closely with the Canadian prototype.

From the topology of the tree and supported by strong bootstrap values (70%–100%), the subgroups can be further divided into genotypes. We attempted to group the G sequences of the 4 subgroups into genotypes by using the criteria previously described for RSV (26), in which sequences were arbitrarily considered a genotype if they clustered together with bootstrap values of 70% to 100% (internal nodes at the internal branches). When these criteria were used (only on South African isolates), subgroup A1 could be divided into 5 genotypes, subgroup A2 into 2 genotypes, B1 into 2 genotypes, and B2 into 6 possible genotypes.

Genetic Variation in South African hMPV Isolates

The estimated nucleotide and amino acid identities showed a high percentage of identity for the F gene and more variability for the G gene (Table). The estimated identities for the F gene between the 2 major groups, A and B, were 83%–85% at the nucleotide level and 93.2%–95.8% at the amino acid level. In contrast, the G gene estimated identities were 45.1%–53.1% at the nucleotide level and 22.4%–27.6% at the amino acid level. There was also a higher percentage of identity between members of the same group (e.g., A1–A2) for the F gene than for the G gene (Table).

Amino acid alignments of hMPV F gene were compared to those of prototype isolates from the Netherlands and Canada (data not shown). Cysteine residues were conserved in all South African strains at positions 60 and 182. Group-specific amino acid residue at positions 122, 135, 139, 167, 175, and 233 differentiated between groups A and B. Further amino acid substitutions at various positions were exclusive to subgroups A1 (amino acids [aa] 61, 82, 143), A2 (aa 61, 143, 185), and subgroups B1 (aa 46, 143, 179) and B2 (aa 143).

The predicted G ORF amino acid alignments of selected South African strains with prototypes from the Netherlands and Canada are shown in online Appendix Figure (available at http://www.cdc.gov/ncidod/EID/vol11no07/05-0050-appG.htm). Sequence variation due to nucleotide substitutions and insertions led to variable lengths in polypeptides, which ranged from 228 aa residues (subgroup A2) to 240 aa residues (subgroup B2). The hMPV G ORFs of subgroups A2 and B1 terminated at the TAA codon, whereas the subgroup B2 isolates terminated at the TAG codon. For both genetic groups A and B, a cysteine residue was present in the intracellular domain. In addition, group B isolates had a cysteine residue in the extracellular domain except in 2 isolates (RSA/71/00 and RSA/90/00).

The region of the predicted G ORF sequenced in this study had a high serine and threonine content (30.7%–34.9% for group A, 30.6%–36.6% for group B isolates). Proline content varied among the subgroups: 7.6%–9.0% for subgroup A2, 9.0%–9.9% for subgroup A1, 7.8%–8.7% for subgroup B1, and 3.7%–5.2%, the lowest content, for subgroup B2. Only 1 potential N-linked glycosylation site was conserved at the junction of the intracellular and transmembrane domains.

Table. Human metapneumovirus F and G gene nucleotide and amino acid identities of South African strains in 3 consecutive years (2000–2002)

Gene	Subgroups	% nucleotide (amino acid) identities			
		A1	A2	B1	B2
F	A1	99–100 (98.4–100)	93–95 (96.3–97.9)	83.8–84.1 (93.2–94.3)	82.7–84.5 (94.3–95.8)
	A2		99–100 (99.4–100)	83–83.8 (94.3)	83.1–85 (95.3–95.8)
	B1			98–100 (100)	93–95 (98.4)
	B2				96–100 (99.4)
G	A1	95.4–100 (87.5–100)	72.8–74.7 (55–63.6)	45.9–47.8 (24.3–26.2)	47.4–48.7 (22.4–26.7)
	A2		95–100 (88.6–98.1)	50.3–51.8 (25.4–27.7)	51–53.1 (23.6–27.6)
	B1			93.2 (87.9)	77.4–80.5 (58.2–62.8)
	B2				93.2–100 (82.8–100)

Discussion

Genetic variability is a strong indicator of positive selection and affects the ability of a virus to continue circulating in a population. This variability poses a challenge for future vaccine development that relies on worldwide molecular epidemiologic studies. Recently, the hMPV G gene was shown to be highly variable, particularly in the extracellular domain, as a result of nucleotide substitutions, insertions, and the use of alternative termination transcription codons (17–19). Limited data have also indicated that the 2 groups of hMPV cocirculate and that different subgroups may predominate from year to year (2,4,17,21).

We report on the largest community-based phylogenetic study of hMPV for both surface glycoproteins and provide evidence on the circulation pattern of hMPV in a single African community in 3 consecutive seasons. We also provide evidence for the presence of multiple lineages and genotypes of hMPV, as has been previously observed for other respiratory viruses such as RSV (26,27).

Phylogenetic analysis based on nucleotide sequences of the F and G ORFs of the South African strains demonstrated the existence of 2 groups (A and B) and 2 subgroups (1 and 2). Using the criteria described for the existence of multiple lineages for RSV (26), we demonstrated that multiple lineages of hMPV are circulating in South Africa; however, these lineages need to be characterized at the antigenic level and the clinical impact characterized.

Strains from both hMPV groups cocirculated in South Africa (Soweto, Johannesburg), but not all 4 subgroup viruses cocirculated in a single year, evidence for a complex circulation pattern that permits hMPV to evade preexisting immunity. In 2000, subgroups A2 and B2 cocirculated; in 2001, A1, A2, and B2 cocirculated; and in 2002, A1 and B1 cocirculated. A switch in predominant subgroup from B2 to A1 was observed from the 2000 to the 2001 epidemic. Subgroups A2 and B2 also declined in subsequent years, and subgroup B1 emerged in 2002. The absence of subgroup B1 in previous years may have been due to preexisting community immunity rather than diagnostic assay limitations, as has been speculated (21). Noting the trend in our results, we speculate that the emergence of subgroup B1 virus may eventually have led to the displacement of subgroup A1 as the dominant viral strain in subsequent years. Similar findings in changes of the dominant group of virus that emerges, fostered by a high prevalence of preexisting community immunity to the other major viral group, have been documented for RSV (26–29). Our study, and another from the Southern Hemisphere (21) showed a high prevalence of subgroup A1 in 2001, a finding that suggests that specific strains may coexist across geographic areas in a given epidemic.

hMPV in this study was sequenced directly from specimens, which avoided any amino acid changes due to cell culture adaptation of the viral surface proteins. Although we only sequenced part of the F gene ORF, our results concur with those of a previous study that sequenced the full-length F gene and showed it to be highly conserved (18). In contrast, a high degree of variation was observed for the G gene at the nucleotide and amino acid levels. The sequence variation in the G gene was due to nucleotide substitutions, in-frame insertions, and the use of alternative termination transcription codons. The in-frame insertions we observed suggest that the nucleotide changes previously seen (17) were not due to the passage of hMPV in cell culture. Structural features of the G protein for both groups of South African strains were similar to those observed by others (17,19) with a high serine-threonine content (31%–36%) and variable numbers and positions of N-linked glycosylation sites. The N-linked glycosylation site at the junction between the intracellular and transmembrane domains (position 30–32) was the only conserved site among all groups. We also only observed 1 conserved cysteine residue in the intracellular domain, at amino acid position 27 of the G gene. The second cysteine residue in the extracellular domain, at position 65, previously reported to be present in all group B isolates (17), was absent from 2 South African group B isolates. Hydrophobicity plot data (data not shown) were also similar for both groups A and B, and as reported by others (17,19).

Although our study is limited by the number of hMPV strains sequenced in the study period and we performed partial sequencing of the F gene, we showed that the circulation pattern of hMPV is complex and that the circulation of multiple lineages may suggest an attempt at evasion of preexisting immunity. Our findings also suggest that extended surveillance, over many years, may be necessary to understand the molecular epidemiology of hMPV in any given geographic area.

Mr. Ludewick is a doctoral student at the University of the Witwatersrand in South Africa. His primary research interest is the molecular epidemiology of respiratory pathogens.

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Address for correspondence: Shabir A. Madhi, PO Bertsham, Chris Hani-Baragwanath Hospital, Department of Pediatrics, Bertsham, Gauteng 2013, South Africa; fax: 27-11-989-9886; email: madhis@hivsa.com

Norovirus Recombination in ORF1/ORF2 Overlap

Rowena A. Bull,* Grant S. Hansman,† Leighton E. Clancy,*‡ Mark M. Tanaka,* William D. Rawlinson,*‡ and Peter A. White*

Norovirus (NoV) genogroups I and II (GI and GII) are now recognized as the predominant worldwide cause of outbreaks of acute gastroenteritis in humans. Three recombinant NoV GII isolates were identified and characterized, 2 of which are unrelated to any previously published recombinant NoV. Using data from the current study, published sequences, database searches, and molecular techniques, we identified 23 recombinant NoV GII and 1 recombinant NoV GI isolates. Analysis of the genetic relationships among the recombinant NoV GII isolates identified 9 independent recombinant sequences; the other 14 strains were close relatives. Two of the 9 independent recombinant NoV were closely related to other recombinants only in the polymerase region, and in a similar fashion 1 recombinant NoV was closely related to another only in the capsid region. Breakpoint analysis of recombinant NoV showed that recombination occurred in the open reading frame (ORF)1/ORF2 overlap. We provide evidence to support the theory of the role of subgenomic RNA promoters as recombination hotspots and describe a simple mechanism of how recombination might occur in NoV.

Noroviruses (NoV) are divided into 5 genogroups (I–V) based on genome sequence (1). NoV genogroups I and II (GI and GII) are now recognized as the predominant worldwide cause of outbreaks of acute gastroenteritis in humans (2,3). NoV are small round virions 27–35 nm in diameter and possess a single-stranded, positive-sense RNA genome of 7.5 to 7.7 kb. The genome includes 3 overlapping open reading frames (ORFs) (4). The first ORF (ORF1) encodes a polypeptide with regions of similarity to helicase, cysteine proteinase, and RNA-dependent RNA polymerase (RdRp)-encoding regions of picornaviruses (5). ORF2 encodes a viral capsid protein (VP1), and ORF3 encodes a minor structural protein (VP2) associated with VP1 stability (6).

*University of New South Wales, Sydney, New South Wales, Australia; †University of Tokyo, Tokyo, Japan; and ‡Prince of Wales Hospital, Randwick, New South Wales, Australia

RNA recombination is among the major driving forces of viral evolution (reviewed in [7,8]). Recombination in viruses can greatly affect phylogenetic groupings, confuse molecular epidemiologic studies, and have major implications in viral vaccine design. A recombinant NoV can be defined as one that clusters with 2 distinct groups of NoV strains when 2 different regions (normally the capsid and polymerase) of the genome are subjected to phylogenetic analysis. The prototype Snow Mountain virus was the first reported naturally occurring recombinant NoV (9). Recently, 4 additional naturally occurring human recombinant strains have been reported: Japanese isolates Saitama U1 and the only reported GI recombinant WUG1 (10), the Thai isolate Mc37 (11), and Arg320 from Argentina (12) (Table). One recombinant strain closely related to Saitama U1 and 2 strains closely related to Mc37 have also recently been reported (13). Furthermore, outside of NoV GII but within the *Caliciviridae*, 2 recombinant NoV genogroup III strains associated with diarrhea in cattle (14,15) and a recombinant sapovirus (16) have also recently been reported. Analysis of these recombinants has suggested that the recombination points (or breakpoints) were near the ORF1/ORF2 overlap (9–12,14–16); however, this hypothesis has not been proven.

The aims of this study were to characterize and compare 3 recombinant NoV sequences isolated in Sydney with other published recombinant NoV and those identified through database searches and phylogenetic analysis. The genetic relationship among all identified recombinants was explored and the recombination breakpoint accurately determined. A model of NoV recombination is proposed.

Methods

Stool samples were thawed on ice from storage at –80°C and a 20% (vol/vol) stool suspension of total volume 1 mL made in water (pH 7.0). The sample was centrifuged for 1 min at 13,000 × *g*; the supernatant was then removed and centrifuged for a further 7 min at 13,000 × *g*.

Table. Norovirus (NoV) recombinant strains and their close relatives

Prototype NoV recombinant strain (ref.)*	Sequence length		Parental strain‡		Genotype of recombinant§		Breakpoint¶	Related strains (>96%)	
	RdRp†	Capsid	RdRp†	Capsid	RdRp†	Capsid		Isolate name	Accession no. (ref.)
Arg320/1995/AR (12)	872	1647	Lordsdale	New Orleans/279	novel	GII.3	4981	Sydney 2212	AY588132 (this study)
Sydney C14/02/AU (this study)	420	550	Hawaii	Mexico	novel	GII.3	5108	Bad Berleberg	AF409067
								Herzberg	AF539439
								Oberhausen 455	AF539440
								Paris Island	AY652979
								OS120458	AB071035
Picton/2003/AU (this study)	420	550	Pont de Roide AY682549	Richmond	novel	GII.1	5039	Gourdon 78	AY580335
Saitama U1/02/JP (10)	1527	1666	Lordsdale	Hawaii	GII.4	GII.12	5038	Honolulu	AF414420
								gifu 96	AB045603
								Schwerin	AF397905
								9912-02F	AB044366 (13)
Mc37/03/TH (11)	1527	1647	Lordsdale	New Orleans/306	GII.4	GII.10	5108	Vietnam 026	AF504671 (13)
								Vietnam 0703	AY237442 (13)
Snow Mountain 1/76/US (9)	420	1629	Hawaii	Melksham	novel	GII.2	4981	None found	NA
E3/1997/Crete (unpub.)	872	564	Lordsdale	Melksham	GII.4	GII.2	5068	None found	NA
VannesL23/1999/FR (unpub.)	815	576	MOH	Richmond	GII.5	GII.1/ GII.12	5039	Tiffin	AY502010
S63/1999/FR (unpub.)	872	576	Melksham	MOH	GII.2	GII.5	5117	None found	
WUGI/02/JP AB081723 (10)	3370	1620	Southampton/ 91 L07418	BS5 AF093797	GI.4	GI.2	5359	None found	

* All strains belong to genogroup II except for WUGI/02/JP, which belongs to genogroup I.
† RdRp, RNA-dependent RNA polymerase.
‡Strain used to determine breakpoint, closest matching strain in the database where enough sequence data were available for analysis. GenBank accession nos. are in Figure 1 unless stated.
§For NoV GI (strain WUGI/02/JP), the classification system of Katayama et al. (10) was used; for GII (all other strains), the classification system of Vinjé et al. (23) was used. Closely related sequences are underlined.
¶Breakpoint determined by using the method of Smith (20) relative to Lordsdale nucleotide position for NoV GII (open reading frame [ORF]1/ORF2 overlap 5085–5104) and Norwalk for NoV GI (ORF1/ORF2 overlap 5358–5374). p value <0.0001.

Viral RNA was extracted by using the QIAmp Viral RNA kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Amplification of the capsid region and a portion of the polymerase region was carried out as described previously (17). Amplification of a 507-bp region of the putative recombinant Sydney 2212/98/AU (corresponding to nucleotides 4610–5117 in Lordsdale virus, GenBank accession no. X86557) encompassing the 3′ end of the polymerase region and the 5′ end of ORF2 was achieved by using a nested reverse transcription–polymerase chain reaction approach. In brief, outer primers CB1 (17) and NoV2oR (5′-GTR AAC GCR TTY CCM GC-3′) (R = A or G, Y = C or T, M = A or C) and inner primer pairs 2212F (5′-GTG AGC ACA GAT ATM AAM TTA-3′) and 2212R (5′-AGA TGG AGY GGC GTC ATT CG-3′) were used in reaction conditions, as described previously (17). The ORF1/ORF2 overlap and flanking polymerase and capsid regions of another 2 suspected recombinants, NoV/Sydney C14/02/AU and NoV/Picton/ 03/AU, were amplified with hep170 (5′-TCH TTY TAT GGT GAT GA-3′) and GV29 (5′-CAA GAM ACW GTR AAM ACA TCA TCM CCA G-3′) (W = A or T) to produce a 1,070-bp product. Products were sequenced directly on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Recombinant NoV were identified by constructing 2 phylogenetic trees, 1 using 420 bp of the 3′ end of the RNA polymerase region and the other using 550 bp of the 5′ end of ORF2. Strains that did not cluster with the same group of viruses in both trees were considered putative recombinant strains. Evolutionary distances between sequences were determined by using the GCG program,

DNAdist (Kimura 2-parameter method) (18). The computed distances were used to construct phylogenetic trees with Fitch (18). To gain an internal estimate of how well the data supported the phylogenetic trees, bootstrap resampling (100 datasets) of the multisequence alignments was carried out with the program Seqboot (18). The consensus tree was calculated with Consense (18). Tree branch lengths were determined by analyzing the consensus tree with Puzzle, and trees were plotted by using the program TREEVIEW (version 1.6.6) (19).

The recombination breakpoint of putative recombinant strains was determined by using 2 methods: the maximum chi-squared method (20) and Simplot (version 2.5) (21). The maximum chi-squared method is recognized as being among the most accurate when compared independently with 13 other methods (22).

Results

Recombinant NoV Strains in Australia

Sydney Cluster Strain NoV/Sydney 2212/98/AU

In 1998, a number of outbreaks of gastroenteritis occurred within daycare centers across Sydney. The etiologic agents were identified as several closely related NoV GII strains, collectively termed Sydney cluster (17). We previously reported that the closest matching strain based on sequence searches using a 298-bp fragment of the RdRp region was the Arg320/95/AR strain (17), a known recombinant NoV (12) (Table). Further sequencing a Sydney cluster isolate, Sydney 2212 (NoV/Sydney 2212/98/AU, GenBank accession no. AY588132) was carried out to determine if this strain, like Arg320, was a recombinant NoV. The collated sequence data from this study and our previous study (17) were 2,446 bp long and encompassed 819 bp of the polymerase gene and the entire capsid region. Phylogenetic analysis of Sydney 2212 placed the polymerase region within the GII.4 cluster (based on the clustering system of Vinjé et al., 2004 [23]), which includes Lordsdale virus, but the capsid region grouped within the GII.3 cluster, which includes the prototype Mexico virus and New Orleans/279 (GenBank accession no. AF414412). Collectively these data demonstrate that Sydney 2212 is also a recombinant GII NoV.

NoV/Sydney C14/02/AU

During February 2002, an outbreak of gastroenteritis occurred at a children's hospital in Sydney; it affected 21 children and staff. Phylogenetic analysis of the NoV strain (NoV/Sydney C14/02/AU, GenBank accession no. AY845056) responsible for the outbreak showed that the capsid clustered in the NoV GII.3 group, which includes prototype NoV strains Mexico and Toronto (Table, Figure 1). The polymerase clustered separately, but it was more closely related to the Melksham (GII.2) virus prototype than Mexico and Toronto viruses. The distinct segregation into 2 different phylogenetic positions strongly suggested that this virus was a recombinant NoV.

NoV/Picton/03/AU

In July 2003, an outbreak of vomiting and diarrhea affecting 71 patients and staff members occurred at an eldercare facility in New South Wales, Australia. The etiologic agent was a NoV GII strain designated NoV/Picton/03/AU (GenBank accession no. AY919139). Phylogenetic analysis of 550 bp of capsid sequence indicated that this strain clustered in the NoV GII.1 group, which includes the Hawaii prototype strain (Table, Figure 1). However, the polymerase did not cluster with Hawaii virus but with the second recombinant that we identified,

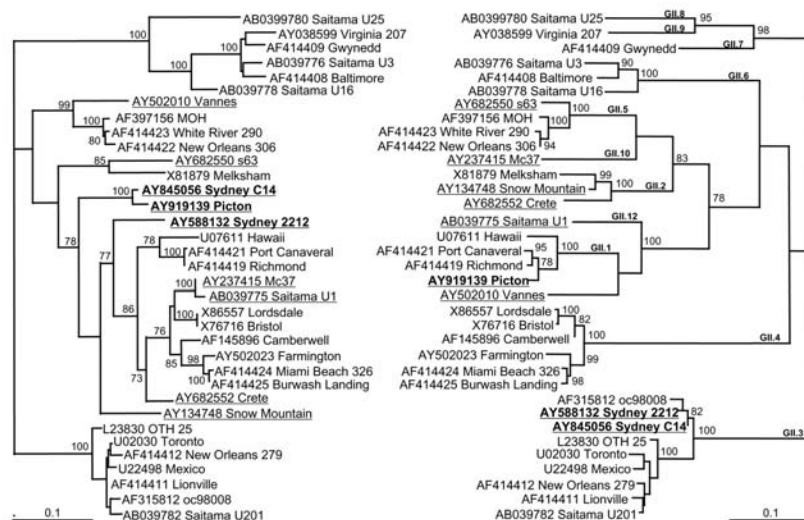

Figure 1. Phylogenetic analysis of the nucleotide sequences of capsid and polymerase regions of 9 identified recombinant norovirus genogroup II strains in relation to 26 known strains and prototype strains. The left tree analyzes the relationship of a 420-bp region of the 3' end of the polymerase region. The right tree shows the relationship of 550 bp of the 5' end of the capsid sequence. Suspected recombinants are underlined to emphasize their different phylogenetic groupings, and strains described in this study are represented in **boldface**. The percentage bootstrap values in which the major groupings were observed among 100 replicates are indicated. The branch lengths are proportional to the evolutionary distance between sequences and the distance scale, in nucleotide substitutions per position, is shown. The capsid clustering is shown in bold and is based on the classification of Vinjé et al. (23) (Table).

namely NoV/Sydney C14/02/AU, and these isolates do not group with any known genotype in the polymerase region. Furthermore, although the capsid region demonstrated 94% sequence identity to Hawaii virus, the polymerase region was unrelated, showing only 85% nucleotide sequence identity. These results indicate that Picton/03/AU was also a recombinant NoV.

Identification and Genetic Relationships

Systematic searches of the GenBank and EMBL databases and phylogenetic analysis identified a number of recombinants (Table). In GII, 9 independent recombinant NoV were identified. Two are published here, 4 were published previously (9–12), and 3 are unpublished. While 3 of the 9 recombinants were unique, 6 had 1–5 close relatives with >96% sequence identity in both polymerase and capsid regions. Sydney 2212/98/AU was closely related to the recombinant Arg320 in both polymerase and capsid regions (Table), with 97% and 96% nucleotide identity, respectively. The second recombinant we identified, Sydney C14/02/AU, was closely related to a number of strains of diverse geographic location, including Oberhausen 455/01/DE, 2 other German strains Bad Berleburg 477/01/DE and Herzberg 385/01/DE, the US isolate Paris Island/03/USA, and the Japanese isolate OS120458/01/JP (Table). The third recombinant NoV identified in the present study, Picton/03/AU, had 1 close relative identified by database searches, Gourdon78/00/FR. Four recombinants were identified that demonstrated >98% identity to the previously published recombinant, Saitama U1 (10), across both the polymerase and capsid regions: Honolulu314/94/US, Schwerin003/00/ DE, Gifu'96/96/JP, and 9912-02F/99/JP. The Thai NoV recombinant Mc37/03/TH (11) had 2 close relatives from Vietnam, while the French recombinant Vannes L23/99/FR was closely related to an isolate from the United States, Tiffin/99/USA. Four additional NoV GII isolates have been reported as recombinants; however, we could not confirm these reports because the polymerase sequence data were not available. These isolates include Seacroft/90/UK (24), Wortley/1990/UK (24), Stepping Hill/01/UK (25), and Harrow/01/UK (25).

For NoV genogroup I, the aforementioned WUG1 (10) was identified as a recombinant (Table), and 2 other strains (NLV/BS5/98/DE, AF093797 and NoV/Saitama KU80GI/99/JP, AB058541) could not be ruled out as recombinants because their polymerase sequences did not cluster with any other strains. Thus, 24 recombinant NoV strains are known to exist: 3 new recombinants identified in the current study, 8 previously published (9–12), and 13 either newly identified or confirmed through database searches and phylogenetic analysis.

Relationships between Regions of Recombinant NoV GII

To determine if genomic regions of the 9 representative recombinant NoV GII sequences (Table) were related to each other, phylogenetic (Figure 1) and pairwise sequence analyses (data not shown) were performed separately for the capsid region and the polymerase regions. Close relationships were found between sections of the identified recombinants (underlined in Table). The 2 Australian recombinants Sydney C14 and Picton were related to each other only in the polymerase region, with 96% nucleotide identity across a 420-bp fragment. However, their capsid regions were unrelated, showing only 73% nucleotide identity. In a similar fashion, the capsid region of Sydney 2212 was 98% identical to the capsid region of Sydney C14, while the polymerase region of Sydney 2212 shared only 85% identity with that of Sydney C14. The Japanese isolate Saitama U1 and the Thai strain Mc37 were related to each other only in the polymerase region, with 97% nucleotide identity across an 819-bp fragment, whereas alignments of their capsid regions demonstrated only 73% nucleotide identity.

Recombination in the ORF1/ORF2 Overlap

By using the maximum chi-squared method (20), the recombination site was placed either immediately upstream (6/9 recombinants) or downstream (3/9 recombinants) of the 20-bp ORF1/ORF2 overlap in genogroup II strains ($p<0.0003$) (Table), and similar results were obtained by using Simplot (Figure 2). Recombination within the ORF1/ORF2 overlap cannot be specifically identified because this region is 100% conserved across all NoV GII sequences. Only 1 recombinant genogroup I strain has been identified, the Japanese isolate WUGI (10). The maximum chi-squared method placed the recombination point within the 17-bp ORF1/ORF2 overlap of this genogroup I isolate ($p<0.0001$) (Table).

Discussion

We identified 3 recombinant NoV GII isolates responsible for outbreaks of acute gastroenteritis in New South Wales, Australia. Phylogenetic analysis of polymerase and capsid sequences of these and other recombinant NoV GII isolates showed 9 recombinant NoV GII sequences. All other recombinant NoV GII were close relatives of these (Table). The 3 NoV GII recombinant sequences identified in this study are constructed from only 2 polymerase sequences and 2 capsid sequences. They share either capsid or polymerase sequences, which suggests that the regions were derived from a pool of circulating viruses. The close geographic relationship of recombinants that share sequences in only 1 part of their genome may indicate the source location of the recombination event. In

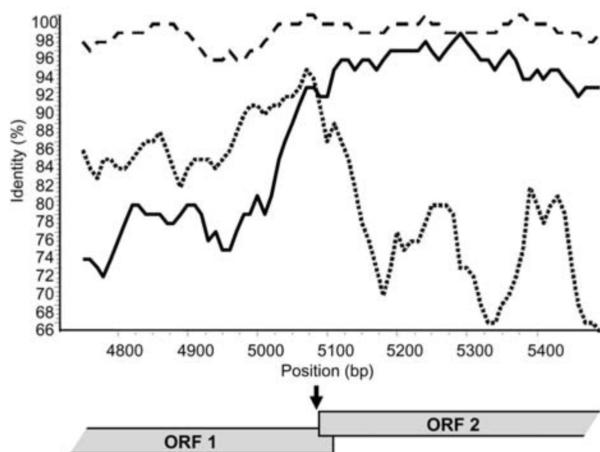

Figure 2. Similarity plot for Sydney 2212. The graph represents as a percentage the identity of the 2 putative parental strains, New Orleans/279 (black line) and Lordsdale (short dash), with the recombinant strain Sydney 2212. The window size was 100 bp with a step size of 10 bp. The site where the 2 parental strains have equal identity to the recombinant (i.e., where the lines cross) is the predicted site of recombination. By varying the window from 20 to 200 bp, the average recombination site was nucleotide 5081 with reference to Lordsdale. The percentage identity of Arg320 (long dash) to Sydney 2212 is also plotted. ORF, open reading frame.

addition to the above example, this phenomenon is seen with 2 isolates from Vietnam, Vietnam 026 and Vietnam 0703, that share polymerase sequence with another Vietnamese isolate 9912-02F (Table) (13). However, the global distribution of recombinants such as Sydney C14, found in Australia, the United States, Germany, and Japan, is evidence against this hypothesis and indicates a much higher prevalence of recombinant strains in relation to other strains than was previously considered.

The putative crossover point was identified on either side of the overlap in 9 recombinant NoV GII (Table). Recombination within the overlap cannot be identified because it is 100% conserved across all NoV GII sequences and masks the breakpoint. This fact and the identification of the breakpoint at position 5359 in the recombinant NoV GI strongly suggest that recombination takes place within the reading frame overlap in NoV. The reading frame overlap and 6–7 bp of downstream sequence are closely related to sequence found at the start of the genome. In NoV GII are 28 bp that are highly conserved at both the 5′ end of ORF1 and around the ORF1/ORF2 overlap, with a consensus sequence of 5′-GTG AAT GAA GAT GGC GTC KAR YGA CGC Y-3′ (bases involved in the formation of stem loop structures are underlined). In NoV GI, 27 bp are highly conserved at the 5′ end of the genome and the ORF1/ORF2 overlap region with a consensus sequence of 5′-GYR AAT GAT GAT GGC GTC KAA RGA CGY-3′. The 2 highly conserved regions for NoV GI and GII contain 2 in-frame and 3 in-frame start codons, respectively. The duplication of a conserved sequence at the start of ORF1 and ORF2 is characteristic of caliciviruses and is seen in all 4 genera, NoV, sapovirus, lagovirus, and vesivirus (5,26–28). This repetition at the 5′ end of the 2 major ORFs led us to consider the role of ORF1/ORF2 as a negative-strand subgenomic RNA promoter site. Indeed, a subgenomic RNA promoter is required for subgenomic RNA synthesis and is often found in close proximity to the 5′ end of subgenomic RNA species (29). The presence of a subgenomic RNA has not been proven in NoV, but it is highly likely based on transcription in related viruses (26,27,30). For example, subgenomic RNA species have been identified, with 5′ ORF2 sequences, in 2 caliciviruses, namely, feline calicivirus (26,27) and rabbit hemorrhagic disease virus (RHDV) (30). The recent and first report of a calicivirus subgenomic RNA promoter in RHDV at the 5′ end of ORF2 provides evidence to support this hypothesis (30). Additionally, RNA promoter regions often have stem loop structures (reviewed in [31]); such structures have been identified within the repeated sequences found at the start of ORF1 and ORF2 of NoV (see sequences above) (28). Taken together, strong evidence exists that the conserved 27/28-bp sequence found at the 5′ end of the NoV genome and ORF2 is part of an RNA promoter sequence.

The primary mechanism involved in recombination in RNA viruses is the copy-choice model (32). In this model homologous recombination is driven by the viral encoded RdRp when pausing occurs during the transcription of a region of secondary structure. The polymerase then loses processivity and switches between RNA templates (reviewed in [7,8]). A number of models of subgenomic synthesis have been proposed, but the most widely recognized is the internal initiation mechanism (33). Here the replicase initiates positive-strand subgenomic transcription internally on a negative-strand copy of genomic RNA (29). Using these 2 well-supported models, we propose a simple mechanism for recombination in NoV (Figure 3). Replication and internal subgenomic RNA synthesis generate 2 positive RNA species. These templates direct RNA synthesis that leads to the generation of both a full-length negative genome and a negative subgenomic RNA species, in the second round of replication. The negative subgenomic RNA is the key player in our proposed model, and such species have been identified in viruses that produce subgenomic RNA (34). We propose that recombination occurs when the enzyme initiates positive-strand synthesis at the 3′ end of the full-length negative strand, loses processivity at the stem loop of the ORF1/ORF2 overlap, then hops across (template switching) to an available negative subgenomic RNA species generated by a co-infecting virus (Figure 3). Alternatively,

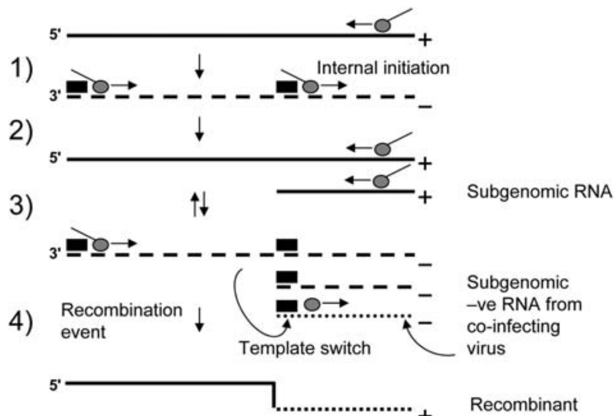

Figure 3. A simple mechanism for recombination in norovirus. 1) RNA transcription by the RNA-dependent RNA polymerase (RdRp) (gray circle) generates a negative-stranded intermediate (dashed line). 2) Binding of the RdRp to the almost identical RNA promoter sequences (filled boxes) generates positive-stranded (straight line) genomes and subgenomic RNA. 3) These templates direct RNA synthesis from the 3´ end that leads to the generation of both a full-length negative genome and a negative subgenomic RNA species. 4) Recombination occurs when the enzyme initiates positive-strand synthesis at the 3´ end of the full-length negative strand, stalls at the subgenomic promoter, and then template switches to an available negative subgenomic RNA species generated by a co-infecting virus. The net result is a recombinant virus that has acquired new open reading frame (ORF)2 and ORF3 sequences.

the RdRp could also template switch directly from 1 genomic RNA to another genomic RNA in the highly conserved ORF1/ORF2 overlap. The net result of both possibilities is a recombinant virus that has acquired new ORF2 and ORF3 sequences.

The decline in the prevalence of previously dominant strains, such as US-95/96 in the United States and Australia (3,17), suggests immunity in the community might be an important factor in reducing further spread of NoV. Recombination offers NoV an attractive mechanism for immune evasion. Subgenomic RNA promoters have been proposed to be recombination hotspots (35,36). In this study we have presented data to support this hypothesis, and we have described a simple mechanism of how recombination might occur in NoV.

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Ms. Bull is a PhD student at the University of New South Wales. She is studying the replication and molecular epidemiology of norovirus.

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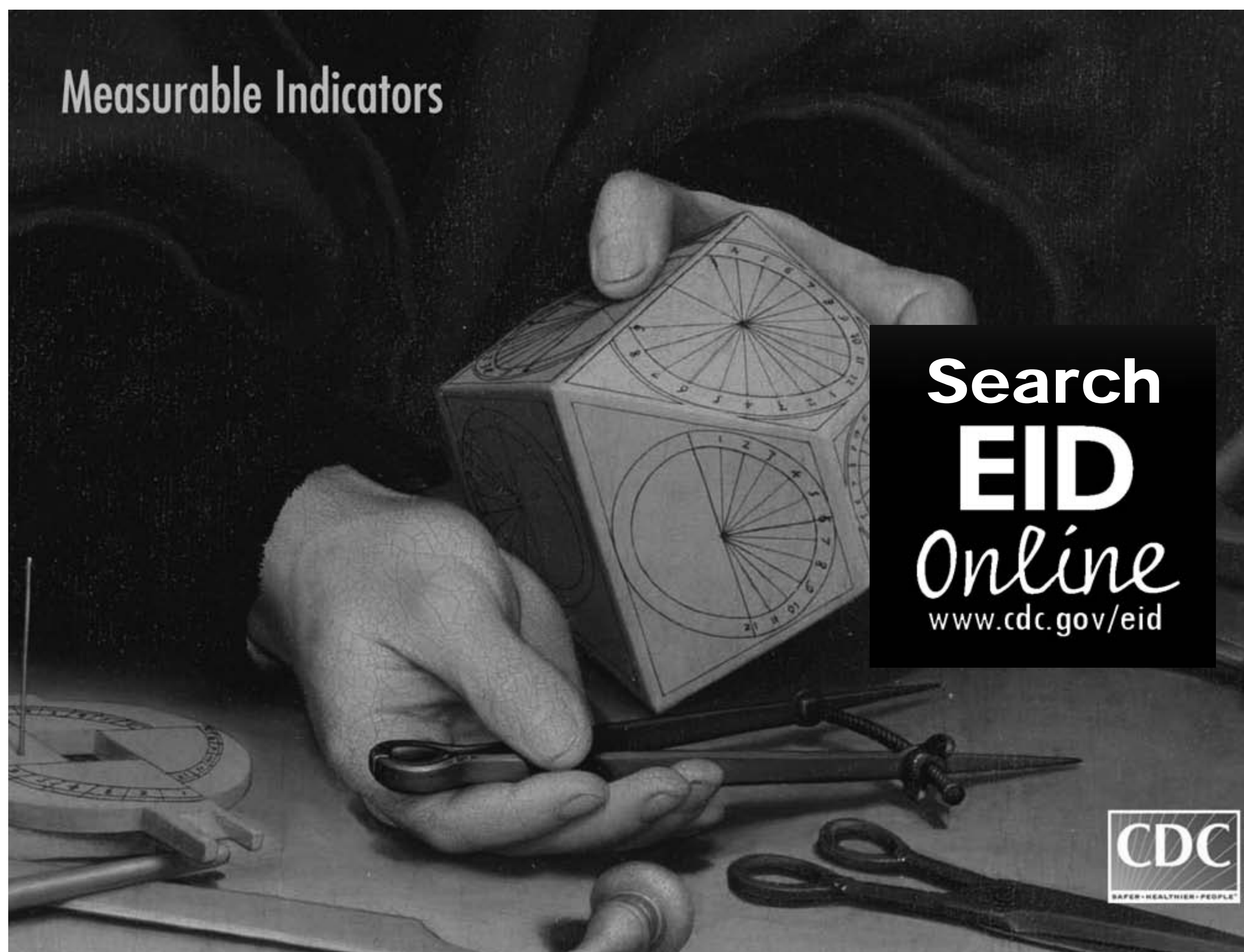
Address for correspondence: Peter A. White, School of Biotechnology and Biomolecular Sciences, Faculty of Science, University of New South Wales, Sydney 2052, New South Wales, Australia; fax: 61-9-385-1591; email: p.white@unsw.edu.au



Adventitious Agents and Smallpox Vaccine in Strategic National Stockpile

Frederick A. Murphy* and Bennie I. Osburn*

In keeping with current standards, we urge that old smallpox vaccines that were made in animal skin and are still a key part of our strategic national stockpile be tested for adventitious infectious agents. The advisory especially applies to viruses that have the potential for zoonotic transmission to human vaccine recipients.

As we studied recent papers on the manufacture and testing of new smallpox vaccine stocks produced for biodefense purposes, we were surprised that the largest part of our national vaccine stockpile, the Wyeth vaccine Dryvax produced in 1980–1982 and the Connaught (now Aventis-Pasteur) vaccine Wetvax produced in the 1950s, has never been scrutinized by modern methods. Of particular concern is the fact that these stocks have never been subjected to testing for adventitious agents, whereas a new vaccine intended to supplement the existing stockpile has been thoroughly tested (1). Testing of these old stocks met the standards of the day. However, if these old vaccines are to be considered valid parts of our national stockpile we should expect not only continuing testing of potency and sterility but also testing for adventitious agents with methods that reflect the standards of today.

This is not to say that the finding of adventitious agents must result in removal of these vaccine stocks. That issue must be a matter for formal risk analysis and consideration by the same experts who review data on new vaccines (e.g., the Advisory Committee on Immunization Practices of the Centers for Disease Control and Prevention) and by officials of the Food and Drug Administration (FDA). However, as greater concern emerges about the potential pathogenicity of infectious agents that might be present in old vaccine stocks, prudence dictates caution and testing.

Such concerns are amplified by the memory of how these old smallpox vaccines were made. Such vaccines were made in the skin of calves and sheep, and seeds and stocks were passaged in tissues of calves, sheep, and rabbits (especially used for seed lot production). Equally important is the fact that for many decades preceding the development of standardized manufacturing methods in the 20th century, the vaccine virus (vaccinia virus) was propagated by serial passages in animals without precise knowledge of the passage history and without use of a seed lot system that stabilized passage level. This uncontrolled system could have allowed amplification of any passenger viruses and could have increased the possibility of untoward changes in their genetic makeup. In addition, since the crude manufacturing methods (Figure) allowed direct contact of the materials harvested from animals with human operators, possibilities existed for contamination of the resulting product with pathogenic human viruses. It was not uncommon practice before the widespread acceptance of vaccine manufacture in animals (at the end of the 19th century) to passage vaccinia virus arm-to-arm between humans (2). Standardized methods for manufacture in animal skin were not initiated until 1925.

Such concerns are further amplified by infectious agents that are targeted by modern vaccine testing protocols. When materials of animal skin origin are used, such agents are *Brucella* spp., *Mycobacterium* spp., *Bacillus anthracis*, *Clostridium* spp., and other bacteria and fungi. Old stocks of vaccine were tested for microbes, and the presence of specific pathogenic species was the basis for rejection of vaccine lots. The specifications for approval of dermal vaccines produced in calves and sheep allowed for the presence of a low concentration of nonpathogenic bacteria and fungi.

However, the dermal vaccines produced from the 1950s to the 1980s and currently in the national stockpile were not tested for mycoplasma or viruses. In the case of vaccines manufactured in calves, the agents of concern include several bovine viruses (bovine viral diarrhea virus,

*University of California, Davis, California, USA

bovine parainfluenza virus type 3, bovine respiratory syncytial virus, bovine adenoviruses, bovine parvovirus[es], bovine herpesvirus 1 [infectious bovine rhinotracheitis virus], other bovine herpesviruses, bovine reovirus[es], rabies virus, bluetongue viruses, bovine polyoma virus, bovine circovirus, Cache Valley virus, and orthopoxviruses other than vaccinia [such as cowpox]). We were unable to find a comprehensive list of possible adventitious agents when ovine materials are used, as is the case for the Lister strain smallpox vaccine produced in Europe and old vaccine stocks held by some European countries for biologic defense. Sheep harbor several members of the same virus groups found in cattle, but they also carry other viruses. Rabbits were sometimes used for intermediate passaging of vaccinia virus stocks and for seed virus production, particularly in Europe. Possible rabbit viruses that could contaminate vaccinia stocks include endogenous retroviruses, papillomavirus, herpesviruses, and leporipoxviruses. Because of research and development of specific pathogen-free swine as special organ and tissue donors for human xenotransplantation over the past decade, the list of possible adventitious agents in materials derived from swine is quite comprehensive (3).

When materials of human origin are used, adventitious agents include HIV-1 and HIV-2; human T cell lymphotropic virus type I (HTLV-I) and HTLV-II; hepatitis A, B, and C viruses; human cytomegalovirus; Epstein Barr virus; human herpesviruses 6, 7, and 8; human parvovirus B19; reoviruses; polyoma (JC/BK) viruses; 5V40 virus; human coronaviruses; human papillomaviruses; influenza A, B, and C viruses; various human enteroviruses; human parainfluenza viruses; and human respiratory syncytial virus.

As mentioned, there is a risk that a human virus could have been introduced into smallpox vaccine seed or vaccine stocks during manufacturing, since barrier methods such as those currently used in all phases of vaccine production were not in place. Although the ability of such human viruses to be propagated in subsequent vaccine lots is uncertain, many human viruses are capable of replicating in animal cells.

When materials from any animal source are used, special consideration is given to exogenous and endogenous retroviruses (e.g., bovine immunodeficiency virus), lymphocytic choriomeningitis virus, adenoassociated viruses, minute virus of mice, and other viruses that are notorious contaminants. However, these special considerations fail to include many infectious agents that should raise concern. However, for old smallpox vaccine stocks, it is enough to question whether any of the infectious agents specifically cited in FDA and European Commission regulations, recommendations, and guidelines are present.

Current regulations, recommendations, and guidelines on testing for adventitious microbial and viral agents from various national and international agencies require nonspecific screening and relevant specific tests. Regulations requiring tests for mycoplasma and viruses came into effect long after old stocks of smallpox vaccine were manufactured. Nonspecific screening tests include classic culture tests for bacteria and fungi (sterility tests), special culture and animal tests for *Mycobacterium* spp., cell culture tests for the presence of certain cytocidal viruses (by inoculation of and blind passage in Vero, MRC-5, HeLa, RK, and A9 cells with observations for cytopathology and tests for hemadsorption and hemagglutination at the end of the culture period), and animal inoculation tests for certain viruses (suckling and adult mice, guinea pigs, and embryonated hen eggs). Electron microscopy is often used to find adventitious agents in cell culture banks.

In the United States, federal regulations specify that products of bovine origin (such as virus preparations, cell lysates, cultured cells, or other reagents) intended for use in the production of human biologics be tested for the presence of specific bovine viruses in accordance with 9 C.F.R. 113.53. Specific tests for adventitious infectious agents are conducted using various polymerase chain reaction (PCR)– and immunochemical–based assays. The extraordinary sensitivity of these assays has served to raise the bar of expectation of test veracity, while improving practicality and containing costs. In many cases, these assays have been validated, that is, proof-tested using salted vaccine and vaccine substrate materials. Since companies exist that conduct these specific tests for vaccine developers and manufacturers, such testing on old smallpox vaccine

Figure. Freeze-dried (smallpox) vaccine being prepared from virus grown on the skin of a calf. The calf is lying on a grate on a table and is bound to the table. Two men in white coverups, 1 of whom has a surgical mask on his face, are performing a procedure on the calf (scarification and introduction of vaccinia virus into the scarified areas). From the record of the US National Library of Medicine; old negative no. 83-168. WHO/11683 SEARO, Smallpox, Bangladesh, SM 5-1 980. (Photograph attributed to J. Mohr, 1980?)

stocks is eminently feasible. The new cell culture-based smallpox vaccine has been tested by using these methods (1). However, considering more advanced PCR-based tests for unknown or unrecognized adventitious agents (e.g., representational difference analysis, use of various degenerate primers) would require extensive research and add substantially to overall costs. For the purpose at hand, we are suggesting only that the battery of specific tests now used on all modern vaccine materials be used.

Concerns about the possible presence of adventitious agents in old smallpox vaccine stocks are amplified further by current concerns about prions and the zoonotic potential of prion diseases. Old smallpox vaccine stocks might not be contaminated by bovine spongiform encephalopathy (BSE) prions, but Lister vaccine stocks that were produced in sheep and vaccine seeds that had been passaged in sheep could be contaminated by scrapie prions. Regulations and guidelines for modern vaccines state that all materials used must come from BSE-free regions but say nothing about scrapie-free regional status. Testing of old vaccines for prions is beyond the sensitivity of any present in vitro prion test, but this issue should be considered (4).

Since no problems related to contamination have been recognized during the long history of smallpox vaccines, or during the intensified program to eradicate smallpox, one might argue that little risk for humans is posed from adventitious agents in old stocks of vaccine. However, it is unlikely that low-incidence untoward events temporally related to adventitious agents have been recognized. It is equally unlikely that diseases that appeared at long intervals after smallpox vaccination would have been associated with the vaccine. Furthermore, since most smallpox vaccine was used in children, we may have less data on its use in adults than we would want. Of note is the recent observation that myopericarditis is a relatively common serious adverse event following smallpox vaccination, but that this complication was not recognized during the era of routine vaccination (5).

Today, the senior guiding document for manufacturers of first-generation smallpox vaccines, i.e., vaccines produced in animal skin, is the World Health Organization (WHO) Recommendations for Production and Control of Smallpox Vaccine, revised 2003 (6). A definitive version of this document will be published in the WHO Technical Report Series (the working version is available from http://www.who.intlbiologicals/Smallpox_final.pdf). FDA documents on this subject are much more general and say little or nothing about adventitious agents (7). The WHO document represents continuation of a series that started in 1956. Several points from the 2003 WHO document (6) are of particular interest here (the chosen points are not meant to be comprehensive or reflect the overall sense of this document).

First, adventitious agent testing for viruses in vaccine virus seeds and product intermediates is complicated and might give ambiguous results. Therefore, newer, more specific tests are planned for the future. Second, testing for viral adventitious agents of animal skin vaccine should take into consideration the source country of the animals. Guidelines for transmissible spongiform encephalopathy testing should be followed. Third, the concentration of nonpathogenic bacteria and fungi in vaccines produced in animal skin may be very difficult to validate, and consistent sterility of the finished product may be difficult to achieve. The use of a nonsterile final product may be justified since smallpox vaccine is administered by scarification rather than by intramuscular or intravenous inoculation, and because its use over many years did not cause problems. Fourth, the general method for testing a live viral vaccine strain for contaminating viruses is to neutralize the vaccine virus and test for adventitious viruses both in vitro and in vivo. However, it is recognized that vaccinia virus is very difficult to neutralize to the extent required for such studies. Additional testing such as nucleic acid amplification techniques for specified viruses and reverse transcriptase assays for retroviruses should complement nonspecific tests. Fifth, in preparing master seed lots, procedures should be used that help remove extraneous agents. Since removal or inactivation of microbial contaminants is unlikely at any downstream level, the presence of extraneous agents in seed lots during the production process must be avoided. Sixth, the absence of specific human pathogens should be confirmed by additional testing, e.g., bacterial and fungal cultures, virus culture, PCR testing for viral agents.

Taken together, these points from the 2003 WHO document make it clear that members of the WHO Expert Committee on Biological Standardization had difficulty dealing with the exceptional problems posed by the first-generation smallpox vaccines, i.e., vaccines produced in animal skin. Their difficulty in producing guidelines also pertained to old lots of such vaccines, such as those that are part of the US and European strategic stockpiles. This seemed most obvious regarding testing for adventitious agents. The limits of such testing seem clear, but so are the practicalities. Standard testing for adventitious agents is practicable and would provide important evidence for risk-benefit considerations if or when old vaccines are used in an emergency situation. Time will eventually obviate such considerations as modern smallpox vaccines replace old vaccines in national stockpiles, but for the present we see the WHO document as another basis for suggesting comprehensive testing of old vaccines.

Since old smallpox vaccine stocks have been in the public domain for many years, we would expect that comprehensive testing would be funded by the same public

agency (the US Department of Health and Human Services) that intends to distribute the vaccines should the need arise. We believe that the testing should be fully transparent, that is, fully open to public scrutiny.

Dr. Murphy is professor in the Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, and professor in the Department of Internal Medicine, School of Medicine, University of California, Davis.

Dr. Osburn is professor in the Department of Pathology, Microbiology and Immunology, and dean of the School of Veterinary Medicine, University of California, Davis.

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Address for correspondence: Frederick A. Murphy, School of Veterinary Medicine, University of California, Surge IV, Room 135, Davis, CA 95616, USA; fax: 530-752-2801; email: famurphy@ucdavis.edu

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New Lymphogranuloma Venereum *Chlamydia trachomatis* Variant, Amsterdam

Joke Spaargaren,* Han S.A. Fennema,* Servaas A. Morré,† Henry J.C. de Vries,‡ and Roel A. Coutinho§

We retrospectively conducted a study of men who have sex with men who visited the Amsterdam, the Netherlands, sexually transmitted diseases clinic from January 2002 to December 2003 and had rectal *Chlamydia trachomatis* infections. We found that symptomatic (73%) as well as asymptomatic (43%) patients were infected with a new *C. trachomatis* LGV variant.

In December 2003, an unusual symptom of early lymphogranuloma venereum (LGV) in a patient infected with HIV-1, who also had proctitis, was reported in Rotterdam (1). In the same city, an outbreak of LGV with similar symptoms, such as proctitis and constipation, subsequently was identified in men who have sex with men (MSM) (2). Here we report 32 patients with, and 13 MSM without, mucous membrane abnormalities in MSM with confirmed LGV in 2002–2003.

LGV is a systemic disease caused by the *Chlamydia trachomatis* serovars L1 to L3. More invasive than disease caused by the urogenital serovars (D–K), LGV can manifest as 1) an inguinal syndrome, with genital ulceration and inguinal lymphadenopathy (buboes) and subsequent suppuration, and 2) an anogenitorectal syndrome, with proctocolitis and hyperplasia of intestinal and perirectal lymphatic tissue. Both syndromes can be accompanied by systemic symptoms including fever, malaise, chills, anorexia, myalgia, and arthralgia. If left untreated, the infection can lead to fistulas, strictures, genital elephantiasis, frozen pelvis, and infertility (3). LGV is endemic in Africa, Southeast Asia, and the Caribbean; it is a sporadic disease in Europe and North America.

*Municipal Health Service, Amsterdam, the Netherlands; †VU University Medical Center, Amsterdam, the Netherlands; ‡ Academic Medical Center, Amsterdam, the Netherlands; and §National Institute of Public Health and Environment, Bilthoven, the Netherlands

The Study

For this study, we selected MSM who were treated at our sexually transmitted disease (STD) clinic (≈20,000 new consultations per year) in 2002 and 2003 with *C. trachomatis* proctitis confirmed by a positive polymerase chain reaction (PCR), COBAS AMPLICOR (Hoffman-La Roche Ltd., Basel, Switzerland). Upon proctoscopic examination by 1 medical practitioner, patients were designated into 2 groups: 1 group with mucous membrane abnormalities (MMA+, n = 44) when mucopurulent anal discharge or bloody, ulcerative rectal lesions were found, and 1 group without MMA (MMA–, n = 30) when those symptoms were not found. Samples were taken by proctoscopic examination. During the study, *C. trachomatis* proctitis was diagnosed in some patients at separate times. Those follow-up samples were excluded from the study. Calculations are based on the number of patients in whom *C. trachomatis* proctitis was diagnosed during their first visit. Patients were treated with a single dose of 1 g azithromycin, the consensus treatment for uncomplicated urogenital *C. trachomatis* infections at that time. Purified *C. trachomatis* DNA obtained from the rectal samples of these 74 patients was used to assess *C. trachomatis* serovars identified by PCR, based on restriction fragment length polymorphism (RFLP) analysis of the *omp*A gene as described previously (4,5). In addition, we sequenced the complete *omp*A gene to identify possible changes at the nucleotide level (ABI 310 automated sequencer, PE Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). The exact sequence methods and primers are described by Morré et al. (5). In short, *omp*A nucleotide sequence analysis was performed in several sequence reactions generating the complete 1.1-kbp order. DNA sequencing was performed in both directions and analyzed by automated DNA sequencing on an ABI 310 sequencer. Sequences were aligned with the BioEdit Sequence Alignment Editor (Ibis Therapeutics, Carlsbad, CA, USA). Reference sequences were derived from GenBank (www.ncbi.nlm.nih.gov/GenBank).

Serum samples from these patients, taken at consultation and stored at –20°C, were used to measure *C. trachomatis*–specific immunoglobulin (Ig) G. This *C. trachomatis*–IgG peptide enzyme-linked immunosorbent assay (pELISA) (Medac Diagnostika mbH, Hamburg, Germany) is based on a synthetic peptide from an immunodominant region of the major outer membrane protein and was performed according to the manufacturer's instructions, as described previously (6). A titer of both ELISAs of ≥1:50 was considered positive, and an arbitrary differentiation was made between low (1:50–1:200) and high titers (>1:200).

Genotyping the *omp*A gene by RFLP of these 74 patients showed that 45 samples were positive for

C. trachomatis all type L2 (Table). Sequencing of the *omp*A gene demonstrated that all L2-positive samples contained a new (based on the National Center for Biotechnology Information BLAST queries) *C. trachomatis* genovariant (Figure), which we designated L2b. The novel sequence was deposited in GenBank (accession no. AY586530).

When the *omp*A sequences of these patients were compared to the prototype sequences of L2 and its variants L2a and L2′, besides 2 already described changes, a new base pair change was found. One change in variable segment 2 was deducted from L2a and L2′, and one from L2. The third change has not been described before. All nucleotide changes resulted in amino acid substitutions. The fourth change was found in constant segment 2 (CS-2) at amino acid 157: the third nucleotide is G in L2b and L1, C in L2, and A in L3. As expected, this mutation is conserved, and all combinations encode for the amino acid glycine. Combining the sequence data with the RFLP typing showed that 32 of 44 samples from MMA+ and 13 of 30 samples from MMA– patients were L2b. In the MMA+ patient group, a positive chlamydia serologic test results mainly an IgG titer 1:≥200, correlated well with the LGV diagnosis. Approximately 80% of all LGV patients had high titers; in the MMA– group, species-specific *C. trachomatis* serologic test results did not correlate with LGV. The patients' characteristics are shown in the Table. Median age of the 45 men with samples positive for *C. trachomatis* was 35.8 years (range 25.9–47.6) compared with 38.1 years (range 25.8–58.2) for the men with samples negative for *C. trachomatis*. All *C. trachomatis*–positive patients lived in the Netherlands, most in Amsterdam, and most were of Dutch ethnic background.

Anal discharge was reported by 15 of 20 patients with LGV. Genital ulcers (all localized to the perianal area) and inguinal lymphadenopathy were found in only a few patients. Ulcers in the 2 patients infected with a non-LGV *C. trachomatis* strain were caused by herpes simplex virus 2 and *Treponema pallidum*. In the 3 ulcers found in the MMA+ patients, the L2b *C. trachomatis* strain was found.

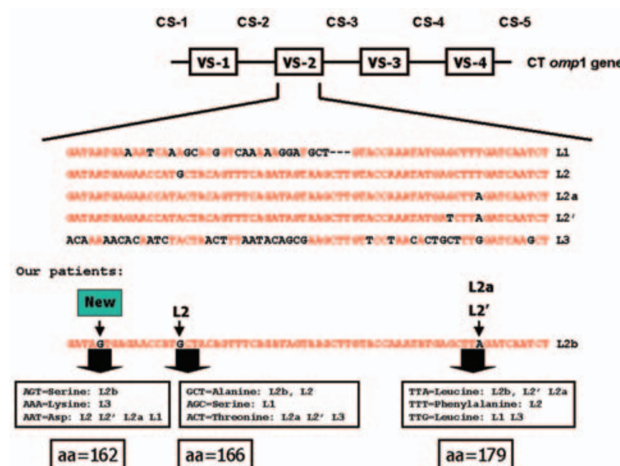

Figure. Schematic representation of the *Chlamydia trachomatis* *omp*A gene. In detail variable segment 2 (VS-2): nucleotide and amino acid (aa) sequence comparison of the prototypes L1, L2, L2 , L2a, and L3 and the newly identified lymphogranuloma venereum (LVG) strain, which we designated L2b. Conserved nucleotides in VS-2 for all LGV strains are shown in red. The nucleotide substitutions in L2b as compared to all LGV strains is indicated by arrows. All aa encoded by the substitution combinations are indicated. CS = constant segment; omp = outer membrane protein.

The mean number of previously documented sexually transmitted infection episodes was 8.3 among the MMA+ LGV patients in contrast to 5.8 episodes in the non-LGV patients. Twenty-four of 30 of the MMA+ LGV patients and 7 of 14 of the MMA– LGV patients were HIV-infected. All patients with a retrospective diagnosis of LGV were

Table. Characteristics of men who have sex with men in retrospective study at sexually transmitted disease outpatient clinic in Amsterdam, the Netherlands*

	MMA+ (2002 and 2003)			MMA– (2003)		
	C. trachomatis			*C. trachomatis*		
Clinical data	L2b	Non-LGV	Total	L2b	Non-LGV	Total
No. patients	32	12†	44	13	17‡	30
Anal discharge	11	3	14	4	2	6
Genital ulcers	3 (L2b)	1 (*Treponema pallidum*)	4	0	1 (HSV-2)	1
Lymphadenopathy	4	0	4	2	2	4
Mean no. infections with sexually transmitted diseases	8.3	5.8	7.8	7	4.6	5.8
Known HIV+	24	6	30	7	7	14
>10 leukocytes by Gram stain	26	3	39	3	6	9
C. trachomatis IgG ≥200	22	2	24	8	6	14
C. trachomatis IgG <200	8§	10	18	4¶	11	15

*MMA, mucous membrane abnormalities; *C. trachomatis, Chlamydia trachomatis;*, LGV, lymphogranuloma venereum; Ig, immunoglobulin.
†2 serum samples were not available for testing.
‡1 serum sample was not available for testing.
§Serogroup B, n = 5; intermediate serogroup, n = 5; serogroup C, n = 2.
¶Serogroup B, n = 10; intermediate serogroup, n = 1; serogroup C, n = 6.

contacted and offered reexamination. If the L2b strain persisted, the patients received doxycycline, 100 mg twice daily for 3 weeks, the consensus treatment for LGV.

Conclusions

We conclude the following: 1) the outbreak of LGV among MSM in the Netherlands expands beyond the cluster reported earlier in Rotterdam and can be traced back to at least January 2002; 2) the outbreak in Amsterdam and possibly the one in Rotterdam was caused by a newly identified L2b variant; 3) both MMA+ and MMA– men are infected with *C. trachomatis* and most of them are HIV-positive; 4) species-specific serology can help support the LGV diagnosis when clinical symptoms are present but cannot be used to detect LGV-infected persons who are asymptomatic.

Although based upon a small, select population, our results justify additional study of high-risk core groups who transmit this LGV genovariant to determine transmission risk factors and diagnostic criteria. The outbreak of LGV is ongoing; we currently see 1–2 new patients per week at our STD clinic.

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Ms. Spaargaren is a medical microbiologist in charge of the public health laboratory of the Muncipal Health Service of Amsterdam. One of her research interests is the epidemiology, pathogenesis, and immunogenetics of *C. trachomatis* infections in the human urogenital tract.

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Address for correspondence: Joke Spaargaren, Public Health Laboratory, Municipal Health Service of Amsterdam, Nieuwe Achtergracht 100, 1018 WT, Amsterdam, the Netherlands; fax: 31-20-555-5629; e-mail: jspaargaren@ggd.amsterdam.nl

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Household Transmission of Gastroenteritis

Sharon Perry,* Maria de la Luz Sanchez,* Philip K. Hurst,* and Julie Parsonnet*

Transmission of infectious gastroenteritis was studied in 936 predominately Hispanic households in northern California. Among 3,916 contacts of 1,099 primary case-patients, the secondary attack rate was 8.8% (95% confidence interval 7.9–9.7); children had a 2- to 8-fold greater risk than adults. Bed-sharing among children in crowded homes is a potentially modifiable risk.

Infectious diarrhea poses a major problem for the US healthcare system and employers. Although <5% of episodes may result in a physician encounter (1), surveillance systems have estimated that 0.72 episodes occur per person-year, and up to 1.1 episodes per year for children <5 years of age (2). Household transmission of infectious gastroenteritis is likely to account for a substantial portion of community incidence. With the exception of a few prospective studies (3,4), studies of household transmission of gastroenteritis have typically reported on community outbreaks of individual pathogens followed up in the home (5–9). In these outbreak settings, secondary attack rates were 4%–20%, depending on pathogen, mode of transmission, and length of time spent in the household. Since household clusters of gastroenteritis may parallel larger community trends (10), information about baseline incidence and risk factors is useful to validate population-based and sentinel surveillance systems.

The Study

Index cases of probable infectious gastroenteritis were identified through 15 participating community health providers; 11 of these were public health clinics serving low-income families. After an initial telephone interview, a home visit was scheduled within 2 weeks of the index episode. Household contacts of the index patient were interviewed regarding symptoms and onset of diarrheal illness. A second visit 3 months later completed documentation of the household episode. The cohort consists predominately of Hispanic families with young children, including a median of 5 persons (range 2–20) per household and a median sleeping density of 2.5 persons/bedroom. Households were excluded if they had <2 participating members or if the living unit was a dormitory or other communal residential arrangement.

Infectious gastroenteritis was defined as 1) diarrheal illness lasting <14 days and marked by symptoms of loose or watery stool occurring at least 5 times per day in a child <2 years of age, or at least 3 times per day in older persons or 2) at least 1 instance of vomiting per day in a person of any age. Illnesses considered noninfectious in origin, such as those due to morning sickness, poisoning, medications, or alcohol, were excluded from the case definition. A primary case was defined as the first household case with onset within 2 weeks of the index referral or onset within 2 days of the first primary case. Secondary gastroenteritis was defined as an illness meeting the case definition of infectious gastroenteritis, beginning at least 2 days after the onset and ≤5 days after the end of an episode in another household contact. A household episode was deemed to have concluded when all members had been symptom-free for at least 5 days. Secondary attack rates were estimated crudely and also modeled by using the life-table method. Risk factors associated with secondary transmission were assessed with logistic regression, with an exchangeable correlation matrix to account for household clusters.

From 1999 to 2004, a total of 3,747 index referrals were received; 2,094 (56%) persons could be contacted by phone and met initial eligibility criteria. Of these, 830 (40%) declined participation, and 1,264 (60%) were scheduled for a home visit. After the initial home visit, 1,154 households were enrolled, and 102 were excluded because the index episode did not meet the case definition (n = 80), the household had <2 interested members (n = 20), or someone in the household had participated in a prior study (n = 2). Of the 1,154 households enrolled, 936 (81%) completed documentation of ≥1 household gastroenteritis episode. These 936 households had 5,783 members. Of these, 5,015 (87%) gave reports sufficient to classify symptoms as primary, secondary, or absent; 557 (9.6%) did not participate in interviews; and 211 (3.6%) reported diarrhea, vomiting, or both that could not be classified temporally. Of participating members, 24% were ≤5 years of age, 18% were 6–17 years of age, and 58% were ≥18 years of age.

Household episodes lasted a median of 9 days (range 5–29 days) and involved 1–6 household members. Of the 1,443 (29%) household members who reported symptoms consistent with infectious gastroenteritis, 1,099 (76%) were classified as primary and 344 (24%) as secondary case-patients (Figure 1). Median age of primary cases was 3.6 years, including 60% <5 years of age (Table 1).

Among 3,916 contacts of the 1,099 primary case-patients, the crude secondary attack rate was 8.8% (95% confidence interval [CI] 7.9–9.7). Household transmission occurred within a median of 4 days (range 2–15 days) of

*Stanford University School of Medicine, Stanford, California, USA

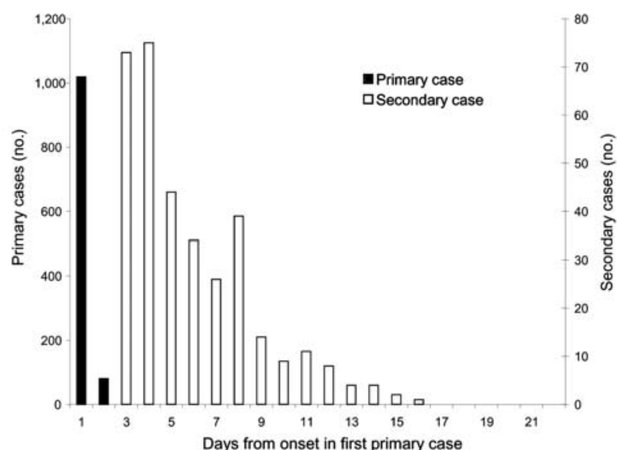

Figure 1. Serial onset of 344 secondary cases in 936 households. A secondary case was defined as onset of symptoms at least 2 days after onset and not more than 5 days after cessation of symptoms in a primary case.

onset of symptoms in the first primary case. Cumulative hazard rates varied substantially by age: 37%, 17%, 15%, and 8% for study participants <2, 2–5, 6–17, and ≥18 years of age, respectively (Figure 2). Secondary transmission occurred in 240 (26%) homes, and households with secondary cases were larger, with a median of 3 versus 2 children.

The crude secondary attack rate of 9% is somewhat lower than, but not inconsistent with, prior estimates. Pickering et al. (5) reported an overall secondary attack rate of 11% among family members of children involved in daycare outbreaks, with rates of 26%, 15%, and 17% for *Shigella*, rotavirus, and *Giardia*, respectively. After a foodborne outbreak of Norovirus, Gotz et al. (6) estimated that 20% of family members of daycare participants became ill. In an investigation of sporadic *Escherichia coli* O157, Parry et al. (9) estimated attack rates of 4%–15% in household contacts. In a small Danish community, 12% of family members became ill after children attending a daycare center in a neighboring town were exposed to a contaminated water supply (11). Similarly, 11% of family members became ill within 3 days of children's return from summer camp, where they were exposed to a suspected viral agent (8).

Since outbreak and surveillance investigations typically focus on highly transmissible agents with more severe illnesses, the somewhat lower secondary attack rate observed in this study of unidentified, mixed agents is not surprising. Risk interval (ours including a 96-hour postsymptomatic period) may affect classification of secondary illness. Among 835 households completing the follow-up visit, 380 (46%) had up to 7 recurrent episodes during a 3-month period, 42 beginning 6–10 days after conclusion of the first episode. Merging these episodes would have increased crude attack rates slightly, to ≈10%. Conversely, >80% of secondary cases occurred within 7 days of primary onset. Thus, our risk interval was likely to capture incubation periods of more common causal agents in the United States, although the fact that these homes were susceptible to recurrent episodes over time cannot be ignored.

The risk factor analysis (Table 2) confirmed the role of age in household transmission; children exhibited a 2- to 8-fold greater risk for secondary gastroenteritis compared with adults. Although 60% of patients with primary cases were <5 years of age, secondary transmission was more likely when the primary case-patient was >5 years (adjusted odds ratio [AOR] 1.7 [95% CI 1.3–2.3]). In addition, being a member of the index family (AOR 2.5 [95% CI

Table 1. Characteristics of 1,099 primary case-patients and 3,916 household contacts

	Primary (n = 1,099) (%)	Secondary (n = 344) (%)	No symptoms (n = 3,572) (%)
No. persons in household, median	5*	6	5*
<18 y	2	3	2
≥18 y	3	3	3
Index case	852 (78)*	79 (23)	–
Member/index family	1,052 (96)*	295 (86)	2,064 (58)*
Age, y, median	3.6*	12.6	24.7*
<2	412 (37)	57 (17)	138 (4)
2–5	254 (23)	50 (15)	303 (8)
6–17	145 (13)	76 (22)	670 (19)
≥18	288 (26)	161 (47)	2,461 (69)
Male	552 (50)*	148 (43)	1,803 (50)*
Daycare (if <6 y)	148 (22)	24 (22)	108 (24)
Symptoms			
Duration, median days	3*	1.3	–
Vomiting with or without diarrhea	799 (73)*	194 (56)	–
Shares a bed with primary case-patient	–	152 (44)	776 (22)*
Exposed to vomiting, primary case-patient	–	87 (25)	649 (18)*

*Univariate analysis, $p<0.05$ for comparison of secondary and primary cases, or secondary cases and other contacts of primary cases; –, not applicable.

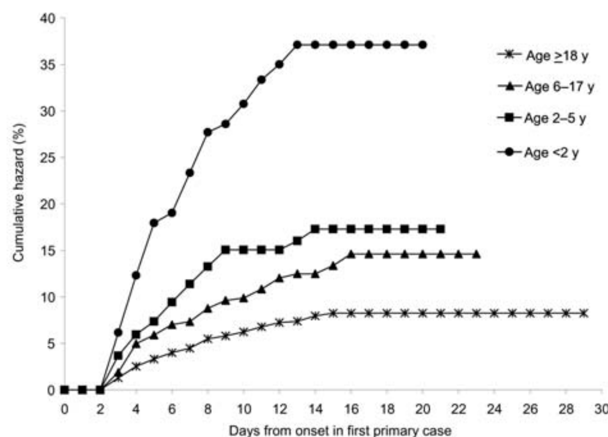

Figure 2. Hazard of secondary gastroenteritis by age group. Cumulative hazard, the cumulative proportion of contacts classified as secondary cases. Household risk periods, defined as ending when all members had been symptom-free for 96 hours, lasted a median of 9 days (interquartile range 7–13).

1.7–3.6]) and sharing a bed with a primary case-patient (AOR 2.0 [95% CI 1.5–2.6]) were independently associated with risk. As observed in some studies of Norovirus (6,12), exposure to vomiting was also associated with household transmission (AOR 1.6 [95% CI 1.2–2.2]), despite the fact that only 1 of 5 contacts reported this exposure.

Conclusions

Viral agents are thought to account for 80% of reported community diarrhea (13), and repeated exposure to agents like rotavirus, as is likely to occur in homes with small children, may be associated with features of acquired immunity (14). Some support for this hypothesis is the fact that, compared with primary cases, secondary cases tended to be in older children, with shorter episodes and fewer vomiting episodes (Table 1). Conversely, children <5 years of age, who constituted nearly one fourth of household members and had more than half of all illnesses, had more protracted episodes, regardless of primary or secondary onset or symptoms. Thus, although age-related immunity may have played a role in modifying duration or severity of secondary illness, the high proportion of young children was more determinative of household risk. Bed-sharing with these children was likely the major factor in propagating infection in these crowded homes.

Although our enrollment rate is similar to that in other population-based studies of gastroenteritis (1,15), we cannot exclude the possibility that participating families had different attack rates than nonparticipants. Approximately 13% of members in participating households were not interviewed or could not be classified as to temporal onset of symptoms; however, ascertainment did not differ between households with and without secondary cases. Although 19% of households were lost to follow-up, most commonly because of relocation, attack rates among completing households available for 1 visit or 2 were not significantly different. Our risk factor model, which assumes that multiple events within households share a common correlation across time, may be strong for longitudinal data, although for more limited risk periods and conditions of intense, close contact, the approach may be plausible.

In conclusion, household transmission continues to play an important role in community rates of acute intestinal infections. Bed-sharing among children in crowded homes constitutes a potentially modifiable risk.

Table 2. Factors associated with secondary gastroenteritis among 3,916 household contacts

Characteristic	Adjusted odds ratio (95% CI)*	p value*
Age, y		
<2	8.0 (5.5–11.4)	<0.0001
2–5	3.0 (2.0–4.3)	<0.0001
6–17	2.0 (1.5–2.8)	<0.0001
≥18	Reference	
Shares bed with primary case-patient	2.0 (1.5-2.7)	<0.0001
Exposure/primary vomiting episode	1.6 (1.2–2.2)	0.001
Member of index family	2.5 (1.7–3.6)	<0.0001
Primary cases, no.	1.5 (1.0–2.2)	0.07
Primary cases, ≥5 y	1.7 (1.3–2.3)	0.0009
Primary cases, duration, days	1.09 (1.0–1.1)	<0.0001

*p values and confidence intervals (CI) obtained by logistic regression for correlated observations, assuming exchangeable correlation within households.

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Dr. Perry is an epidemiologist with the Parsonnet Laboratory in the Division of Geographic Medicine and Infectious Diseases at Stanford University School of Medicine. Her research interests include immunoepidemiology of *Helicobacter pylori*, *Mycobacterium tuberculosis*, and intestinal co-infection.

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Address for correspondence: Sharon Perry, Stanford University School of Medicine, 300 Pasteur Dr, HRP (Redwood) Bldg, Room T225, Stanford, CA 94305, USA; fax: 650-725-6951; email: shnperry@stanford.edu

Nosocomial Malaria and Saline Flush

Sanjay K. Jain,* Deborah Persaud,* Trish M. Perl,* Margaret A. Pass,* Kathleen M. Murphy,* John M. Pisciotta,* Peter F. Scholl,* James F. Casella,* and David J. Sullivan*

An investigation of malaria in a US patient without recent travel established *Plasmodium falciparum* molecular genotype identity in 2 patients who shared a hospital room. *P. falciparum* can be transmitted in a hospital environment from patient to patient by blood inoculum if standard precautions are breached.

Almost all of the 1,400 cases of malaria reported each year in the United States are acquired by mosquito bite during travel in malaria-endemic areas (1). However, mosquito transmission in the United States accounts for a few cases each year (2). Nosocomial malaria represents person-to-person transmission of parasite-infected erythrocytes through blood transfusion, needlestick injury, improper use of blood glucometers, multidose heparin vials, organ transplantation, contaminated catheters that deliver contrast medium, or rarely, open wounds (3–6).

Nosocomial transmission of malaria secondary to inpatient nursing practices has never been reported in the United States. We report nosocomial transmission of *Plasmodium falciparum*, confirmed by molecular genotyping, by improper use of saline flush syringes in a tertiary care hospital in the United States.

The Study

Abdominal pain, emesis, and a high fever developed in patient 1, a 9-year-old Gambian boy with sickle cell disease residing in the United States, during the flight home after a month in the Gambia; he had taken no malaria prophylaxis drugs. After diagnosis of *P. falciparum* malaria with 4% parasitemia and transfer to unit A of a tertiary care hospital, he responded well to antimalarial therapy and was discharged 2 days later.

Seven days before patient 1's admission, patient 2, a 14-year-old girl, with severe developmental delay, was admitted to unit A for placement of a surgical feeding tube. Patients 1 and 2 shared a unit A semiprivate room for ≈24 hours. Patient 1 received a continuous quinidine gluconate infusion through a peripheral intravenous line. Chart review and interviews indicated that neither patient had glucose monitoring by glucometer, blood transfusions, common infusions such as contrast material, or a needlestick injury report, all events that have been previously implicated in nosocomial malaria. Medicine doses given and blood samples drawn were documented with a separation time >50 minutes. A week after discharge and 17 days after sharing the semiprivate room, patient 2 was admitted to another hospital (3-day stay), and a viral illness was diagnosed after all cultures were negative for pathogens. Persistent fever and pancytopenia developed and resulted in her readmission to unit A of the tertiary care hospital, now 23 days after sharing the room with patient 1. Patient 2 was febrile and pale, with no notable change in her baseline neurologic state. The leukocyte count was 1,890 cells/mm^3, hematocrit was 35.2%, platelets were 47,000/mm^3, and total bilirubin was 1.5 mg/dL. Despite administration of antimicrobial drugs for presumed microbial sepsis, the following conditions developed over the next 72 hours: persistent spiking fevers; loose, nonbloody stools; splenomegaly; abdominal distension; and bilateral lower extremity edema. Formal review of her peripheral blood smear by the hematology consultation service showed intraerythrocytic ring forms indicative of *P. falciparum* malaria, with 12% parasitemia and ≈200 gametocytes/mm^3. After 3 blood transfusions and quinidine gluconate and doxycycline therapy, the patient was discharged on hospital day 14, with a stable hematocrit, persistent gametocytemia, and splenomegaly. Initial and follow up tests on patient 2 were negative for viral bloodborne pathogens. The patient lives with her parents in the Baltimore-Washington area ≈12 miles from the nearest international airport. She had no history of recent travel to any areas that are endemic for malaria. No locally acquired cases of malaria had been reported in the regional area. This prompted an investigation to look for a nosocomial route of transmission. Approval for the study was obtained from Johns Hopkins University

Two potential sources of nosocomial transmission in unit A were identified: 1) heparin syringes, which were filled from multidose vials, and 2) factory preloaded 10-mL saline flushes, of which up to 3 mL was used per flush for intravenous lines not in use. Hospital policy had strict guidelines for using multidose devices and did not allow reusing single use devices. However, interviews of 7 nurses, including those who cared for the 2 patients, showed that 2 nurses admitted reusing saline flushes on the same patient, and 4 nurses had observed saline flushes being reused in unit A. Reusing multidose heparin vials was not documented.

To determine whether used saline flush syringes that were visibly clear contained blood, we tested saline from 8 used syringes and 2 unused controls. The contents of only 1 used saline flush syringe visibly contained blood. After the contents were centrifuged to concentrate erythrocytes,

*Johns Hopkins University, Baltimore, Maryland, USA

blood was found in 4 of 8 of the used syringes (Figure 1) by using matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (7). No blood was found in the unused syringes.

Polymerase chain reaction (PCR) amplification of *P. falciparum* genomic DNA, isolated with DNAzol (Invitrogen, Carlsbad, CA, USA), was successful from the archived Giemsa blood films from both patients and 2 other patients (not related in time or travel history) infected with *P. falciparum* malaria seen at our institution in the past year. Figure 2 shows that the PCR product restriction digests for the polymorphic *P. falciparum* merozoite protein 2 (*Pfmsp2*) (8) and the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) (9) gene fragments were identical for patients 1 and 2. This identity was also confirmed by sequence analysis.

We also examined 4 *P. falciparum* microsatellite alleles (TA81, PFPK, C13M30, C4M8) at independent chromosomal loci by a fluorescent-tagged heminested PCR amplification (10). Patients 1 and 2 had identical *Plasmodium* microsatellite lengths at all 4 loci; each of the positive controls was different as shown in Figure 2, the online Appendix Figure (available at http://www.cdc.gov/ncidod/EID/vol11no07/05-0092_appG.htm), and the Table. With the simplistic assumption of $\geq$10 alleles at each microsatellite loci and *Pfmsp2*, the chance occurrence of identity is <1:100,000. By using the AmpFLSTR Profiler kit (Applied Biosystems, Foster City, CA, USA), subsequent forensic analysis of the human genomic DNA from leukocytes in the same isolated *P. falciparum* DNA samples from the blood films demonstrated the sex difference between patients 1 and 2. The analysis also demonstrated the differences at 9 human microsatellite loci, which indicated that cross-contamination during processing was not a likely source for the similarities detected.

Conclusions

Natural transmission of malaria between patients 1 and 2 by a mosquito is not possible within 24 hours; the maturation cycle from ingestion of viable gametocytes in a blood meal to infective sporozoite stage in the mosquito salivary glands is 8–35 days (11). Direct mechanical transmission of parasitized blood by an arthropod vector is a theoretical possibility, but no such transmission has ever been reported, and the female *Anopheles* mosquito does not regurgitate blood upon refeeding. Another remote possibility, luggage malaria, is the introduction of an already infectious mosquito from the Gambia brought into unit A through patient 1's luggage (12). If this were the case, a different parasitic genotype would be expected in patients 1 and 2 because the wild mosquito that infected patient 1, from 1 to 2 weeks before onset of symptoms, would have died 2–6 days after delivering the infective bite. Likewise, an asymptomatic traveling companion of patient 1 would have a distinct parasite genotype.

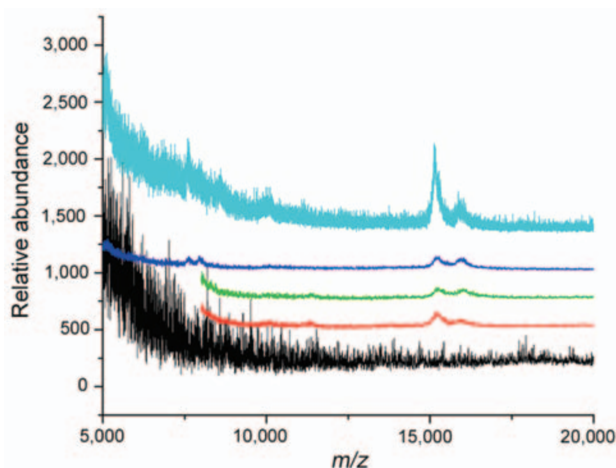

Figure 1. Mass spectroscopic analysis of sterile saline flush syringes after routine use. The contents of the used syringes were concentrated by centrifugation. Matrix-assisted laser desorption ionization detected the α and β chains of hemoglobin as the ions at mass/charge (*m/z*) 15,126 and 15,867, respectively, in samples A (red), B (green), C (blue), and J (aqua) that were absent in the matrix alone (black). The lower limit of sensitivity with matrix-assisted laser desorption ionization is ≈0.5 erythrocytes per mL.

In this report, epidemiologic investigation excluded many nosocomial routes of transmission. Potential sources not excluded were multidose heparin vials and saline flush syringes. However, identifying reuse of saline flush syringes in the unit and detecting blood in clear, used saline flush syringes suggested that this was the most likely source. Finally, molecular identity of *P. falciparum* isolated from patients 1 and 2, as assessed by restriction fragment length polymorphism of *Pfmsp2*, and forensic microsatellite analysis of *P. falciparum* genotypes confirmed that contaminated fluid from patient 1 was the source of the infection in patient 2. Together, these data strongly suggest that apparently clear solution in a used

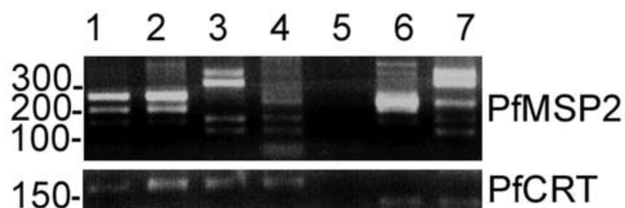

Figure 2. Genotype analysis of patient blood films. Restriction fragment length polymorphism are shown for *Pfmsp2* and *Pfcrt*. Patient 1 and 2 are identical at the polymorphic *Pfmsp2* (lanes 1 and 2), while unrelated patient controls (lanes 3 and 4) are different. One of the 2 negative controls is shown (lane 5). Genomic DNA from clone HB3 (lane 6) and isolate NF54 (lane 7) are also included as additional positive controls. All recent patient samples have the *Plasmodium falciparum* chloroquine-resistant genotype.

Table. *Plasmodium falciparum* microsatellite lengths in number of base pairs

Patient	TA81	PFPK	C13M30	C4M8
1	114	178	97	132
2	114	178	97	132
3	111	196	115	138
4	126	178	97	154
HB3	126	190	89	151

saline flush syringe containing infected *P. falciparum* erythrocytes, was reused on a neighboring patient, leading to nosocomial transmission of malaria. Saline flush vials and bags have been implicated in nosocomial transmission of hepatitis B and microbes, respectively (13,14).

Theoretically, a single infected erythrocyte is able to transmit malaria by inoculation. Patient 1 had almost 200 million infected erythrocytes per milliliter of blood based on parasitemia level. Based on our data, apparently clear saline can contain ≈1 million erythrocytes per milliliter. Therefore a single microliter of patient 1's blood diluted into 5 mL of saline would be almost 40,000 infected erythrocytes per milliliter of an apparently clear solution.

Our report illustrates that nosocomial transmission can occur in a tertiary care setting in the United States, despite healthcare workers' access to gloves, disposable needles, intravenous devices, and flushes. The common perception that flushing practices are not associated with the aspiration of minute amounts of blood contributed to this occurrence of nosocomial malaria. Our institution, like many others, has used such episodes to facilitate change and refocus on patient safety. The interventions initiated specifically included retraining of pediatric staff about 1) no reuse of single use saline flushes, 2) transmission of bloodborne pathogens with an emphasis on nonviral pathogens, and 3) removal of multidose vials, including those with heparin, from pediatric units. Warnings about the potential risks of reusing saline flush syringes have been incorporated into institution-wide safety training. Ongoing rigorous review of healthcare practices and strict adherence to body and fluid precautions are essential for minimizing patient exposure to highly infectious pathogens, even in resource-rich settings.

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Dr. Jain is a senior clinical and research fellow in pediatric infectious diseases at Johns Hopkins University. His research interests include tropical diseases and central nervous system tuberculosis.

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Address for correspondence: David J. Sullivan, W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, 615 North Wolfe St, E5628, Baltimore, MD 21287, USA; fax; 410-955-0105; email: dsulliva@jhsph.edu

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West Nile Virus Surveillance, Guadeloupe, 2003–2004

Thierry Lefrançois,* Bradley J. Blitvich,† Jennifer Pradel,* Sophie Molia,* Nathalie Vachiéry,* Guillaume Pallavicini,* Nicole L. Marlenee,† Stéphan Zientara,‡ Martial Petitclerc,§ and Dominique Martinez*

We conducted extensive surveillance for West Nile virus infection in equines and chickens in Guadeloupe in 2003–2004. We showed a high seroprevalence in equines in 2003 related to biome, followed by a major decrease in virus circulation in 2004. No human or equine cases were reported during the study.

The recent introduction of West Nile virus (WNV, family Flaviviridae, genus *Flavivirus*) into the Caribbean region is a major public health concern, particularly because transmission of this virus probably occurs year-round in the neotropics. Since 2002, WNV activity has been detected in Guadeloupe (1), Mexico (2,3), the Dominican Republic (4), and Jamaica (5). The objectives of this study were to determine the spatial distribution of WNV in Guadeloupe, establish a surveillance system in humans and animals to detect clinical cases, and increase our understanding of WNV epidemiology in the neotropics.

The Study

The investigation was conducted in the Guadeloupe archipelago, which includes Guadeloupe (the main island), Marie Galante, Saint Martin, and Saint Barthelemy. Surveys of domestic birds (chickens) were performed in July 2003 and 2004 on 25 to 27 farms selected to cover the whole island; each farm contained 15–20,000 chickens from 1 month to 2 years of age. Exhaustive surveys were also conducted on equines in July 2003 and August 2004 (46 survey locations, 1–68 equines each, mean 10 equines).

Epitope-blocking enzyme-linked immunosorbent assays (ELISA) were performed by using the WNV-specific monoclonal antibody 3.1112G and flavivirus-specific monoclonal antibody 6B6C-1 (Chemicon, Temecula, CA, USA) as previously described (6,7). The ability of the test sera to block the binding of the monoclonal antibodies to WNV antigen was compared to the blocking ability of horse or chicken serum without antibody to WNV. An inhibition value >30% was considered to indicate the presence of viral antibodies. Plaque reduction neutralization tests (PRNTs) were performed as described previously (3) on serum samples that had antibodies to flaviviruses. PRNTs were performed with WNV and St. Louis encephalitis virus (SLEV, family Flaviviridae, genus *Flavivirus*). A serum sample was considered to have antibodies to WNV if it significantly inhibited the binding of monoclonal antibody 3.1112G by blocking enzyme-linked immunosorbent assay (ELISA) and had a 90% plaque reduction ($PRNT_{90}$) titer to WNV that was at least 4-fold greater than the corresponding SLEV $PRNT_{90}$ titer. A serum sample was considered to have antibodies to SLEV if it inhibited the binding of monoclonal antibody 6B6C-1 and had a $PRNT_{90}$ titer to SLEV that was at least 4-fold greater than the corresponding WNV titer. A serum sample was considered to have antibodies to a flavivirus of undetermined origin if it contained epitope-blocking ELISA or neutralizing antibodies but did not meet the criteria for a WNV or SLEV infection.

Out of 487 equines (437 horses, 34 donkeys, 16 ponies) sampled in July 2003, 94 (19.3%) had antibodies to WNV, and 10 (2.1%) had antibodies to a flavivirus of undetermined origin (Table 1). In August 2004, of 431 equines (386 horses, 27 donkeys, 18 ponies), 70 (16.2%) had antibodies to WNV, and 11 (2.6%) had antibodies to a flavivirus of undetermined origin. WNV $PRNT_{90}$ titers were between 1:20 and 1:1,280 (mean 1:260).

In 2003, WNV seroprevalence in the 46 equine centers was highly heterogeneous (0%–100%, chi-square test $p<0.001$, Figure 1). This heterogeneity was also found by county (0%–71%, $p<0.001$) and by island ($p<0.001$, Table 2).

Figure 2 shows each equine center in relation to the ecologic area. Most WNV seropositive equines were located in evergreen forests characterized by a low altitude ($\leq$100 m) and intensive farming of sugar cane in the vicinity of mangroves (*Rhizophora mangle*, *Avicennia germinans*, *Laguncularia racemosa*, *Conocarpus erectus*), back mangroves (marshy forests with *Pterocarpus* spp.), and swamps. Within Grande Terre, seropositive equines were mostly identified in the humid plains of Abymes or near small mangrove areas. In Marie Galante, nearly all seropositive equines were located near swamps, temporary ponds, or rivers. Conversely, no seropositive equines were found in the semideciduous forest (dry leeward coast of Basse Terre) and very few in the dry regions of Grande Terre.

*Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Prise d'Eau, Guadeloupe, French West Indies; †Colorado State University, Fort Collins, Colorado, USA; ‡Agence Française de Sécurité Sanitaire des Aliments, Paris, France; and §Direction des Services Vétérinaires de Guadeloupe, Basse-Terre, Guadeloupe, French West Indies

Table 1. West Nile virus (WNV) seroprevalence in equines and chickens in Guadeloupe, 2002–2004

Sample	Equines, n (%)			Chickens, n (%)		
	July 2002*	July 2003	August 2004	December 2002*	July 2003	July 2004
WNV positive	10 (2.8)	94 (19.3)	70 (16.2)	11 (52.4)	11 (1.7)	5 (0.6)
Unknown flavivirus positive	0	10 (2.1)	11 (2.6)	0	1 (0.2)	2 (0.2)
Total tested	360	487	431	21	656	801

*Data from the serosurveys conducted in 2002 have been presented elsewhere (1).

Sixty-two equines that were WNV seronegative in December 2002 (1) were retested in July 2003, and all remained seronegative. Because of changes in the whole population (e.g., death, exportation), only 328 equines were tested in both July 2003 and August 2004. Of the 257 seronegative equines identified in 2003, 256 were seronegative in 2004, and 1 had been infected with an undetermined flavivirus. Thus, no WNV seroconversions were observed in equines between 2003 and 2004. Conversely, all the WNV-seropositive equines in 2003 were still positive in 2004.

In July 2003, of 656 chickens, 11 (1.7%) had antibodies to WNV, and 1 had antibodies to an undetermined flavivirus. Three (0.5%) WNV-seropositive chickens were found on the main island of Guadeloupe and 8 (14.8%) on Marie Galante (Figure 1). In July 2004, of 801 chickens, 5 (0.6%) had antibodies to WNV, and 2 had antibodies to an undetermined flavivirus. All seropositive chickens were on the main island of Guadeloupe (Figure 1). WNV $PRNT_{90}$ titers were between 1:20 and 1:1,280 (mean 1:519).

Figure 1. Results of West Nile virus (WNV) serosurveys in chickens and equines in Guadeloupe, 2003–2004. Equine centers are represented by circles, the sizes of which are proportional to the numbers of equines. The proportion of WNV-seropositive animals is represented in red. Chicken farms are represented by triangles (pointing down for 2003 survey, pointing up for 2004 survey). Red triangles denote farms where at least 1 seropositive chicken was identified. All other farms are denoted by yellow triangles. Ab, antibody.

A surveillance system was established in equines, domestic and wild birds, and humans. Equine surveillance was performed by veterinarians. Awareness of WNV was increased in the public, hunters, and horse and poultry owners with educational leaflets and with the help of veterinarians and natural reserve wardens. In 2003 and 2004, veterinarians reported 4 horses that exhibited signs of WNV-like illness (ataxia, weakness of limbs); the horses were tested at the onset of clinical signs and 15 days later. All were negative for WNV by epitope-blocking ELISA and PRNT, and other causes were eventually determined. Clinical signs were not observed in any equines with antibodies to WNV. Abnormal death and paralysis were reported in domestic chickens in 5 farms. In these farms, serum specimens from 23 chickens were analyzed for evidence of WNV infection, and 1 chicken was seropositive.

Human surveillance was performed by the public hospitals of Guadeloupe. In humans, suspected cases were defined as neurologic symptoms of encephalitis or meningoencephalitis or acute neurologic symptoms with fever. In 2003, the hospital reported 9 suspected cases of clinical WNV infection in Guadeloupe (Cecile Herrmann-Storck, pers. comm.). Antibodies to WNV were not detected in the sera of any of these patients, and another cause was determined.

Conclusions

A high seroprevalence (19.3%) for WNV was detected in equines in 2003. Seropositive equines and chickens were commonly found near mangroves, which contain many species of wild birds and mosquitoes. *Culex nigripalpus* and *Ochlerotatus taeniorhynchus* are common in Guadeloupe and are particularly abundant in mangrove

Table 2. West Nile virus (WNV) seroprevalence in equines by island, July 2003

Island	WNV antibody positive, n (%)	No. tested
Guadeloupe main island	80 (19.6)	409
Marie Galante	13 (43.3)	30
Saint Martin	1 (2.9)	34
Saint Barthelemy	0	14

areas. *Culex* species are the major amplification vectors of WNV in the United States (8,9) and may also be vectors of WNV in Guadeloupe.

Results of the equine and avian serosurveys suggest that transmission of WNV decreased dramatically in 2003 and 2004 in comparison to 2002. No equine seroconversion occurred between January 2003 and August 2004, and seroprevalence in chickens was low in 2003 (1.7%) and 2004 (0.6%). In comparison, 10 of 21 chickens in Goyave were seropositive for WNV in 2002, although the sample size was small (1). In the tropics, where temperature is favorable year-round, changes in rainfall can substantially affect the size of vector populations (10). *Cx. nigripalpus* and *Oc. taeniorhynchus* need heavy rains or changes in water level to develop (11). Therefore, the 7-month drought (half of the usual rainfall) observed between November 2002 and May 2003 was probably responsible for a major decrease in the mosquito population. If *Cx. nigripalpus* is involved in WNV transmission in Guadeloupe, a decrease in its population could explain reduced virus circulation. Alternatively, the number of nonimmune resident birds may have decreased.

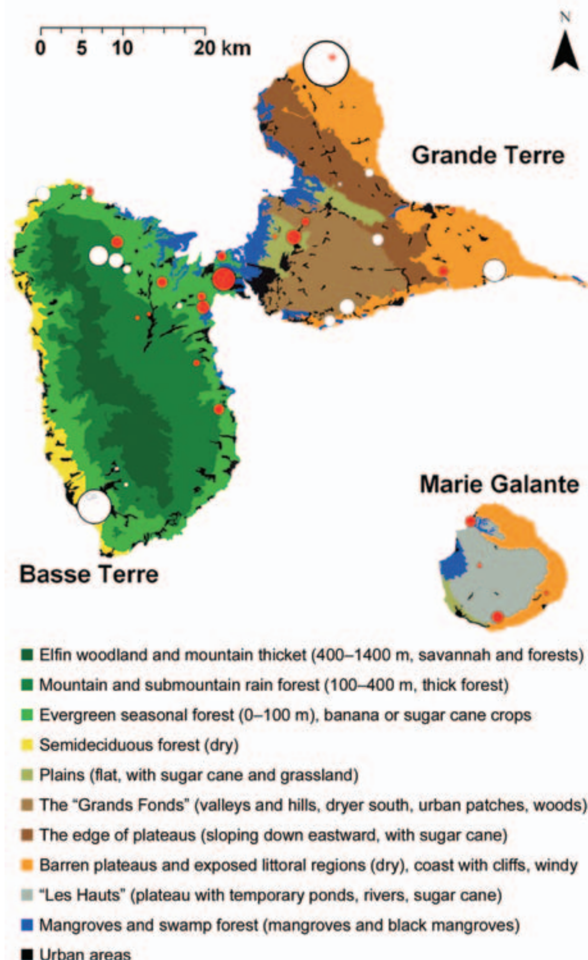

Figure 2. Ecologic map of Guadeloupe and West Nile virus (WNV)-positive equine centers. Basse Terre (southwest) is mainly mountainous (volcanic, highest point 1,467 m) and wet. Grande Terre (northeast) is flat (mainly <100 m) and dry. Marie Galante is flat (plateaus <200 m) but has more water than Grande Terre. The ecologic map was derived from "Carte écologique de la Guadeloupe," created by Alain Rousteau, University Antilles-Guyane. Equine centers with WNV-seropositive equines are represented by red circles (the size of each circle is proportional to the number of seropositive equines). Centers without WNV-seropositive equines are represented by white circles (the size of each circle is proportional to the number of equines tested). The red asterisk shows a site that contained 3 seropositive equines. All were race horses that travel frequently and thus may have been infected elsewhere.

No dead wild bird was reported to the veterinary services in Guadeloupe in 2003 or 2004, although dead bird carcasses are presumably difficult to find in areas with dense vegetation. No abnormal death rate was noted in anthropophilic birds either.

Although an efficient passive surveillance system was implemented in humans and equines, no clinical cases of WNV infection were observed. This situation is considerably different from that observed in the United States but mimics the situation in Mexico, where only a few human and equine cases were observed, despite a seroprevalence of 29% in equines in 2004 (12). Cross-protection conferred by other flaviviruses could explain the difference with the North American situation. In fact, we found some equines that were considered to have been infected with an unknown flavivirus. Alternatively, virus mutations could explain a change in virulence and the absence of clinical cases. Indeed, recent studies identified attenuated WNV in Texas (13) and southern Mexico (14). Isolation and characterization of WNV in Guadeloupe would help clarify these issues.

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Dr. Lefrançois is a veterinarian and member of the Control of Exotic and Emerging Animal Diseases research unit at the Centre de Coopération Internationale en Recherche Agronomique pour le Développement–Département Délevage et Médecine Vétérinaire research center in Guadeloupe. His research focuses on diagnosis and surveillance of several animal diseases in the Caribbean.

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Address for correspondence: Thierry Lefrançois, CIRAD-EMVT Guadeloupe, Domaine de Duclos, Prise d'eau, 97170 Petit Bourg, Guadeloupe, FWI; fax: 590-590-94-0396; email: thierry.lefrancois@cirad.fr

Landscape

Mary Oliver

Isn't it plain the sheets of moss, except that
they have no tongues, could lecture
all day if they wanted about

spiritual patience? Isn't it clear
the black oaks along the path are standing
as though they were the most fragile of flowers?

Every morning I walk like this around
the pond, thinking: if the doors of my heart
ever close, I am as good as dead.

Every morning, so far, I'm alive. And now
the crows break off from the rest of the darkness
and burst up into the sky—as though

all night they had thought of what they would like
their lives to be, and imagined
their strong, thick wings.

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Enterotoxigenic *Escherichia coli* and *Vibrio cholerae* Diarrhea, Bangladesh, 2004

Firdausi Qadri,* Ashraful I. Khan,* Abu Syed G. Faruque,* Yasmin Ara Begum,* Fahima Chowdhury,* Gopinath B. Nair,* Mohammed A. Salam,* David A. Sack,* and Ann-Mari Svennerholm†

Flooding in Dhaka in July 2004 caused epidemics of diarrhea. Enterotoxigenic *Escherichia coli* (ETEC) was almost as prevalent as *Vibrio cholerae* O1 in diarrheal stools. ETEC that produced heat-stable enterotoxin alone was most prevalent, and 78% of strains had colonization factors. Like *V. cholerae* O1, ETEC can cause epidemic diarrhea.

In July 2004, Bangladesh experienced devastating floods, which also affected the capital, Dhaka, and outbreaks of diarrheal diseases occurred throughout the city. As a result, a steep increase was seen in patient admissions, which reached epidemic numbers around July 20, when >350 patients were admitted every day to the hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). During the peak period, >700 patients were seen per day, and the total number seen during the epidemic was >17,000.

Diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) is highly prevalent in young children in developing countries as well as in travelers to these areas (1). In Bangladesh, *Vibrio cholerae* is the bacterial pathogen that most frequently necessitates hospitalization (2). ETEC is also commonly isolated from patients seeking treatment in hospitals (3–5), but it is not actively screened for during natural disasters. However, reports have suggested that ETEC, in addition to cholera, is a predominant cause of diarrhea in Bangladesh (6,7). Since ETEC spreads though contaminated water and food (8,9), we analyzed diarrheal stools for this pathogen to assess the prevalence of ETEC during the epidemic.

ETEC causes diarrhea by producing different combinations of the heat labile (LT) or heat stable (ST) enterotoxins and 1 or more of at least 22 different colonization factors, which contribute to the virulence of the pathogen (10). Since genes for these factors are predominantly present on plasmids, which may be lost on storage, we tested for phenotypic expression of these factors by using freshly cultured isolates. For this purpose, diarrheal stools were collected from patients in a 2% systematic routine surveillance system; every 50th patient attending the hospital is routinely screened for *V. cholerae*, *Shigella* spp., and *Salmonella* spp. (4) at the Clinical Research and Service Centre of the ICDDR, B. The study was approved by the institutional review board of ICDDR,B.

The Study

Only samples negative for *V. cholerae* were tested for ETEC, starting from July 20, 2004, when the patient numbers increased at the ICDDR,B hospital for ≈6 weeks, until the patient numbers decreased and the floods had receded. Information, including age, sex, fever, vomiting, dehydration status, and related clinical features, was also collected from patients. For ETEC surveillance, we used lactose-fermenting *E. coli* colonies cultured on MacConkey agar plates that had been cultured from fresh stool specimens (4). Six lactose-fermenting individual colonies of *E. coli* were tested for the presence of LT, ST, and colonization factors. Detection of LT and ST was carried out with ganglioside GM1 enzyme-linked immunosorbent assays (4). The colonies that tested positive for the toxins were also plated onto colonization factor antigen (CFA) agar plates with and without bile salts for testing colonization factors (4). Trypticase soy agar containing 5% sheep blood (TSA) was used to test for the colonization factor CS21 (5).

The strains were cultured at 37°C overnight; those grown on CFA agar without bile were tested for colonization factors CFA/1, CSI, CS2, CS3, CS4, and CS6, and those on CFA agar plus bile were tested for CS5, CS7, CS17, CS8, CS12, and CS14 (4). Those strains grown on TSA were tested for CS21 only (5). Of the patients included in this study, 67% had severe-to-moderate dehydration; of these, 51% were children <5 years of age, while 39% were >15 years of age. They were treated for diarrhea with oral (61%) or intravenous (39%) rehydration therapy and other medications as needed.

Of 350 stool specimens tested during the epidemic, 78 (22.2%) were positive for *V. cholerae* O1 (22 Ogawa and 56 Inaba serotype), and 63 (18.0%) were positive for ETEC. *Shigella* spp. (3.4%, n = 11) and *Salmonella* spp. (1.7%, n = 5) were seen at lower rates. Children with ETEC diarrhea were negative for *V. cholerae* O1 as well as *Shigella* spp. and *Salmonella* spp. We did not test *V. cholerae*–positive samples for ETEC and therefore cannot rule out possible concomitant infection with ETEC in these 78 cholera patients (4).

*International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh; and †Göteborg University, Göteborg, Sweden

Isolation of ETEC and *V. cholerae* O1 remained high throughout the epidemic (Figure), and during 1 week, comparable numbers of ETEC and *V. cholerae* were isolated from stools of patients. We compared demographic and clinical features of patients with ETEC and *V. cholerae* infections (Table 1). Most patients with ETEC diarrhea were <2 years of age (56%) or >15 years of age (36%) (median 1.5 years), whereas those with *V. cholerae* O1 infection were mostly >5 years of age (median age 15.5 years). Although more cholera patients had severe dehydration (60%), 22% of the patients with ETEC diarrhea also had severe dehydration (p<0.001). Intravenous rehydration was needed for both ETEC- and *V. cholerae*–infected patients, but it was more frequently used in the latter.

With regard to toxin profile, ETEC expressing ST alone was the most common (67%), followed by strains producing both ST and LT (19%) and LT alone (14%). Dominance of the ST-expressing ETEC has been documented earlier during seasonal outbreaks and epidemics in Bangladesh (4) and in Egypt and the Middle East (11,12). Patients infected with the different toxin phenotypes of ETEC had dehydration status ranging from severe to none, although no significant association was seen between toxin phenotype and degree of dehydration.

A high proportion of the ETEC strains (78%) expressed 1 or more colonization factors (Table 2), a much higher frequency than that seen in other hospital or community-based studies (10,12). In earlier studies in Bangladesh, we found 56% of strains positive for these colonization factors (4). In the present study, ≈92% of ST/LT-, 79% of ST-, and 56% of LT-expressing ETEC expressed 1 or more colonization factors. CFA/I was the most common phenotype, followed by the strains expressing CS4 + CS6 or CS5 + CS6, followed by others. Thus, most of the colonization factor types were those known to be present in clinical strains and those that have previously been isolated from hospitalized patients (4,5). These antigens have been given priority for designing vaccines to protect against a wide range of colonization factors (10). In addition, 3 strains co-expressed CS21, a type IV pilus antigen (4). Of these, 2 strains expressed CFA/I and CS21, and 1 was positive for CS1, CS3, and CS21.

We used 13 colonization factor–specific monoclonal antibodies in testing; however, >22 colonization factors have been described, not all of which could be tested in this study. In addition, although precautions were taken to rule out the loss of phenotypic properties of colonization factors, some may have been lost on culture. By using

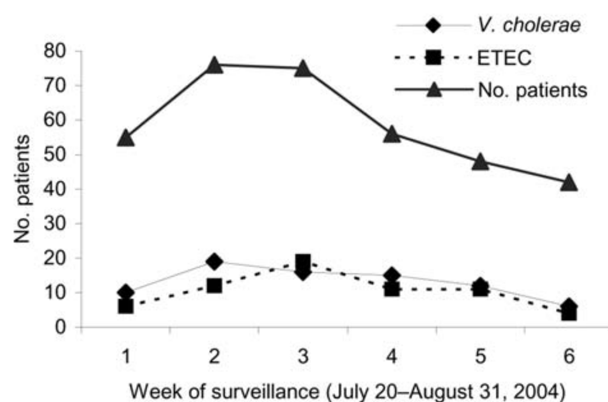

Figure. Weekly distribution of patients with *Vibrio cholerae* O1 or enterotoxigenic *Escherichia coli* (ETEC) infections during the study period from July 20 to August 31, 2004. The total number of patients who underwent stool analyses at the treatment center each week during the diarrheal epidemic is also shown.

Table 1. Characteristics of the patients with enterotoxigenic *Escherichia coli* (ETEC) and *Vibrio cholerae* O1 infection during the diarrheal epidemic, July–August 2004, Bangladesh

Parameter	No. ETEC* (n = 63) (%)	No. *V. cholerae** (n = 78) (%)	All patients† (N = 350) (%)
Age			
≤2 y	35 (56)	9 (12)	159 (45)
3–4 y	3 (5)	9 (12)	22 (6)
5–15 y	2 (3)	21 (27)	33 (9)
>15 y	23 (36)	39 (50)	136 (39)
Median (mo)	18	186	48
Range (mo)	1.9–600.0	4.9–780.0	0.67–960
Sex			
Male	35 (56)	41 (53)	198 (57)
Female	28 (44)	37 (47)	152 (43)
Dehydration status			
No sign	29 (46)	4 (5)	115 (33)
Some	20 (32)	27 (35)	133 (38)
Severe	14 (22)	47 (60)	102 (29)
Intravenous rehydration needed	18 (29)	56 (72)	137 (39)

*No. patients infected with respective bacterial pathogens seen at the International Centre for Diarrhoeal Disease Research, Bangladesh treatment center during the epidemic.
†Total patients with specimens tested during the study period.

Table 2. Colonization factor (CF) types of enterotoxigenic *Escherichia coli* (ETEC) isolated from patients during diarrhea epidemic, Bangladesh*

Toxin produced	CF type(s) produced	No. isolates (%)
ST (n = 42)	CFA/I	9 (21.4)
	CFA/I, CS21	1 (2.38)
	CS1 + CS3, CS21	1 (2.38)
	CS4 + CS6	7 (16.67)
	CS5 + CS6	7 (16.67)
	CS6	6 (14.29)
	CS14	1 (2.38)
	CS17	1 (2.38)
LT/ST (n = 12)	CFA/	1 (8.33)
	CFA/I, CS21	1 (8.33)
	CS1 + CS3	2 (16.67)
	CS2 + CS3	1 (8.33)
	CS4 + CS6	2 (16.67)
	CS5 + CS6	3 (25.00)
	CS14	1 (8.33)
LT (n = 9)	CS7	3 (33.3)
	CS6 + CS8	1 (11.1)
	CS17	1 (11.1)

*Of the 63 ETEC strains isolated, 78% were positive for 1 or more of the 12 CFs tested. Those that were positive are shown above. ST, heat stable; LT, heat labile

polymerase chain reaction or DNA hybridization procedures, more colonization factor–specific genes and those that have undergone phenotypic changes could have been detected (13).

Conclusions

We hypothesize that contaminated water during floods can be a cause of ETEC diarrhea. Flood waters may be contaminated by sewage, increasing transmission by the fecal-oral route. Our recent studies have also shown that ETEC can be isolated relatively frequently from surface water samples in Bangladesh (14).

Although diarrhea can be prevented by improving water quality, sanitation, and overall hygiene, these improvements will not be possible in the near future in densely populated areas with limited resources. Thus, developing vaccines that can prevent such epidemics is a goal. Such vaccines should include at least the most prevalent colonization factors, such as those found on the ETEC strains we isolated, to provide protection against the virulent, colonization factor–expressing, ST-positive ETEC strains.

This article emphasizes that ETEC can be a major source of acute watery diarrhea in epidemics caused by floods. This report is the first to show that during waterborne natural disasters, ETEC can also cause dehydrating diarrhea severe enough to require clinical care and, in many instances, intravenous rehydration. During epidemics, focus on ETEC should be on pediatric patients <2 years of age, since ETEC was the most prevalent bacterial enteropathogen identified in this age group. The treatment strategy should be designed accordingly, since ETEC strains are becoming increasingly resistant to erythromycin (15), the drug usually used for young children with acute watery diarrhea, irrespective of diagnosis.

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Dr. Qadri is senior scientist and head of the Immunology Unit, ICDDR,B. Her major research interests are studies of enteric pathogens, especially enterotoxigenic *Escherichia coli* and *Vibrio cholerae*, with emphasis on immunologic and epidemiologic studies of natural infections and vaccine development.

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Address for correspondence: Firdausi Qadri, Laboratory Sciences Division, ICDDR,B, GPO Box 128, Dhaka 1000, Bangladesh; fax: 880-2-8802-8823116; email: fqadri@icddrb.org

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SARS Coronavirus Detection Methods

Susanna K.P. Lau,* Xiao-Yan Che,† Patrick C.Y. Woo,* Beatrice H.L. Wong,* Vincent C.C. Cheng,* Gibson K.S. Woo,* Ivan F.N. Hung,‡ Rosana W.S. Poon,* Kwok-Hung Chan,* J.S. Malik Peiris,* and Kwok-Yung Yuen*

Using clinical samples from patients with severe acute respiratory syndrome, we showed that the sensitivities of a quantitative reverse transcription–polymerase chain reaction (80% for fecal samples and 25% for urine samples) were higher than those of the polyclonal (50% and 5%) and monoclonal (35% and 8%) antibody-based nucleocapsid antigen capture enzyme-linked immunosorbent assays.

The epidemic of severe acute respiratory syndrome (SARS) in 2003, caused by SARS-associated coronavirus (SARS-CoV), has affected 30 countries, with 8,098 cases and 774 deaths (1–8). Early diagnosis of SARS-CoV infection, which involves viral detection, is important for preventing future epidemics. Since culturing of SARS-CoV is difficult and insensitive, the reverse transcription–polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) has been the working standard in diagnosis (2,9). Nevertheless, these techniques are relatively expensive and rely on the availability of equipment and expertise. We recently reported the development of 2 sandwich enzyme-linked immunosorbent assays (ELISAs) for detection of SARS-CoV nucleocapsid protein in clinical specimens of SARS patients (10,11). However, no studies have been conducted to compare the sensitivities of ELISA with those of RT-PCR. Although PCR assays are generally more sensitive, ELISAs are less expensive and easier to conduct (12,13). To evaluate the potential usefulness of ELISA in diagnosing SARS-CoV infections, we compared the performance of ELISA and qRT-PCR and studied the correlation between their results.

The Study

Fecal specimens (n = 40, from 40 patients 1–27 days after symptom onset) and urine specimens (n = 133, from 101 patients 2–57 days after symptom onset) were collected from SARS patients hospitalized in Hong Kong from March to May 2003. SARS was confirmed by the presence of serum immunoglobulin (Ig) G against SARS-CoV by an immunofluorescence assay (4). Specimens were tested with polyclonal and monoclonal antibody–based capture ELISAs for SARS-CoV nucleocapsid protein and real-time qRT-PCR. Control urine (n = 100) and fecal (n = 100) specimens were obtained from hospitalized patients without SARS.

SARS-CoV nucleocapsid protein was detected by polyclonal antibody–based ELISA according to published protocols (7,11). SARS-CoV nucleocapsid protein was detected by monoclonal antibody–based ELISA using a modified protocol for serum samples (10). Briefly, fecal and urine specimens were inactivated with 2% and 0.5% phenol, respectively, for 15 min before centrifugation and dilution in phosphate-buffered saline with 2% skim milk. One hundred microliters of 1:10 diluted fecal specimens or 1:2 diluted urine specimens was added to wells previously coated with antinucleocapsid monoclonal antibodies. Plates were incubated, washed, treated with antinucleocapsid rabbit monoclonal antibodies, and analyzed as described previously (10,11). RNA extraction and real-time qPCR assay specific for the 1b region of SARS-CoV were conducted as described previously (3,9).

We compared the detection rates of 2 ELISAs and real-time qRT-PCR using the McNemar test and studied the correlation between the optical density values at 450 nm (OD_{450}) of the 2 ELISAs and $\log_{10}$ viral concentrations, as determined by real-time qRT-PCR, by linear regression (SPSS version 11.0, SPSS Inc., Chicago, IL, USA). A p value <0.05 was regarded as significant.

A comparison of the 2 ELISAs is shown in the Figure and Table 1. The cutoffs of the polyclonal antibody–based ELISA have been determined previously, with specificities of 96% and 99% for fecal and urine specimens, respectively (11). The baselines of the monoclonal antibody–based ELISA were determined by using 100 control fecal and urine specimens, with mean OD_{450} values of 0.089 and 0.05 and standard deviation (SD) values of 0.074 and 0.03, respectively. The specificities of the monoclonal antibody–based ELISA were 93% for fecal specimens and 98% for urine specimens, as determined using cutoffs defined as the mean + 2 SD. Of 40 fecal samples obtained from SARS patients, 20 (50%) obtained on days 9 to 23 after onset of symptoms were positive by the polyclonal antibody–based ELISA, and 14 (35%) obtained on days 2 to 21 were positive by the monoclonal antibody–based ELISA. Of 133 urine samples, 6 (5%) obtained on days 16 to 32 after onset of symptoms were positive by the polyclonal antibody–based ELISA, and 11 (8%) obtained on days 6 to 45 were positive by the monoclonal antibody–based ELISA. Results of the polyclonal antibody–based ELISA were comparable with our previous

*University of Hong Kong, Hong Kong Special Administrative Region, People's Republic of China; †First Military Medical University, Guangzhou, People's Republic of China; and ‡Queen Mary Hospital, Hong Kong Special Administrative Region, People's Republic of China

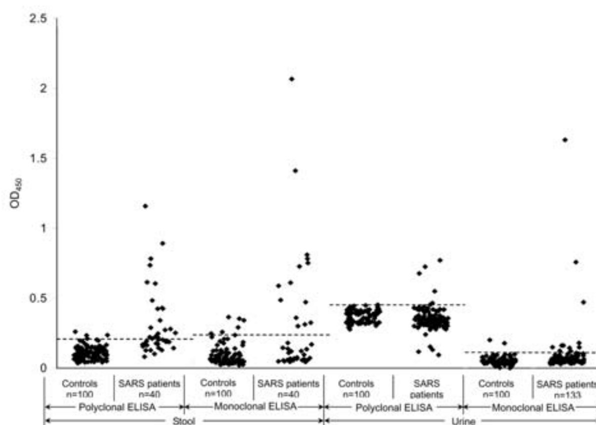

Figure. Evaluation of polyclonal and monoclonal antibody-based enzyme-linked immunosorbent assays (ELISAs) for detecting nucleocapsid protein in fecal and urine specimens. The dashed horizontal lines represent the corresponding cutoff optical density values at 450 nm (OD_{450}). SARS, severe acute respiratory syndrome.

data on different specimens (11). The OD_{450} values of both fecal (Pearson correlation 0.610, p<0.0005) and urine specimens (Pearson correlation 0.475, p<0.0005) detected by the 2 ELISAs were significantly correlated.

Conclusions

The method of choice for early diagnosis of SARS-CoV infection should be the qRT-PCR. The sensitivity of qRT-PCR is superior to that of both ELISAs. Moreover, qRT-PCR can detect SARS-CoV earlier in fecal specimens (Tables 1 and 2). Among the 40 fecal samples from SARS patients, 32 (80%) were positive by qRT-PCR, which was significantly higher than that of the polyclonal (50%) and monoclonal (35%) antibody-based ELISAs (McNemar test, p<0.005 and p<0.001, respectively). Of the 133 urine samples from SARS patients, 33 (25%) were positive by qRT-PCR, which was also significantly higher than that of the polyclonal (5%) and monoclonal (8%) antibody-based ELISAs (McNemar test, p<0.001 for both comparisons). When qRT-PCR was used as a standard, the sensitivities of the polyclonal and monoclonal antibody–based ELISAs were 53.1% (17/32) and 43.8% (14/32) in fecal specimens, and 12.1% (4/33) and 15.2% (5/33) in urine specimens, respectively. The qRT-PCR can detect SARS-CoV in fecal specimens obtained on days 1 to 27 after onset of symptoms and in urine specimens obtained on days 9 to 45. Moreover, 6 (75%) of the 8 fecal specimens obtained on days 1 to 10 were positive by qRT-PCR. All 3 tests had the highest detection rates in fecal specimens collected on days 16 to 20, which suggested that this was the period of peak viral shedding in stool. The detection rates in urine specimens were much lower than those in fecal specimens in all 3 assays.

SARS-CoV can be detected during the late phase of illness. Since SARS-CoV cannot be readily isolated from SARS patients after week 3 of illness (14), the detection of SARS-CoV beyond this time may be due to prolonged shedding of nonviable viruses in these patients or the presence of neutralizing immunoglobulins in clinical specimens, which has prevented viral replication in cell cultures.

SARS-CoV RNA concentration and ELISA results were correlated. Higher detection rates by both ELISAs

Table 1. Detection of SARS-CoV in clinical specimens by qRT-PCR and ELISA in relation to time from onset of symptoms*

Days from onset of symptoms	No. of specimens	No. of positive specimens (%)		
		qRT-PCR	Polyclonal antibody–based ELISA	Monoclonal antibody–based ELISA
Fecal specimens				
1–5	4	3 (75)	0	1 (25)
6–10	4	3 (75)	2 (50)	0
11–15	13	9 (69)	7 (54)	5 (38)
16–20	14	13 (93)	9 (64)	7 (50)
21–25	4	3 (75)	2 (50)	1 (25)
26–30	1	1 (100)	0	0
Urine specimens				
1–5	1	0	0	0
6–10	13	1 (8)	0	2 (15)
11–15	19	3 (16)	0	0
16–20	67	24 (36)	5 (7)	7 (10)
21–25	10	1 (10)	0	1 (10)
26–30	11	2 (18)	0	0
31–40	5	0	1 (20)	0
41–50	5	2 (40)	0	1 (20)
51–60	2	0	0	0

*SARS-CoV, severe acute respiratory syndrome coronavirus; qRT-PCR, quantitative reverse transcription–polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

Table 2. Detection of SARS-CoV by qRT-PCR and ELISA in clinical specimens of patients with SARS*

	Fecal specimens			Urine specimens		
RNA concentration (copies/mL)	No. specimens	No. positive by polyclonal antibody–based ELISA	No. positive by monoclonal antibody–based ELISA	No. specimens	No. positive by polyclonal antibody–based ELISA	No. positive by monoclonal antibody–based ELISA
$<3 \times 10^2$	8	3	0	100	2	6
3×10^2–$<10^4$	5	3	3	16	1	1
10^4–$<10^6$	3	0	0	10	1	1
10^6–$<10^8$	9	5	0	7	2	3
10^8–$<10^{10}$	13	8	10	0	0	0
$\geq 10^{10}$	2	1	1	0	0	0
Total	40	20	14	133	6	11

*SARS-CoV, severe acute respiratory syndrome coronavirus; qRT-PCR, quantitative reverse transcription–polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

were found in specimens with higher viral concentrations (Table 2). There was also a significant correlation between viral load and ELISA OD_{450} values in fecal specimens tested with the monoclonal antibody–based ELISA (Pearson correlation 0.424, $p = 0.003$), and in urine specimens tested with both the polyclonal and monoclonal antibody–based ELISAs (Pearson correlation 0.386 and 0.331, respectively, $p<0.0005$ in both analysis). Although the correlation between viral load and ELISA OD_{450} values in fecal specimens tested with the polyclonal antibody–based ELISA was not significant, there was a trend for such a correlation (Pearson correlation 0.229, $p = 0.078$).

In this study, fecal and urine samples were used because they are easier and safer to obtain and more readily available. In our previous reports, nucleocapsid protein was detected by the polyclonal antibody–based ELISA in 83% of nasopharyngeal aspirates collected on days 11 to 15 after symptom onset and by the monoclonal antibody–based ELISA in 85% of serum obtained during the first 10 days (10,11). These findings suggest that ELISA may be more useful when used with nasopharyngeal aspirate and serum specimens. However, these specimens were not included in the current study because only small amounts were available. Similar studies should be conducted if such samples are available.

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Dr. Lau is assistant professor in the Department of Microbiology, University of Hong Kong. Her research interests include emerging infectious diseases and novel pathogens.

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Address for correspondence: Kwok-Yung Yuen, Department of Microbiology, University of Hong Kong, University Pathology Building, Queen Mary Hospital, Hong Kong, People's Republic of China; fax: 852-2855-1241; email: hkumicro@hkucc.hku.hk

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Veillonella montpellierensis Endocarditis

Clarisse Rovery,* Anne Etienne,* Cédric Foucault,* Pierre Berger,* and Philippe Brouqui*

Veillonella spp. rarely cause infections in humans. We report a case of *Veillonella* endocarditis documented by isolating a slow-growing, gram-negative microbe in blood cultures. This microbe was identified as the newly recognized species *Veillonella montpellierensis* (100% homology) by 16S RNA gene sequence analysis.

Veillonella are anaerobic, gram-negative cocci, part of the normal flora of the mouth, gastrointestinal tract, and vaginal tract. *Veillonella dispar*, *V. atypica*, and *V. parvula* have been cultured from human specimens. They are infrequently isolated in human infections. Rarely, *Veillonella* species have been the only etiologic agents identified in serious infections such as meningitis, osteomyelitis, prosthetic joint infection, pleuropulmonary infection, endocarditis, and bacteremia. A new species, *V. montpellierensis*, has recently been isolated from the gastric fluid of a newborn and from the amniotic fluid of 2 women (1). Its pathogenic role is still debated.

The Study

A 75-year-old woman was admitted to the intensive care unit with septic shock. She had a history of diabetes mellitus. A cardiac murmur had been noted 8 years earlier but was not investigated further. On physical examination, the patient had aortic and mitral murmur. Reagent strip for urinalysis detected leukocytes and nitrites. After 3 blood cultures and urinalysis, the patient was treated for septic shock secondary to upper urinary tract infection with ceftriaxone, 2 g/day intravenously. The patient's condition rapidly improved with antimicrobial drugs and dopamine. Three days after admission, she was afebrile and hemodynamically stable; she was transferred to the urology department for acute pyelonephritis, which had not been confirmed by computed tomographic (CT) scan. Urine culture yielded *Gardnerella vaginalis*. Chest radiograph showed a patchy density of the right inferior pulmonary lobe confirmed by chest CT scan that suggested either pneumonia or neoplasia. On day 6, a transesophageal echocardiograph, performed because of the cardiac murmur, showed oscillating intracardiac masses on the aortic and mitral valves. Because the blood cultures were still negative, we determined that the patient had culture-negative endocarditis and replaced ceftriaxone with amoxicillin, 12 g/day for 6 weeks, in addition to gentamicin, 3 mg/kg/day for 3 weeks. On day 26, another transesophageal echocardiograph was performed and showed that the vegetation on the aortic valve had disappeared and the mitral vegetation was greatly reduced. The patient was discharged after 42 days of antimicrobial drug treatment, and follow-up was not possible.

On day 14 after sampling, 2 of 3 anaerobic blood cultures (automated blood culture BACTEC 9240 system (Becton Dickinson, Le Pont de Claix, France) yielded a slow-growing, gram-negative microbe. Blood was subcultured onto Columbia agar with 5% sheep blood (Mérieux, Marcy l'Etoile, France) under 5% CO_2 and anaerobic atmosphere and resulted in small colonies. This slow-growing microbe was lost after 2 subcultures, and no isolate is available for further description. The isolate retrieved in the blood culture was identified by 16S rRNA gene sequence analysis. The template DNA was prepared from a few colonies that were isolated on the blood agar incubated anaerobically. DNA was extracted by using Fastprep DNA extraction kit (Ozyme, St Quentin en Yvelines, France) according to the manufacturer's recommendations and was subjected to polymerase chain reaction (PCR) targeting the 16S rRNA gene as previously described (2). Sequencing the PCR product (2) showed a 1,531-nucleotide sequence. This sequence shared 100% homology with that of *V. montpellierensis* (GenBank accession no. AY244769) and was already reported (GenBank accession no. AY244769) in a previous article (3). In this article, the isolated *Veillonella* strain (that was isolated from our patient) was first identified as "candidatus *V. atypica*" since the sequencing of the amplicon disclosed 94% sequence similarity with that of *V. atypica* (3). *V. montpellierensis* had not yet been described. PCR contamination was unlikely since this organism had never been amplified in our laboratory and negative controls remained negative.

Conclusions

According to the modified Duke criteria (4), our patient had definite endocarditis. Anaerobic microbes do not commonly cause endocarditis (5). Most cases of anaerobic endocarditis are caused by anaerobic cocci, *Propionibacterium acnes*, and *Bacteroides fragilis* group (5). We describe the seventh reported case of well-documented infectious endocarditis in which a *Veillonella* species was the sole pathogen and the first due to *V. montpellierensis.* Characteristics of the 7 *Veillonella* endocarditis patients are summarized in the Table. Five of them fulfilled the Duke modified criteria for definite endocardi-

*Hôpital Nord, Marseille, France

Table. Summary of 7 reported patients with endocarditis due to *Veillonella* species*

Veillonella species isolated	Age	Sex	Infected valve	Preexisting valvular disease	Echo vegetation	Specimen site	Duration of illness before diagnosis	Outcome
V. alcalescens (6)	51	Male	Prosthetic mitral	Y	Y	Blood†	–	Cured
V. alcalescens (7)	60	Male	Native aortic	Benign heart murmur	N	Valve‡	6 mo	Cured
V. alcalescens (8)	35	Male	Native mitral	N	–	Blood	7 mo	Cured
V. dispar (9)	56	Male	Prosthetic mitral	Y	Y	Blood	2 wk	Cured
V. dispar (10)	57	Female	Prosthetic mitral	Y	Y	Blood†	3 wk	Cured
V. parvula (11)	49	Male	Prosthetic mitral	Y	N	Valve‡	36 h	Cured
V. montpellierensis (present work)			Native mitral and aortic	Y	Y	Blood	6 d	Unknown

*Y, yes; N, no; echo, echocardiography.
†Negative culture of valve specimens.
‡Negative culture of blood specimens.

tis; the 2 others were possible endocarditis. All previously reported cases of *Veillonella* endocarditis were due to either *V. dispar* (9,10), *V. parvula* (11), or *V. alcalescens* (6–8), currently considered *V. parvula* (12). One patient had no history of fever (7), and 1 patient had no preexisting valvular disease (8). Five patients had an infected mitral valve; 4 of the 5 had prosthetic valves. Our patient had mitral and aortic endocarditis. All patients had positive blood culture except 2, for whom the diagnosis was made by culturing the valve (6,11). *Veillonella* spp. had also been isolated from intravenous drug users with polymicrobial endocarditis (13); *V. parvula* was isolated from a lung abscess in a patient with echocardiographic vegetations, but blood cultures were negative (14). We could not test the susceptibility of the organism because the bacterium was lost on subculture. In treating infections with *Veillonella* species, penicillin has been the antimicrobial agent of choice (10). However, recent studies found a notably high resistance to penicillin G (MIC ≥2µg/mL) (15). These penicillin G–resistant isolates showed generally reduced susceptibility to ampicillin or amoxicillin but remained susceptible to amoxicillin and clavulanate (15). We treated our patient for culture-negative endocarditis with amoxicillin. As the clinical state of our patient improved, we did not change antimicrobial agents.

Our isolate has recently been compared with 3 other isolates and classified as a new *Veillonella* species named *V. montpellierensis* by Jumas-Bilak et al. (1). We demonstrate here that *V. montpellierensis* is pathogenic for humans and may be included as a new agent of endocarditis caused by fastidious pathogens.

We report here the seventh case of endocarditis due to *Veillonella* spp. identified by PCR amplification and sequencing of 16S rDNA gene and the first case of endocarditis due to *V. montpellierensis*. This case reemphasizes the usefulness of molecular methods in identifying fastidious microorganisms and in describing new clinical entities (3).

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Dr. Rovery is a physician who specializes in infectious disease and tropical medicine in Marseille. Her research interests include rickettsial diseases and emerging pathogens.

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Address for correspondence: Philippe Brouqui, Service des Maladies Infectieuses et Tropicales, Hôpital Nord, Chemin des Bourrelys, 13915 Marseille Cedex 20, France; fax: 33-0-4-91-96-89-38; email: philippe.brouqui@medecine.univ-mrs.fr

Burkholderia fungorum Septicemia

G. Peter Gerrits,*† Corné Klaassen,* Tom Coenye,‡ Peter Vandamme,‡ and Jacques F. Meis*†

We report the first case of community-acquired bacteremia with *Burkholderia fungorum*, a newly described member of the *Burkholderia cepacia* complex. A 9-year-old girl sought treatment with septic arthritis in her right knee and ankle with soft tissue involvement. Commercial identification systems did not identify the causative microorganism.

The genus *Burkholderia* contains >30 species, of which *Burkholderia pseudomallei*, *B. mallei*, and members of the *B. cepacia* complex are the most well known. The species *B. fungorum* was recently proposed for isolates recovered from the environment, and animal and human clinical samples (1,2). Here we describe the first case of community-acquired bacteremia with *B. fungorum* in a 9-year old girl with the clinical features of septic arthritis.

Case Report

A previously healthy 9-year-old girl had pain, swelling, and redness of the right foot and ankle 4 days before hospital admission, and similar symptoms of the right knee, 2 days before admission. One day before admission, a temperature of 39°C developed. She and her family had no history of arthritis, rheumatic arthritis, or other autoimmune disorders.

On physical examination, she had a body temperature of 38.8°C. Except for some slight swelling, pain, and redness of the right ankle, no other abnormalities or suspected source of the fever was apparent. She did not allow pressure on the calcaneus, which was painful.

Laboratory investigations demonstrated a C-reactive protein level of 262 mg/L, an erythrocyte sedimentation rate of 125 mm in the first hour, and a leukocyte count of 12.6×10^9/L with 9.1×10^9/L neutrophils. Levels of serum electrolytes, creatinine, and hepatic enzymes were within normal limits. Tests for antinuclear antibodies and antineutrophilcytoplasmic antibodies were negative. Serologic results for cytomegalovirus, *Toxoplasma gondii*, *Borrelia burgdorferi*, and Epstein-Barr virus were unremarkable. Throat and feces samples were negative for any virus. Feces cultures were negative for *Salmonella*, *Shigella*, *Yersinia*, and *Campylobacter*.

Radiologic examination of the right lower leg demonstrated no abnormalities of knee, foot, and ankle. A magnetic resonance imaging scan of the right lower leg showed a modest fluid collection in the soft tissues between the calcaneus and fascia plantaris, but no signs of osteomyelitis. An echogram of the abdomen was unrevealing, and a computed tomographic scan of the cerebrum showed no signs of an intracranial or sinus infection.

A bone scintigraph demonstrated a slight asymmetric increased signal in the epiphysial disc of the femur at the right knee. Because osteomyelitis with soft tissue involvement was suspected, she was empirically treated with intravenous cefuroxime (800 mg 3x/day), which was continued when the blood cultures became positive with gram-negative rods (on the fourth day of admission). Defervescence was initially seen within 24 h, but her temperature rose again to 38.5°C on the day 3 and up to 40.5°C on the day 7 of antimicrobial therapy. Because the cultured gram-negative rod was susceptible to cefuroxime in vitro, antimicrobial treatment was not changed. C-reactive protein level initially diminished gradually to 162 mg/L, but increased to 310 mg/L. Several diagnostic efforts, including a cardiac echogram, electrocardiogram, and an ear, nose, and throat workup, were carried out to determine the focus of infection but without convincing results. Although the patient had a clinical course of recurrent fever, she was otherwise stable, and we therefore decided to discontinue cefuroxime on day 9. At that time, the gram-negative rods in blood cultures, obtained before and during cefuroxime treatment, were reported to be *B. fungorum*. Intravenous ciprofloxacin, 170 mg 2x/day (15 mg/kg/day), was started; within 24 h, she became afebrile, and the C-reactive protein level became normal within 1 week. After 10 days, intravenous administration of ciprofloxacin was changed to oral administration (180 mg 2x/day) and continued for another 4 weeks. She recovered completely without sequelae.

On 3 following days, before and during treatment with cefuroxime, 5 blood cultures were collected in BACTEC aerobic pediatric resin bottles (Becton Dickinson, Sparks, MD, USA) and incubated in the BACTEC 9240 (Becton Dickinson). All blood cultures yielded gram-negative rods after 4 days of incubation. Subcultures produced a fine growth after 1 day of incubation.

The organism was positive for catalase and oxidase, and routine identification procedures with the API 20NE kit and database release 6.0 (bioMérieux, Marcy l'Etoile, France) produced the numerical code profile 1066157 which, according to the database, was a good identification (98.9% T 0.42) as *B. cepacia*. Antimicrobial susceptibility testing was performed with broth microdilution in accordance with Clinical and Laboratory Standards Institute

*Canisius-Wilhelmina Hospital, Nijmegen, the Netherlands; †Sint Maartenskliniek, Ubbergen, the Netherlands; and ‡University of Ghent, Ghent, Belgium

(formerly NCCLS) protocols (3). The organism was susceptible to amoxicillin (MIC 1 mg/L), cefotaxime (MIC 0.5 mg/L), ceftazidime (MIC <0.5 mg/L), cefuroxime (MIC 1 mg/L), trimethoprim/sulfamethoxazole (MIC <1 mg/L), meropenem (MIC 0.25 mg/L), tobramycin (MIC <0.25 mg/L), and ciprofloxacin (MIC 0.5 mg/L), but resistant to cefazolin (MIC >32 mg/L). This rather susceptible microorganism prompted us to repeat the identification with partial sequencing of the 16S rRNA gene. DNA was isolated from a pure culture by using established protocols (4). Amplification primers 5′-CCTAACACATG-CAAGTCGARCG-3′ (forward) and 5′-CGTAT-TACCG-CGGCTGCT-3′ (reverse), both from Eurogentec (Seraing, Belgium) were used in a standard polymerase chain reaction (PCR) to generate a 490-bp fragment from the 5′ end of the 16S gene. The PCR (25 µL) consisted of 1 µL DNA, 0.5 µmol/L of both PCR primers, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, and 1 U FastStart Taq DNA polymerase (Roche Diagnostics, Almere, the Netherlands) in 1 x reaction buffer. Cycling conditions were as follows: 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C, repeated 30 times, preceded by a 10-min activation step at 94°C and followed by an additional 10 min elongation step at 72°C. The obtained amplicon was purified by using High Pure chemistry (Roche Diagnostics) and sequenced with the reverse amplification primer using a MegaBACE DYEnamic ET Dye terminator Kit as suggested by the manufacturer (Amersham Biosciences, Roosendaal, the Netherlands). Reaction products were purified by ethanol precipitation, dissolved in distilled water, and analyzed on a MegaBACE 500-capillary DNA analysis platform (Amersham Biosciences) under standard electrophoretic conditions. The obtained DNA sequence was compared to the public DNA databases by using the BLAST interface (http://www.ncbi.nlm.nih.gov/BLAST/) (5) and proved to be 100% identical to previously reported *B. fungorum* sequences.

To confirm this molecular identification, cellular protein and fatty acid analyses were also performed. Whole-cell proteins were prepared and evaluated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (1). The identification of the isolate as *B. fungorum* was subsequently confirmed by comparing it to a large database, which contained profiles of all *Burkholderia*, *Ralstonia*, and *Pandoraea* species and various other gram-negative nonfermenters (2). After aerobic growth for 24 h on Trypticase soy agar (Becton Dickinson, Erembodegem, Belgium), a loopful of well-grown cells was harvested, and fatty acid methyl esters were prepared, separated, and identified by using the Microbial Identification System (MIDI, Inc., Newark, DE, USA). By using the commercially available database (MIDI, Inc.), the isolate was again falsely identified as *B. cepacia* with a score of 0.611.

The oxidase-positive isolate reduced nitrate and assimilated glucose, mannose, mannitol, N-acetyl-glucosamine, adipate, malate, citrate, and caprate. Beta-galactosidase activity was present.

Conclusions

To the best of our knowledge, this case is the first description of bacteremia and invasive infection due to *B. fungorum*. The name *B. fungorum* was recently proposed for a group of 9 *B. cepacia*–like isolates recovered from the environment and human and animal clinical samples (1). The only 2 strains from human clinical samples in that study were recovered from the vaginal secretions of a pregnant woman (22 weeks) with *Candida* sp. vaginitis and preterm labor, and the cerebrospinal fluid of a 66-year-old woman, respectively. No clinical data were available from these patients; therefore, the clinical significance could not be determined. Since the original report was made, *B. fungorum* has been identified in a range of soil- and plant-associated samples, in infections of the central nervous system of a pig and a deer (H. Scholz and P. Vandamme, unpub. data), and in the respiratory secretions of people with cystic fibrosis (1,2). However, in these cases, the clinical significance of isolation of *B. fungorum* was also unclear.

Since *B. fungorum* was only described recently and has not been found frequently in clinical samples, the organism is not included in most commercial biochemical databases used for identification, and it has previously been shown that *B. fungorum* isolates can easily be misidentified as *B. cepacia* complex organisms (1,2). A similar misidentification with conventional commercial biochemical tests was recently described in a case of *B. cenocepacia* vaginal infection (6). In the present case, the antimicrobial susceptibility profile, the patient's history, and the clinical findings suggested that this isolate did not belong to the *B. cepacia* complex, and the identification as *B. fungorum* was confirmed by using a polyphasic approach. Most *B. cepacia* complex infections in non–cystic fibrosis patients are nosocomial in origin, but severe community-acquired infections (including endocarditis, brain abscesses, and pneumonia) have also been reported (7). As this case illustrates, *B. fungorum* may pose a challenge to many clinical microbiology laboratories, and infections with this organism may be erroneously diagnosed as *B. cepacia* complex infections. A complete immunologic workup for our patient did not give any indication of why this child experienced this infection. The source of the *B. fungorum* bacteremia in our patient remained elusive, but, undoubtedly, it was a community-acquired infection manifested as a soft tissue infection of her leg. Of interest was the clinical failure of cefuroxime therapy, despite the isolate's in vitro susceptibility, and the rapid response to ciprofloxacin treatment.

Dr. Gerrits is pediatric pulmonologist at Canisius-Wilhelmina Hospital, Nijmegen, the Netherlands, and consultant at Sint Maartenskliniek, Ubbergen, the Netherlands. His major research interests are infectious diseases in general and pulmonary diseases due to asthma and cystic fibrosis specifically.

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Address for correspondence: Jacques F. Meis, Department of Medical Microbiology and Infectious Diseases, Canisius-Wilhelmina Hospital, PO Box 9015, 6500 GS Nijmegen, the Netherlands; fax 31-24-365-7516; email: j.meis@cwz.nl

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Blackwater Fever in Children, Burundi

Federico Gobbi,* Sabrina Audagnotto,* Laura Trentini,* Innocent Nkurunziza,† Manuel Corachan,‡ and Giovanni Di Perri*

Blackwater fever is characterized by acute intravascular hemolysis with hemoglobinuria in patients with *Plasmodium falciparum* malaria. Its pathogenesis and management are still debated. Nine cases of this syndrome occurred in 2003 at Kiremba Hospital in Burundi in children receiving multiple quinine treatments.

Blackwater fever (BWF) is a clinical entity well known only in long-term residents in *Plasmodium falciparum*–endemic areas who take quinine irregularly. This syndrome became less frequent when chloroquine was the drug of choice for malaria from 1950 until the 1990s (1). Glucose-6-phosphate dehydrogenase (G6PD) deficiency is also frequently associated with the syndrome; however, its role is not determinant, as BWF is frequently described in patients with normal erythrocyte G6PD levels who are receiving quinine for severe malaria (2). Isolated cases have also been described with other antimalarials, such as halofantrine and mefloquine, which belong to the aminoalcohol drug family (3–5).

The pathogenesis of BWF thus remains unclear (4,6,7). Its management changed with the introduction of artemisinin derivates but is still debated. White and other researchers (2,8) state that parenteral quinine can be stopped when artemisinin derivatives are available because they seem to be safe and well tolerated.

Clinical features defining BWF are well established (2,9). The syndrome is characterized by severe intravascular hemolysis and anemia producing dark urine in patients with severe malaria. Abdominal pain, jaundice, hepatosplenomegaly, vomiting, and renal failure (especially in adults) have also been reported.

As *P. falciparum* resistance to chloroquine developed, quinine was increasingly used in clinical practice for treating intermittent malaria infections. BWF seemed to reappear at the end of the 1990s, according to descriptions in several European clinics of imported diseases (3–5). It particularly affected European missionaries with years of previous residence in malarious areas. In fact, some of the classical definitions of the syndrome described it in expatriate populations only (9). Cases of BWF in autochthonous populations have recently been described in the literature from Southeast Asia (10) and in African children in Senegal (7). We describe a large number of BWF cases in the pediatric ward of a hospital in the Burundi highlands, where no case has been observed in the previous 10-year period (1992–2002).

The Study

Since January 1992, a hospital-based survey of malaria has been conducted at Kiremba Hospital in Ngozi Province. This 140-bed facility is located 1,540 m above sea level in the Burundi highlands; it serves a population of 75,000 (11).

For each case of malaria, laboratory data and clinical findings are recorded. Rising illness and death rates are being reported throughout Burundi, where *P. falciparum* accounts for most cases (12). According to the Kiremba Hospital registry, a 2-fold increase in admissions for malaria in the pediatric ward (children ≤14 years of age) was recorded from 1997 (658 cases) to 2002 (1,343 cases).

From February to December 2003, a period when 1,039 malaria patients were hospitalized, we observed 9 cases of severe intravascular hemolysis with dark urine in pediatric patients who had been treated with quinine. These children were all male with a mean age of 8.2 years (range 3–14 years). According to patients' health cards, all had been previously treated with quinine, either parenterally or orally according to Burundi's national policy for treating severe malaria (10 mg/kg 3×/day for 7 days). Clinical and laboratory data are presented in the Table.

When BWF occurred, quinine was stopped and artemether (3.2 mg/kg on day 1, then 1.6 mg/kg from day 2 to day 5), was administered intramuscularly in association with 3 days of corticosteroid therapy. All patients had severe anemia requiring blood transfusion according to hospital policy (hemoglobin <4.5 g/dL or <6 g/dL with accompanying dyspnea). Four patients needed 1 U of blood; 5 other patients needed >1 U. No deaths were recorded, and clinical outcome on discharge was satisfactory: thick smears were negative and hemoglobin levels had improved in all patients.

Conclusions

In Burundi, chloroquine was replaced by sulfadoxine-pyrimethamine (SP) alone as firstline treatment for uncomplicated malaria in 2001. However, the rapid development of resistance to SP brought back the use of oral quinine, a drug still available in health centers as well as in hospital settings. Since November 2003, artesunate and amodiaquine have replaced SP as firstline treatment in Burundi (13).

The result of the new treatment guidelines was a considerable reduction in the number of hospitalized malaria

*Clinica Universitaria Malattie Infettive, Turin, Italy; †Hopital de Kiremba, Ngozi, Burundi; and ‡University Hospital, Barcelona, Spain

Table. Clinical data from children at the onset of blackwater fever

Data	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9
Age	13	3	5	14	7	12	14	3	3
Hemoglobin (g/dL)	4.2	3.8	2.2	4.6	5.8	4.3	2.5	2.5	3.1
Parasites/µL	4,700	6,800	8,100	1,680	0	7,600	11,480	5,450	0
Fever*	+	+	+	–	+	+	+	+	+
Jaundice†	+	–	+	+	–	–	–	–	–
Hepatomegaly‡	–	+	–	–	–	–	–	+	+
Splenomegaly‡	–	+	+	–	–	+	+	+	+
Vomiting	–	–	+	+	+	+	–	–	–
Oligoanuria	–	–	–	–	–	–	–	–	–
Abdominal pain	+	–	–	+	–	–	–	+	–

*Axillary temperature >37.5°C.
†Total bilirubinemia >1.5 mg/dL.
‡Manually assessed, >2 cm by costal margin.

cases in 2004 (671 cases from January 1 to October 31, 2004). No cases of BWF were observed in this period. Despite changes in policy for the use of firstline antimalarial drugs, however, parenteral quinine continued to be the drug of choice for severe cases throughout this period.

All 9 patients with BWF seen in 2003 (with 1 exception) lived in the area served by Kiremba Hospital and were recorded during an 11-month period. This number represents an incidence of 11.5 cases/100.000 population/ year.

In reviewing recent literature, we found only 1 publication on BWF involving an African population (7). The study was carried out at the Dielmo village in Senegal, where 3 cases were detected in a 10-year prospective study in a small population (315 inhabitants). All 3 cases were in children who suffered several malaria attacks and were treated with oral or parenteral quinine, depending on the severity of the case. As a consequence, quinine was withdrawn as the drug of firstline therapy for uncomplicated cases of malaria. No more cases of BWF were recorded during the subsequent 6-year follow-up period.

In our study, patients were all boys admitted to the pediatric ward. No cases of oligoanuria were seen, which is not surprising in pediatric patients (14). At the onset of severe intravascular hemolysis, the blood smears of 2 children were negative for malaria; parasitemia was low in the others. These findings agree with the definition of BWF as being characterized by scanty or absent parasitemia (4,6,9). We were unable to determine G6PD levels in our patients, which is a major limitation of our study. However, in view of the overlap between malaria, quinine administration, and G6PD deficiency, the hemoglobinuria triggered by this deficiency should not be seen as a separate syndrome (10).

The management of our cases included 3 components: First, treatment with parenteral (intramuscular) artemether (3.2 mg/kg on day 1, then 1.6 mg/kg from days 2 to 5) after stopping quinine, according to recent trends in the literature (3–5). Artemisinin derivates have not been implicated in BWF episodes unless given in combination with mefloquine (8). Second, blood transfusion for severe anemia was performed according to the above described hospital policy. And finally, a short course of corticosteroid therapy was administered.

Our experience suggests the need to review the definition of BWF since the syndrome affects not only adult expatriates but also African children. All reported African children with cases of BWF had frequently received oral quinine therapy. African adults seem to be only occasionally affected. This finding suggests that BWF occurs in nonimmune persons or those who have not yet gained immunity. This statement is supported by the lack of cases in adults cured in the same hospital.

To reduce hemolysis, we treated BWF with corticosteroids, even though this step is not recommended by the World Health Organization. Our reasoning was that the phenomenon could be related to immune mechanisms in quinine-sensitized erythrocytes (14).

The influence of quinine seems to be an important factor in the pathogenesis of BWF. Other amino-alcohol drugs such as mefloquine or halofantrine have never been used intensively in Africa, principally because they are expensive. When policy changes lead to less use of oral quinine, BWF syndrome tends to disappear. Further similar reports from other areas in the African continent that would confirm our findings could have important implications on national policies for treating malaria in African children.

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Dr. Gobbi is a specialist in infectious diseases at Turin University in Italy. Much of his research has been conducted in African countries (Burundi, Kenya, Democratic Republic of Congo, Uganda, Mozambique). His primary research interests are tropical diseases, in particular, malaria.

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Address for correspondence: Federico Gobbi, Clinica Universitaria Malattie Infettive, Ospedale Amedeo di Savoia, Corso Svizzera 164, 10149, Torino, Italy; fax: 39-01-1439-3972; email: fedgobbi@tin.it

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Cervids as Babesiae Hosts, Slovenia

Darja Duh,* Miroslav Petrovec,*
Andrej Bidovec,† and Tatjana Avsic-Zupanc*

We describe cervids as potential reservoir hosts of *Babesia* EU1 and *B. divergens*. Both babesial parasites were found in roe deer. Sequence analysis of 18S rRNA showed 99.7% identity of roe deer *Babesia* EU1 with the human EU1 strain. *B. divergens* detected in cervids was 99.6% identical to bovine *B. divergens*.

Human babesiosis is an emerging tick-transmitted disease caused by intraerythrocytic parasites of the genus *Babesia*. A bovine parasite, *Babesia divergens* has been implicated as the most common agent of this dangerous zoonosis in Europe (1). The life cycle of *B. divergens* is determined by cattle, the vertebrate host, and by European sheep ticks, *Ixodes ricinus*. Ticks are not only the vectors of *B. divergens* but also its most important nonbovine reservoir (2). Many questions regarding parasite epidemiology and biology and the host response to infection remain to be answered. Furthermore, molecular data for *B. divergens* are scarce; only 1 DNA sequence of this parasite from humans from mainland Europe has been recently deposited (3). Recently, 2 cases of human babesiosis have been reported in Italy and Austria. The etiologic agent was identified as *Babesia* EU1, a pathogen closely related to, but clearly distinct from, *B. divergens* (4). The distinction was based on analysis of the complete babesial 18S rRNA gene, which also showed that EU1 is most closely related to *B. odocoilei*, a parasite of white-tailed deer (*Odocoileus virginianus*) in the United States (5). *I. ricinus*, the most prevalent and widely distributed tick species in Europe, has already been implicated as the vector of EU1 (4,6). Moreover, *I. ricinus* has a wide range of vertebrate hosts and readily bites humans. Rapidly and accurately identifying the reservoir of *Babesia* EU1 will enable appraisal of the full range of disease control options.

The Study

We investigated 2 species of cervids shot by professional hunters from 1996 to 2000 in the vicinity of Ljubljana, Slovenia. DNA was extracted from spleen samples of 51 roe deer (*Capreolus capreolus*) and 30 red deer (*Cervus elaphus*), as previously described (6). Babesiae were detected in cervids by using specific nested polymerase chain reaction (PCR) that allowed discrimination between *B. divergens* and EU1. Primers were designed on the basis of alignment of complete 18S rRNA gene sequences of EU1, *B. divergens,* and *B. odocoilei*. With primers PIRO-A (7) and BABSr, a 600-bp babesial 18S rRNA gene was amplified with 5 µL of DNA and PCR Master Mix (Promega, Madison, WI, USA). One microliter of PCR product was used for nested PCR with either primer set PIRO-B/BOD and PIRO-B/BDV to detect 240 bp of 18S rRNA of EU1 and *B. divergens*, respectively. Both babesial parasites were detected in roe deer (76.5%); however, more animals were infected with *B. divergens* (54.9%) than *Babesia* EU1 (21.6%). Only 16.7% red deer were infected with *B. divergens* alone. Infection with babesial parasites did not differ significantly between sexes in either roe or red deer.

To assess DNA sequence homologies with EU1 from human and ticks, distinctive amplicons of the complete babesial 18S rRNA gene derived from cervids were cloned and sequenced. Parasite DNA from 2 roe deer that were found positive with a different set of nested primers was used in PCR with CRYPTO F and CRYPTO R (6). Amplicons were ligated into a plasmid vector (TOPO TA Cloning Kit for Sequencing, Invitrogen, Groningen, the Netherlands), and *Escherichia coli*–competent cells were transformed as instructed by the manufacturer. Plasmid DNA was purified from overnight cultures of selected colonies (Wizard Plus Minipreps DNA Purification System, Promega) and analyzed for inserts by restriction analysis with EcoR1 (Promega). Sequencing on both strands was carried out in an automated sequencer using BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). Two clones were included in reactions with T3, T7, and internal primers to obtain complete gene sequence. All primers designed and used for this study are listed in the Table. Sequences were analyzed with computer programs of the Lasergene 1999 software package (DNASTAR, Madison, WI, USA) and submitted to GenBank to determine accession numbers. Homology search and alignment of the complete sequence of the babesial 18S rRNA gene from 1 roe deer showed 99.7% (5 nucleotide [nt] differences) identity with EU1 from a human patient and 99.8% (4 nt differences) identity with EU1 present in *I. ricinus* ticks from Slovenia. The complete sequence of the babesial 18S rRNA gene from another roe deer was, however, nearly identical (99.6%, 7 nt differences) to babesial parasite MO1 and *B. divergens*. Phylogenetic relationships of babesiae from roe deer and from other sources are shown the Figure. By using TREECON software (8), a phylogenetic tree was constructed with the neighbor-joining

*Institute of Microbiology and Immunology, Ljubljana, Slovenia; and †Institute of Wildlife Pathology, Ljubljana, Slovenia

Table. Nucleotide sequences and optimum annealing temperatures of the primers designed and used for nested polymerase chain reaction (PCR) and those for amplifying and sequencing the complete babesial 18S rRNA gene

Primer	Nucleotide sequence (5′→3′)	Annealing temperature (°C)
BABSr*	CTC CAA TCC CTA GTC GGC A	60
BOD*	GTT ATT GAC TCT TGT CTT TAA	53
BDV*	AAT ATT GAC TGA TGT CGA GAT	53
CRYPTO F	AAC CTG GTT GAT CCT GCC AGT AGT CAT	60 (6)
PIRO A*	AAT ACC CAA TCC TGA CAC AGG G	(7)
PIRO B*	TTA AAT ACG AAT GCC CCC AAC	(7)
PIRO C	GTT GGG GGC ATT CGT ATT TAA	60
1055 F	GGT GGT GCA TGG CCG	60
1055 R	AAC GGC CAT GCA CCA C	60
1200F	CAG GTC TGT GAT GCC	60
1200 R	GGG CAT CAC AGA CCT G	60
CRYPTO R	GAA TGA TCC TTC CGC AGG TTC ACC TAC	60 (6)

*Primers used for nested PCR.

method, and topology of the tree was obtained with the K80 model. Support for the tree nodes was calculated with 1,000 bootstrap replicates.

Conclusions

Babesia EU1, a zoonotic pathogen, was the cause of human babesiosis recently reported by Herwaldt et al. (4). While *I. ricinus* was already implicated as a vector of EU1, no other information about biology, ecology, or geographic distribution of EU1 exists (4,6). Phylogenetic analysis based on comparing the complete 18S rRNA gene sequence of EU1 derived from humans and ticks with other babesial parasites showed that EU1 is more closely related to *B. odocoilei* than *B. divergens* (4). *B. odocoilei*, which is transmitted by *I. scapularis*, primarily infects white-tailed deer in the United States (5). Cases of fatal babesiosis were described in 2 other species of cervids, namely a zoo-housed caribou (*Rangifer tarandus caribou*) and an elk (*C. elaphus elaphus*) (9). Therefore, we tested 2 species of cervids from Slovenia as potential reservoir hosts of EU1. By using specific nested PCR, the presence of EU1 was established in roe deer (21.6%) but not in red deer.

In Slovenia, roe deer are widely distributed, and the population density is high. Their pasture comprises woodland, bushes, and even open meadows and fields (10). However, red deer were nearly extinct in Slovenia in the beginning of the 19th century. Although they were later imported from Austria, Poland, and Hungary, they are still less numerous and therefore harbor fewer ticks (10). The identity of babesial parasites from roe deer from Slovenia with EU1 was confirmed by cloning and sequencing the complete babesial 18S rRNA gene. The sequences obtained were 99.8% and 99.7% identical to the 18S rRNA genes of EU1 from ticks and humans, respectively. Since the habitat of roe deer is expanding in other European countries (10), additional studies are needed to determine whether roe deer are reservoir hosts of EU1 elsewhere in Europe.

Whereas the presence of EU1 in cervids was anticipated, detection of *B. divergens* in roe and red deer was surprising. With the exception of a single report of naturally acquired babesiosis caused by *B. divergens* in reindeer (*R. tarandus tarandus*), no data about cervids as reservoirs of *B. divergens* were available at the time of our research (11,12). Although *B. divergens* can infect cervids experimentally, animals experience only mild infections with low parasitemia (2,11). However, 54.9% of roe deer and 16.7% of red deer were infected with *B. divergens* in this study. Further cloning and sequencing of the complete 18S rRNA gene of the parasite indicated 99.6% (7 nt differences) identity with babesial parasite MO1 and *B. divergens*. MO1 was described as an etiologic agent of human babesiosis acquired in Missouri and was genetically almost identical to *B. divergens* (99.9% identity, 2 nt

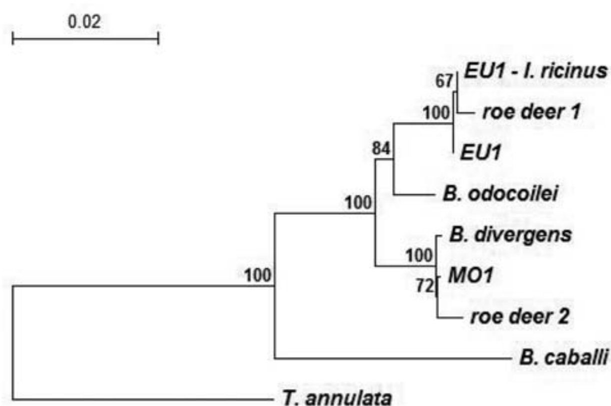

Figure. Phylogenetic relationships of representative babesiae deposited in GenBank and detected in this study, inferred from multiple sequence alignment of complete 18S rRNA gene. Accession numbers of babesiae: *Babesia* EU1 from *Ixodes ricinus* ticks, AY553915; babesiae from roe deer 1, AY572457; babesiae from roe deer 2, AY572456; *Babesia* EU1 from human, AY046575; *B. divergens*, AY046576; *B. odocoilei*, AY046577; *Babesia* MO1, AY048113; *B. caballi*, Z15104; and *Theileria annulata*, M64243. The number on each branch shows the percent occurrence in 1,000 bootstrap replicates.

differences), but the authors claimed that the parasites probably differ (13). However, piroplasms in abnormal hosts or hosts that are not generally considered primary hosts may have morphologic differences (12). In addition, high molecular identity of piroplasms does not necessarily mean that they have the same infectivity for different hosts.

A *Babesia* sp., tentatively called *B. capreoli*, was observed and described in red deer in Scotland (14) and sika deer (*C. nippon*) in Ireland (15). The parasite resembled *B. divergens* morphologically and antigenically. *B. capreoli* was suggested to be transmitted by *I. ricinus* ticks. The main difference between bovine *B. divergens* and these deer parasites is in their host specificity. Whereas *B. divergens* can infect a wide range of animals after splenectomy, including some deer species, various nonhuman primates, gerbils, and humans, *B. capreoli* can apparently infect only cervids and perhaps sheep (15). Nevertheless, with no deposited sequence of 18S rRNA of *B. capreoli*, the identity of *B. divergens* from roe and red deer from Slovenia is uncertain.

The finding that roe and red deer may be reservoirs for *B. divergens* has serious implications. Future research must determine if parasites from cervids share biologic characteristics with *B. divergens*, such as infectivity to cattle and humans and transmission by *I. ricinus*.

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Ms. Duh is a PhD student at the Medical Faculty, Ljubljana, Slovenia. She is currently working at the Institute of Microbiology and Immunology. Her research is primarily based on babesiae and other tickborne pathogens.

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Address for correspondence: Darja Duh, Institute of Microbiology and Immunology, Medical Faculty, Zaloška 4, 1000 Ljubljana, Slovenia; fax: 386-1-543-7401; email: darja.duh@mf.uni-lj.si

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Salmonella Agona Outbreak from Contaminated Aniseed, Germany

Judith Koch,* Annette Schrauder,* Katharina Alpers,* Dirk Werber,* Christina Frank,* Rita Prager,† Wolfgang Rabsch,† Susanne Broll,* Fabian Feil,‡ Peter Roggentin,§ Jochen Bockemühl,§ Helmut Tschäpe,† Andrea Ammon,* and Klaus Stark*

A nationwide outbreak of *Salmonella* Agona caused by aniseed-containing herbal tea occurred from October 2002 through July 2003 among infants in Germany. Consumers should adhere strictly to brewing instructions, although in exceptional cases this precaution may not be protective, particularly when preparing tea for vulnerable age groups.

Salmonella enterica serotype Agona is rarely isolated from humans in Germany (1). In other countries, *S.* Agona outbreaks among humans have been traced back to contaminated animal feed (2), dried milk, a peanut-flavored snack (3), and a cereal product (4).

In February 2003, a cluster of *S.* Agona infections was observed among children (median age 13 years, age range 3–20) receiving parenteral nutrition in an institution for handicapped persons in Lower Saxony, Germany. Analysis of national surveillance data showed a strong increase in *S.* Agona case reports in January and February 2003 compared to the same periods in 2001 and 2002. The increase was almost entirely attributable to infants ≤13 months of age. An outbreak investigation was conducted to identify risk factors and the vehicle of infection among infants.

The Study

From October 2002 through July 2003, a total of 42 *S.* Agona cases among infants ≤13 months of age were reported compared with 3 infections in this age group during the same period in the previous year (Figure 1). Cases occurred sporadically and were reported in 12 of the 16 German federal states. No substantial increase was found in the number of persons >13 months of age infected with *S.* Agona.

*Robert Koch Institute, Berlin, Germany; †Robert Koch Institute, Wernigerode, Germany; ‡Public Health Department, State of Lower Saxony, Hannover, Germany; and §Institute for Hygiene and the Environment, Hamburg, Germany

Among the 39 infants for whom data were available, 21 (54%) were girls, 23 (59%) were ≤6 months of age (median age 4.0 months), 35 (90%) had diarrhea, and 23 (59%) had fever. Twenty-one infants (54%) required hospitalization. No invasive disease was diagnosed and no deaths occurred. None of the patients had traveled abroad.

Exploratory interviews with the parents showed 2 common exposures: case-patients had received various brands of infant teas (n = 32, 82%) and had also consumed various milk powder products (n = 27, 69%) in the week before disease onset. Drinking teas containing herbs and spices such as fennel, aniseed, or caraway was reported for 27 (69%) infants. Because *S.* Agona had also been isolated from 2 aniseed samples in routine food safety monitoring in 2002 by the National Reference Center for Salmonella (Hamburg branch), a case-control study was launched to test the hypothesis that aniseed-containing herbal teas were the source of infection.

Patients ≤13 months of age with onset of diarrhea (defined as >2 soft stools in 24 hours) from October 1, 2002, to July 6, 2003, and *S.* Agona (outbreak strain) cultured from their stool were considered case-patients. Eight infants were excluded: 2 were from a set of triplets of whom only 1 was included, 3 had parents who could not be reached or refused to participate in the case-control study, 2 had an *S.* Agona pulsed-field gel electrophoresis (PFGE) patterns different from that of the outbreak strain, and 1 did not fulfill the case definition because of asymptomatic chronic infection. Controls were randomly selected from community population registries and frequency-matched by age group of the case-patients at time of illness (≤6 months or >6–≤13 months). The questionnaire elicited information on types of herbal teas, milk powder formulas, and other food consumed by the infants; tea preparation habits of parents; and breast-feeding history. For patients, details of usual food consumption were obtained for the 7-day period before disease onset. Parents of controls were

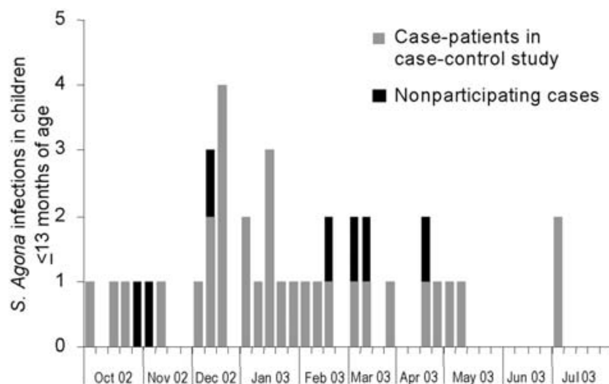

Figure 1. Epidemic curve showing week of onset of illness for confirmed cases of *Salmonella* Agona outbreak in infants ≤13 months of age, Germany, October 2002–July 2003 (n = 42).

asked about specific 7-day periods selected to match the distribution of the 7-day periods of the patients.

Overall, 31 patients and 130 controls were included in the study. Patients were significantly more likely than controls to have consumed any tea, any herbal tea from tea bags, and tea made from tea bags that contained aniseed (Table). Consumption of other types of tea bag products without aniseed or instant tea products was not associated with illness. Significantly fewer patients than controls were breast-fed. All other factors investigated were not significantly associated with illness.

Restricting analysis to those case-patients (n = 24) and controls (n = 41) who drank tea made from tea bags showed that the consumption of tea containing aniseed remained strongly associated with *S*. Agona infection (odds ratio [OR] 24.9, 95% confidence interval [CI] 6–102). Case-patients had consumed tea bag products containing aniseed from 12 different producers, and controls had consumed products from 8 different producers. Sixty-seven percent of the parents of patients reported always using boiling water for preparation of tea compared to 85% of control parents (p = 0.1).

When age in months was controlled in multivariable logistic regression, the consumption of tea from tea bags containing aniseed remained the only risk factor for *S*. Agona infection (OR 30.9, 95% CI 10.1–95.0). Breast-feeding was inversely associated with infection (OR 0.2, 95% CI 0.1–0.7).

The food safety authority in the state of Saxony-Anhalt collected 18 brands of teas containing aniseed from store shelves. One sample tested positive for *S*. Agona. In a subsequent nationwide sampling, various *Salmonella* serotypes were isolated from 61 (11%) of 575 tea and other aniseed-containing products. Tea from several of the contaminated tea brands had been drunk by affected infants. Among 44 *S.* Agona positive samples (8%), 41 were tea products containing aniseed and 3 were pure aniseed.

S. Agona isolates for subtyping were available from 17 patients, 6 different tea brands containing aniseed, and 3 samples of unprocessed aniseed. Molecular typing was performed at the National Reference Center for Salmonella (Wernigerode branch) by phage typing and PFGE. These methods are described elsewhere in detail (5). All isolates had phage type 02, identical PFGE patterns (Figure 2), and identical antimicrobial drug sensitivity patterns, but they were different from historical isolates. Four *S*. Agona–positive tea samples were quantitatively examined by using the most-probable-number method (6). This method yielded an estimated concentration of 0.036 salmonellae per gram of sample.

Products from all 12 producers of aniseed-containing herbal teas implicated in the study were traced back to a single importing company that had received the implicated large lot (≈15 metric tons) from Turkey. The company declared that the source of contamination of the raw product resulted from a batch of aniseed cultivated in Turkey that had been fertilized with manure.

All producers of tea products contaminated with *S*. Agona were notified by the food safety authorities. Unsold portions of contaminated production lots were recalled. The public was informed about the possibility that herbal tea may not be free from microbial contamination. Health authorities stressed that boiling water should be used in preparing tea and that a high steeping temperature should be maintained for at least 5 min before cooling the tea.

Conclusions

This investigation provides strong epidemiologic and microbiologic evidence that herbal tea containing aniseed caused this diffuse outbreak of *S*. Agona among infants. Tea consumption was the only factor associated with illness in the study. Strains of *S*. Agona isolated from patients, aniseed-containing herbal tea, and unprocessed aniseed imported from Turkey showed an identical PFGE pattern. To our knowledge, this description is the first of a *Salmonella* outbreak caused by herbal tea.

Parents interviewed in this study indicated that herbal tea was not perceived as a product at risk for contamination with enteric pathogens, particularly since hot water is typically used in tea preparation. In Germany, aniseed-

Table. Univariate analysis of exposure factors for *Salmonella* Agona infection among infants ≤13 months of age, Germany, October 2002–July 2003

Exposure	Case-patients (n = 31) No. (%)	Controls (n = 130) No. (%)	Odds ratio	95% CI*
Any tea	30 (97)	67 (52)	28.2	3.7–213.1
Any herbal tea from tea bags	24 (77)	41 (32)	7.4	2.9–18.0
Tea from tea bags with aniseed	21 (68)	9 (7)	28.2	10.3–77.7
Tea from tea bags without aniseed	3 (10)	33 (25)	0.3	0.1–1.1
Instant tea	9 (29)	34 (26)	1.2	0.5–2.9
Breast feeding	6 (19)	67 (52)	0.2	0.1–0.7
	Subgroup cases† (n = 24)	Subgroup controls† (n = 41)		
Always used boiling water for tea preparation	16 (67)	35 (85)	0.3	0.1–1.2

*CI, confidence interval.
†Subgroup analysis on case-patients and controls who drank tea made from tea bags.

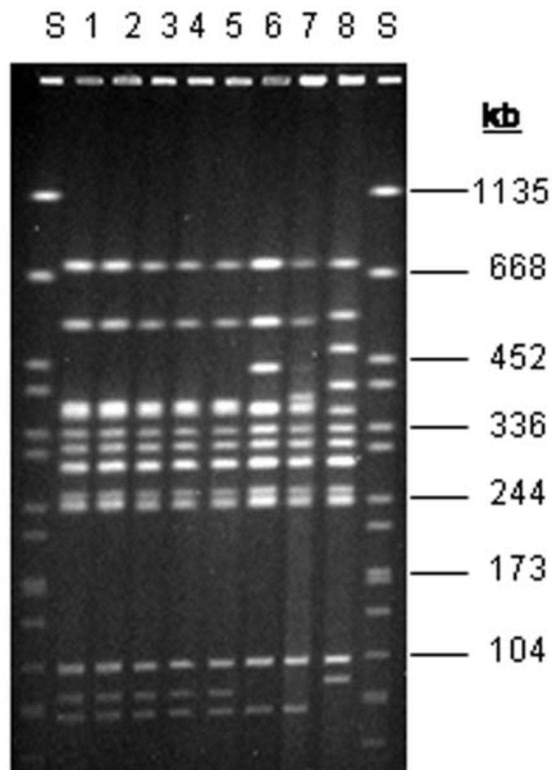

Figure 2. Pulsed-field gel electrophoresis patterns of XbaI-digested DNA from *Salmonella* Agona strains. Lanes 1 and 2, pattern SAX0001 (outbreak strain from tea); lanes 3-5, pattern SAX0001 (outbreak strain from humans); lanes 6-8 (nonoutbreak strains), lane S, molecular mass standard (S. Braenderup). kb, kilobases.

containing herbal teas, often in combination with fennel, are formulated and marketed specifically for their supposed antiflatulence and antispasmodic effects in infants. This may be 1 explanation of why this age group was particularly affected.

Aside from consumption patterns, host susceptibility likely played a role in this outbreak. Infants are particularly vulnerable to enteric pathogens because of factors such as gastric hypochlorhydria and insufficient mucosal immunity (7). Breast-feeding is known to reduce the severity of gastrointestinal infections among infants (8,9), which may explain the inverse association between a history of breast-feeding and *S.* Agona infection in this study.

Results of quantitative microbiologic investigations suggested low-level contamination of aniseed-containing teas with *S*. Agona. In previous outbreaks, similar low concentrations of salmonellae in foods (e.g., chocolate, cheddar cheese, and paprika-powdered potato chips) were reported, suggesting a low infectious dose (10–13). In dried food products such as aniseed, salmonellae can adapt to the dry state and may become resistant to environmental stress (e.g., heat, lack of nutrients) (14).

Two thirds of the parents of case-patients reported the consistent use of boiling water. Some parents reported quickly cooling the tea (e.g., by adding cold water). However, even if fewer parents of case-patients had always used boiling water (e.g., inaccurate recall), the use of boiling water may not have been sufficient to kill all viable salmonellae. Factors such as the addition of sugar, storage temperature, and elapsed time would have influenced the amount of salmonellae at the time of tea consumption. Further microbiologic studies on the heat resistance of salmonellae and desiccated strains are needed to provide information on how tea products can be rendered microbiologically safe through appropriate heat treatment during production and preparation at home.

Diffuse outbreaks may only be detected by demonstration of an identical pathogenic clone or when rare serotypes such as *S*. Agona are involved (15). Because of their wide distribution and long shelf-life, the implicated tea products could be sampled and linked to the human infections.

Because of underdiagnosis, the *S*. Agona cases in this outbreak most likely represented only a fraction of all infections due to contaminated herbal tea. Importing and tea-producing companies need to develop procedures to ensure microbiologic safety of their products. Brewing instructions on packages of tea should inform the consumer about potential microbiologic risks and the importance of following brewing instructions, especially in view of vulnerable populations such as infants and persons with weakened immune systems.

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Dr. Koch is a physician and epidemiologist at the Department for Infectious Disease Epidemiology of the Robert Koch Institute in Berlin, Germany. Her current research interests focus on surveillance and control of gastrointestinal infections and zoonoses.

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Address for correspondence: Judith Koch, Department of Infectious Disease Epidemiology, Robert Koch Institute, Seestrasse 13, 13353 Berlin, Germany; fax: 49-30-4547-3533; email: KochJ@rki.de

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Yersinia pseudotuberculosis Septicemia and HIV

Maria Grazia Paglia,* Silvia D'Arezzo,* Anna Festa,* Cosmo Del Borgo,* Laura Loiacono,* Andrea Antinori,* Giorgio Antonucci,* and Paolo Visca *†

Two cases of community-acquired septicemia caused by serotype-O1 *Yersinia pseudotuberculosis* were diagnosed in middle-aged, HIV-positive, immunodeficient patients during an 8-month period. Bacterial isolates were genetically indistinguishable, but no epidemiologic link between the 2 patients was established. HIV-related immunosuppression should be regarded as a risk factor for *Y. pseudotuberculosis* septicemia.

Yersinia pseudotuberculosis is a rare cause of disease in humans. Animals, food, and the abiotic environment are *Y. pseudotuberculosis* reservoirs from which epizootic and human infection may arise (1). A geographic gradient of *Y. pseudotuberculosis* isolation rates has been reported in Europe (1,2), with a 0.05% recovery rate from stools of patients with acute enteritis in Italy (3). The organism can also cause mesenteric lymphadenitis, which mimics appendicitis, or infection at other body sites that occasionally leads to postinfectious sequelae such as reactive arthritis and erythema nodosum (1). About 60 cases of *Y. pseudotuberculosis* septicemia have been reported thus far, mainly in patients with underlying conditions such as hepatic cirrhosis, malignancy, diabetes, aplastic anemia, thalassemia, and iron overload (1,4,5). We recently reported the first case of *Y. pseudotuberculosis* septicemia in a severely immunocompromised, HIV-positive patient (6). Here, a second case of *Y. pseudotuberculosis* septicemia in an HIV-infected outpatient attending the same hospital is described. The unique molecular type of both *Y. pseudotuberculosis* isolates and the atypical clinical course of infection will be comparatively discussed.

The Study

The first case has recently been reported (6) and is briefly reviewed here. Patient 1 was a 42-year-old woman with HIV infection since 1987 (Centers for Disease Control and Prevention [CDC] Classification C3). In June 2003, she was admitted to the National Institute for Infectious Diseases, Rome, from prison because of high fever and confusion. Physical examination showed a temperature of 39.5°C, abnormal mental status, and oral candidiasis, but no gastrointestinal symptoms. Her history included lack of response to highly active antiretroviral therapy (HAART), and she exhibited HIV viremia of 413,624 copies/mL, a low CD4+ cell count (5/mm^3), and leukopenia ($3.0 \times 10^3/mm^3$). Laboratory values were altered for aminotransferases (aspartate aminotransferase, 273 U/L; alanine aminotransferase, 77 U/L), hemoglobin (7.0 g/dL), erythrocyte sedimentation rate (136 mm in the first hour), and platelet count ($34 \times 10^3/mm^3$). Multiple blood cultures yielded growth of *Y. pseudotuberculosis*. Stool cultures and test results for antibodies against *Y. pseudotuberculosis* were negative. Intravenous ceftriaxone therapy was started at admission, with total remission of symptoms in 4 days. No recurrence of *Y. pseudotuberculosis* infection was observed during a 1-year follow-up period.

Patient 2 was a 54-year-old man who was admitted to the same hospital in February 2004 because of sudden fever and confusion. He tested HIV-positive in 1993 (CDC Classification B3), and his CD4+ cell count nadir was 121 cells/mm^3. His recent antiretroviral therapy was a combination of stavudine, lamivudine, and nelfinavir. HCV-related liver cirrhosis was diagnosed 3 years before this admission. His condition was routinely followed up in the outpatient unit, and he had been hospitalized 1 month earlier for culture-negative pneumonia in the left lower lobe. On admission, the patient had a high fever (41°C), chills, and abnormal mental status with somnolence. Results of a chest radiograph, nuclear magnetic resonance imaging of the brain, and echocardiogram were normal. HIV viremia level was <50 copies/mL and CD4+ cell count was 204 cells/mm^3. Laboratory values were notable for leukocytosis (leukocytes, $14 \times 10^3/mm^3$), anemia (hemoglobin, 10 g/dL), and thrombocytopenia (platelets, $69 \times 10^3/mm^3$). Levels of C reactive protein, aminotransferases, blood glucose, creatinine, urea nitrogen, and electrolytes were within the normal range. Liver cirrhosis was classified as Child-Pugh group B, score 7. Urine was negative for opioids and cocaine metabolites. On second day postadmission, multiple blood cultures became positive for *Y. pseudotuberculosis*, while stool cultures were negative. Intravenous ceftriaxone therapy was begun at admission, with total remission of symptoms within 3 days. He was discharged on day 10 and continued intravenous ceftriaxone therapy for 4 weeks (2 g daily) as an outpatient. At the final clinical observation, 6 months later, the patient was free of clinical symptoms and had no recurrence of *Y. pseudotuberculosis* infection.

Blood culture isolates from both patients were identified with >99% confidence as *Y. pseudotuberculosis* by using both the API20E-Rapid test (bioMérieux, Marcy l'Etoile, France) and the BD Phoenix system (Becton

*National Institute for Infectious Diseases "Lazzaro Spallanzani," Rome, Italy; and †University "Roma Tre," Rome, Italy

Dickinson, Sparks MD, USA). Isolates showed an extended spectrum of antimicrobial susceptibility (sensitive to β-lactams, monobactams, aminoglycosides, quinolones, and sulfonamides). O-serotyping with commercial antisera (Denka Seiken, Tokyo, Japan) identified both organisms as *Y. pseudotuberculosis* serotype O1, consistent with the high frequency of this serotype among strains isolated from patients with septicemia in Europe (2).

The 16S ribosomal DNA (rDNA) sequences were identical in the 2 isolates and optimally matched (>99% identity) the 16S rDNA of the *Y. pseudotuberculosis* type strain ATCC 29833 (GenBank accession no. AF366375). To differentiate the strains at the genome level, amplified fragment length polymorphism and arbitrarily primed polymerase chain reaction (PCR) were performed (7–9). While these methods are highly discriminatory and detect DNA polymorphisms to the strain level, no differences between the 2 clinical isolates were observed (Figure, panels A and B).

To gain insight into the pathogenic potential of the 2 isolates, we assessed the presence of pYV (70-kb virulence plasmid), high pathogenicity island (HPI), and *Y. pseudotuberculosis*–derived mitogen (YPM) genetic markers by PCR (2,6). Both isolates harbored a characteristic repertoire of virulence determinants. Correctly sized PCR products were obtained for *inv* (chromosome-borne), *irp1*, *irp2*, IS*100* (HPI-borne), *lcrF*, and *yadA* (plasmidborne) genes (Figure, panel C). The identity of PCR products was confirmed by direct DNA sequencing. Interestingly, both isolates were negative for the *ypmA* (chromosome-borne) gene, consistent with the genetic instability of this marker which is typically absent in strains from Western countries (2,10). Reducing PCR stringency for *ypmA* yielded a 237-bp amplicon, which resulted from mispriming of oligonucleotides on the *Y. pseudotuberculosis hmp* flavohemoprotein gene (data not shown).

Molecular typing and virulence gene probing indicate that both cases resulted from indistinguishable *Y. pseudotuberculosis* strains, which raises the suspicion of a common source of infection. However, no other cases of *Y. pseudotuberculosis* infection were diagnosed in our institute or reported to the National Center for Enteropathogenic Bacteria, Istituto Superiore di Sanità, Rome, from January 2003 to June 2004 (I. Luzzi, pers. comm.). In September 2004, patients were interviewed about their lifestyle, illness, food and fluid consumption, or animal exposure in the 2 weeks preceding hospitalization. Nosocomial infection and direct contact between patients were ruled out, which suggested that infection had been independently acquired in the community. Both patients denied any contact with wild or domestic animals. Patient 1 had not been released from detention in the 6 months preceding hospitalization and regularly consumed collective meals in prison. However, prison infirmary records did not show an increased frequency of abdominal symptoms or fever among ≈500 inmates from May to July 2003. Patient 2 was a heavy smoker who had been abusing alcohol and illicit drugs until 2001. He lived alone and had a seafood meal 4 days before admission. Thus, although infection may have been acquired from contaminated food or fluid, questions regarding the actual source of bacteria and the extent of exposure remain unanswered.

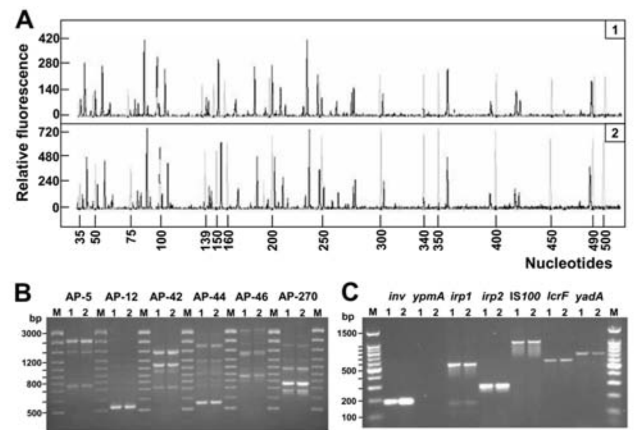

Figure. Genetic fingerprinting and detection of virulence genes of *Yersinia pseudotuberculosis* isolates. A) Fluorescent amplified fragment length polymorphism (AFLP) analysis of *Y. pseudotuberculosis* DNA (black electropherogram; 1 and 2 refer to patient number). Reactions were performed as indicated in the AFLP Microbial Fingerprinting kit (Applied Biosystems, Foster City, CA, USA). Reference DNA from *Escherichia coli* W3110 (Applied Biosystems) was used as internal control (gray electropherogram). Separation and detection of the AFLP fragments were performed with the Applied Biosystems model 3100 capillary electrophoresis system equipped with a 36-cm capillary loaded with the POP-4 polymer. Size determinations of the labeled DNA fragments were performed automatically with the Genescan Analysis 3.0 software (Applied Biosystems). B) Arbitrarily primed polymerase chain reaction (AP-PCR) analysis with a set of 6 oligonucleotides: AP5 (5´-TCCCGCTGCG-3´), AP12 (5´-CGGCCCCTGC-3´), AP42 (5´-AACGCGCAAC-3´), AP44 (5´-AGCCAGTTTC-3´), AP46 (5´-GAGGACAAAG-3´), and AP270 (5´-TGCGCGCGGG-3´) (8,9). Amplification patterns of DNA from the 2 clinical isolates are shown: lane 1, patient 1; lane 2, patient 2. M, molecular weight marker. The numbers on the left indicate the length (in base pairs) of the reference ladder. Primers are indicated on top. C) Detection of *Y. pseudotuberculosis* virulence genes. Primers and PCR conditions have been described elsewhere (2,6). Lane 1, patient 1; lane 2, patient 2. Lane M, molecular weight marker. The numbers on the left indicate the length (in base pairs) of the reference ladder. Target genes are indicated on top.

Conclusions

AIDS is a known risk factor for *Y. enterocolitica* infection (11), but no link between HIV and *Y. pseudotuberculosis* infection has yet been proposed. Yersiniae have an

impaired iron metabolism, and for this reason, they rarely cause sepsis in patients without iron overload, which is often secondary to alcoholism, asplenia, hemochromatosis, thalassemia major, or tobacco smoking (1). In our 2 patients, no clinical evidence indicated iron overload, and a diagnosis of hemochromatosis was excluded. The most important risk factor was HIV-related severe immunodeficiency. Patient 1 was severely immunocompromised despite HAART, while patient 2 responded poorly to HAART and had hepatitis C–related liver cirrhosis.

Although the clinical management of *Y. pseudotuberculosis* septicemia is often difficult and mortality rates are high (≈75%), despite antimicrobial drug therapy (1), both our patients responded unexpectedly well to ceftriaxone therapy and promptly recovered. During infection, *Y. pseudotuberculosis* directly manipulates lymphocyte signaling and activation by expressing different virulence factors (12). The pYV plasmid-encoded Yop proteins have been implicated in lymphocyte suppression through down-regulation of co-stimulatory molecules (13) and impairment of nitric oxide, tumor necrosis factor (TNF)-α, and proinflammatory cytokine production (14). Conversely, YPM superantigen(s) contribute to systemic illness by activating a large proportion of T cells (essentially CD4+) and inducing proinflammatory cytokines such as TNF-α, TNF-β, γ-interferon, and interleukins 1 and 6, as in toxic shock syndrome (15). Our experimental data show that the 2 clinical strains were positive for all the virulence genes tested, except for *ypm*A. Even considering the different degree of immunodeficiency between the 2 patients, we speculate that impairment of immune response secondary to HIV infection may have increased the susceptibility to *Y. pseudotuberculosis* infection while mitigating the septic shock sequelae. Accordingly, the inflammatory response consequent to T-cell activation may have been attenuated by the deficiency of CD4+ cells in both patients, concomitant with the lack of YPM expression by both *Y. pseudotuberculosis* isolates. In conclusion, these 2 cases indicate that *Y. pseudotuberculosis* is an emerging pathogen in HIV patients and remind us that septicemia in these patients can exist without prodromic symptoms. They also alert us to the local circulation of a pathogenic *Y. pseudotuberculosis* strain whose natural reservoir remains so far unknown.

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Dr. Paglia is senior scientist at the National Institute for Infectious Diseases "Lazzaro Spallanzani," Rome, Italy. Her research interests include the development of DNA-based microbial identification tools and molecular epidemiology.

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Address for correspondence: Paolo Visca, Molecular Microbiology Unit, National Institute for Infectious Diseases "Lazzaro Spallanzani" I.R.C.C.S., Via Portuense 292, 00149 Rome, Italy; fax: 39-6-55176321; email: visca@uniroma3.it

Bordetella petrii Clinical Isolate

Norman K. Fry,* John Duncan,* Henry Malnick,* Marina Warner,* Andrew J. Smith,† Margaret S. Jackson,† and Ashraf Ayoub†

We describe the first clinical isolate of *Bordetella petrii* from a patient with mandibular osteomyelitis. The only previously documented isolation of *B. petrii* occurred after the initial culture of a single strain from an environmental source.

A 67-year-old man visited an emergency dental clinic, where he complained of toothache in the lower right mandibular quadrant. Examination showed a root-filled lower right canine tooth that was mobile and tender to percussion. The tooth was extracted uneventfully under local anesthesia. The patient returned after several days with pain at the extraction site. A localized alveolar osteitis was diagnosed, and local debridement measures were instituted. These measures proved unsuccessful, and repeat examination showed submandibular lymphadenopathy and tenderness to palpation in the buccal sulcus in the extraction site. Radiographs showed no abnormality at this stage.

A course of oral amoxicillin (250 mg, every 8 h for 5 days) followed by oral metronidazole (200 mg every 8 h for 5 days) was prescribed. Symptoms persisted, with increasing severity of pain in the affected area, and the patient was referred to a tertiary referral center. On examination there, the patient had normal full blood count, hematinics, and glucose levels. Ultrasound examination of the submental soft tissue region did not indicate any abnormal pathology. Radiographs showed radiolucencies in the bone surrounding the extraction site. During this period a 3-week course of oral co-amoxiclav (375 mg, every 8 h) was prescribed. A bone biopsy was performed under local anesthesia, and a diagnosis of mandibular osteomyelitis was made.

A portion of the bone biopsy specimen was cultured in 10 mL Fastidious Anaerobe Broth (FAB) (BioConnections, Wetherby, UK) and incubated for 48 h at 37°C in air. After 48 h, the FAB was subcultured onto 1) Fastidious Anaerobe Agar (FAA, BioConnections), incubated for 72 h at 37°C under anaerobic conditions, and 2) Columbia Blood Agar (CBA, BioConnections), incubated for 72 h at 37°C under 5% CO_2 atmospheric conditions. Culture on both FAA and CBA showed a pure growth of a facultative gram-negative bacillus that had not been identified with routine laboratory protocols. Initial susceptibility testing using disk diffusion indicated apparent susceptibility of the isolate to erythromycin, gentamicin, ceftriaxone, and piperacillin/tazobactam. The isolate was resistant to amoxicillin, co-amoxiclav, tetracycline, clindamycin, ciprofloxacin, and metronidazole. After initial sensitivity results, a 6-week course of oral clarithromycin (500 mg, 8 hourly) was begun.

At follow-up appointments 3 months and 6 months after antimicrobial drug therapy ceased, clinical and radiographic findings were not unusual, and the infected area healed successfully. Despite the successful clinical outcome, the isolate was subsequently shown to be resistant to clarithromycin in vitro (Table). Improvement of the osteomyelitis may also have been facilitated by the biopsy procedure, during which a sequestrum of bone was removed.

The gram-negative bacillus (designated strain GDH030510) was submitted to the Health Protection Agency, Centre for Infections, London, for identification. Preliminary tests results were consistent with those described for members of the genus *Bordetella*. Colonies had the following phenotypic characteristics: positive reaction for oxidase and negative reaction for urease production, motility using the hanging-drop method at 37°C, and slide agglutination with *B. pertussis* and *B. parapertussis* antiserum (Difco, Shannon, Ireland). The organism could be cultured on MacConkey agar and was nonhemolytic on blood agar. Genomic DNA was extracted by using the InstaGene Purification Matrix (BioRad, Hercules, CA, USA). DNA amplification of small-subunit (SSU) rRNA genes was performed by using primers 27f and 1525r (1). Amplification and sequencing of the gene for the *Bordetella* outer membrane protein A (*ompA*) and the RisA response regulator (*risA*) were as described by von Wintzingerode et al. (2). Reaction mixes contained the following: 2 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 0.05% W-1 (Invitrogen, Paisley, UK), 0.2 µmol/L of each deoxynucleotide (Roche Applied Science, Lewes, UK) 20 pmol of each primer (MWG Biotech, Milton Keynes, UK), 2.5 U of Taq DNA polymerase (Invitrogen), 1.0 mol/L betaine (Sigma-Aldrich, Gillingham, UK), and 10 µL template DNA. Amplification was performed in a DNA Engine (MJ Research, Bio-Rad) by using 35 cycles of denaturation of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C and a final step at 72°C for 3 min. Amplicons from triplicate samples were pooled and purified with Montage PCR96 filter plates (Millipore, Watford, UK). For the rRNA gene, nucleotide sequence was determined by using the primers used for amplification, together with internal primers (1). Sequencing was performed with the Dye Terminator Cycle Sequencing kit (Beckman Coulter, High Wycombe, UK) and analyzed on

*Health Protection Agency, London, United Kingdom; and †Glasgow Dental Hospital and School, Glasgow, United Kingdom

Table. Results of antimicrobial drug–susceptibility testing for strain GDH030510

Antimicrobial agent	MIC (μg/mL)
Penicillin	>32
Ampicillin	>256
Piperacillin/tazobactam	2
Ceftriaxone	>32
Cefotaxime	>32
Ceftazidime	32
Imipenem	>32
Meropenem	>32
Ertapenem	>32
Amikacin	>256
Gentamicin	4
Tobramycin	16
Ciprofloxacin	>32
Erythromycin	128
Azithromycin	4
Clarithromycin	64
Clindamycin	>256
Chloramphenicol	>256
Cotrimoxazole	8
Rifampin	>32
Tetracycline	>256

a CEQ 8000 Genetic Analysis System (Beckman Coulter), according to the manufacturer's instructions. Contig assembly and sequence analyses were performed with Kodon, version 2.0 (Applied Maths, Kortrijk, Belgium) and BioNumerics, version 3.5 (Applied Maths). Consensus sequence was compared with public databases with the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST) (3), and sequences with the greatest similarity were downloaded for further analysis.

The nucleotide sequences of the 16S rRNA gene, *risA* gene, and *ompA* gene of strain GDH030510 have been submitted to the EMBL Nucleotide Sequence Database under accession numbers AJ870969, AJ920265, and AJ920264, respectively. The 1,486 nucleotides (nt) of the SSU rRNA gene sequence that were determined showed a maximum similarity of 99.3% (1,468/1,479 nt) with the 16S rRNA gene from the type strain of *B. petrii* (GenBank accession no. AJ249861). For all sequence analyses, regions not present in all sequences and ambiguous bases were excluded. The species with the next highest similarities were *B. parapertussis*, 98.4% (1,455/1,479 nt), and *B. bronchiseptica*, 98.3% (1,454/1,479 nt). The secondary structure of the SSU rRNA from both strain GDH030510 and the submitted sequence for the type strain of *B. petrii* were compared to that proposed for bacteria (4). Where possible (i.e., in stems), supportive evidence from base-pairing was sought. This analysis indicated that the assigned nucleotides at 3 separate locations in the GenBank *B. petrii* sequence were not supported, as they formed noncanonical base-pairing (C-U vs. C-G [156, 165], U-U vs. A-U [824, 875], A-C vs. G-C [838, 848]; *Escherichia coli* numbering [5]). Exclusion of these 3 bases increased the similarity to 99.5% (1,468/1,476 nt). The 445 nt of the *risA* gene sequence of strain GDH030510 that were determined showed a maximum similarity value of 93.9% (418/445 nt) with the *risA* gene from the *B. petrii* type strain (AJ242553). The species with the next highest similarities were *B. parapertussis*, *B. avium*, *B. bronchiseptica*, and *B. pertussis*, all with similarities of 88.3% (393/445 nt). The 414 nt of the *ompA* gene sequence that were determined showed maximum a similarity value of 92.0% (381/414 nt) with the *ompA* gene from the *B. petrii* type strain (AJ242599). The species with the next highest similarities were *B. bronchiseptica* and *B. parapertussis*, both with similarities of 87.9% (364/414 nt).

Further susceptibility testing was undertaken, with MICs determined by agar dilution on diagnostic sensitivity test agar (Oxoid, Basingstoke, UK) supplemented with 5% lysed horse blood, with inocula equivalent to 0.5 and 2 McFarland standards, and with incubation at 37°C in air for 24 to 36 h to ensure adequate growth. No inoculum effects were noted on MICs for any antimicrobial agents. MICs are shown in the Table.

Currently, the genus *Bordetella* comprises 8 species (2), 7 of which have been isolated from humans and a variety of warm-blooded animals. Three of these species, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, are often referred to as the classic *Bordetella* species (6); they are closely related phylogenetically but have distinct host ranges. *B. pertussis* is an obligate pathogen for humans and is the etiologic agent of pertussis. *B. parapertussis* causes a similar, usually milder, infection in humans, and *B. parapertussis* strains may also be isolated from sheep with chronic pneumonia. Human isolates of *B. parapertussis* are highly clonal and appear distinct from sheep isolates. *B. bronchiseptica* is known to infect many mammals, including humans, although human infection is rare and usually occurs in immunocompromised hosts. *B. avium* is pathogenic for birds, including poultry, and causes coryza or rhinotracheitis in turkeys. Three other species of *Bordetella*, *B. hinzii*, *B. holmesii*, and *B. trematum*, have subsequently been described (7–9). *B. hinzii* is a commensal of the respiratory tract of fowl but has some pathogenic potential in immunocompromised humans (10) and was implicated as the causative agent in a fatal case of septicemia (11). *B. holmesii* has been isolated from blood of young adults, and nasopharyngeal specimens (9,12,13). *B. trematum* has been isolated from human ear infections and wounds, but its pathogenicity remains unknown (7). The most recently recognized species, *B. petrii*, was described in 2001 (2) and was isolated from an anaerobic bioreactor culture enriched from river sediment. This description was based on a single isolate and, to our knowledge, no further

isolates of this species have been previously reported from any source.

The source of infection of the strain described here and the pathogenic role of *B. petrii* are currently unknown. However, the identification and characterization of further clinical isolates should help determine the reservoir and virulence potential of this intriguing species.

Addendum

A clinical isolate of a new species of *Bordetella*, *Bordetella ansorpii* sp. nov, has recently been reported (14). Phylogenetically, it appears to be more closely related to *B. petrii* than to other members of the genus.

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Dr. Fry is a principal clinical scientist in the Respiratory and Systemic Infection Laboratory, Centre for Infections, London. His interests include the detection, diagnosis, epidemiologic typing, and analysis of virulence factors of microorganisms, in particular, those belonging to the genera *Bordetella*, *Legionella*, and *Bartonella*.

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Address for correspondence: Norman K. Fry, Health Protection Agency, Respiratory and Systemic Infection Laboratory, Centre for Infections, 61 Colindale Ave, London NW9 5HT, United Kingdom; fax: 44-20-8205-6528; email: Norman.Fry@HPA.org.uk

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Caliciviruses and Foodborne Gastroenteritis, Chile

Roberto Vidal,* Veronica Solari,† Nora Mamani,* Xi Jiang,‡ Jimena Vollaire,† Patricia Roessler,* Valeria Prado,* David O. Matson,§ and Miguel L. O'Ryan*

Human caliciviruses caused 45% of 55 gastroenteritis outbreaks occurring in Santiago, Chile, during 2000–2003. Outbreaks affected ≈99 persons, occurred most commonly in the home, and were associated with seafood consumption. Thirteen outbreak strains sequenced were noroviruses, including 8 GII, 2 GI, and 3 belonging to a novel genogroup.

Human caliciviruses (HuCVs), especially noroviruses, are a major cause of food- and waterborne outbreaks in industrialized countries. Their role as a cause of gastroenteritis outbreaks in economically developing areas is unclear because little information is available (1–3). Five norovirus genogroups have been described, with serogroup II (GII) prevailing in outbreaks worldwide since ≈1990 (3–6). Strains differing significantly from GI and GII prototypes are being increasingly reported since detection methods have improved (5,6).

Chile is a rapidly developing country. Studies have shown seroprevalence for HuCVs of >70% for children 5 years of age and incidence of 8% in acute sporadic cases of diarrhea in children (7–9). A small number of norovirus-associated outbreaks have been reported but information is scarce because no surveillance system for gastroenteritis exists (8). The capital city of Chile, Santiago, with ≈6.1 million persons, contains ≈40% of the country's population. Ninety-six public hospitals, private clinics, and emergency outpatient clinics distributed within 6 healthcare services centers are responsible for notifying the Health Ministry when infectious diseases that are on the National Mandatory Notification List are identified.

The Study

In 1994, the Metropolitan Area Environmental Health Service (health service) began a gastroenteritis outbreak surveillance program in the centers. This program was improved in 2000 by using a standard protocol for pathogen detection. This study was to determine the role of HuCVs as a cause of gastroenteritis outbreaks from June 1, 2000, to January 30, 2003, in Santiago, Chile, by using recently improved antigen and genome detection assays, and to characterize genetically the circulating strains.

Sentinel sites were instructed to report gastroenteritis outbreaks ≤48 hours after detecting the sentinel case. A health service epidemiologist would initiate an investigation and make home visits to identify all persons possibly involved in the outbreak. Specific attack rates for implicated food products were calculated.

Stools samples for pathogen detection were collected during home visits from affected persons and were cultured for *Salmonella*, *Shigella*, *Campylobacter*, and *Vibrio* spp., according to standard techniques using selective media (10). Enteropathogenic *Escherichia coli*, enterotoxigenic *E. coli*, and enterohemorrhagic *E. coli* were studied by multiplex polymerase chain reaction (11) and enzyme-linked immunosorbent assay (ELISA). Rotavirus and enteric adenoviruses were detected by ELISA or by commercial kits (SAS Rota Test, SA Scientific Inc., San Antonio, TX, USA; Premier Adenoclone, Meridian Diagnostics Inc., Cincinnati, OH, USA; 40/41 Adeno-Strip, Coris Bioconcept, Gembloux, Belgium) and parasites were detected by Burrows technique.

All samples were tested for HuCV by a novel ELISA specific for noroviruses based on pools of sera obtained from rabbits and guinea pigs hyperimmunized with a total of 9 different norovirus capsids (12) and by reverse transcription–polymerase chain reaction (RT-PCR) targeting conserved sequences in the polymerase region of HuCVs (9). Primers used for RT-PCR were 289 (RT)/290 (PCR) or a pool of degenerate primers of last generation, 289hi for RT and 290hijk for PCR, that detect norovirus and sapovirus (13,14). RT-PCR products were cloned by using pGEM-T Easy vector system (Promega, Madison, WI, USA). The 327-base nucleotide sequences that encode for the polymerase dependent RNA were aligned by using OMIGA 2.0 (Oxford Molecular, Madison, WI, USA) software and compared with 21 prototype sequences retrieved using BLAST searches from the GenBank database. Phylogenetic distances were calculated by Kimura 2-parameter method and a phylogenetic tree was plotted by the neighbor-joining method using MEGA, version 2.1 (15). Bootstrap values were based on 1,000 generated trees.

Conclusions

During the 30-month study, a total of 82 outbreaks affecting ≤100 persons in the Santiago metropolitan area were reported properly to the health service and investigated. In each outbreak, a rectal swab from ≥1 person was

*University of Chile, Santiago, Chile; †Health Ministry of Chile, Santiago, Chile; ‡University of Cincinnati College of Medicine, Cincinnati, Ohio, USA; and §Eastern Virginia Medical School and Children's Hospital of The King's Daughters, Norfolk, Virginia, USA

collected for microbial studies. In each of 55 outbreaks, ≥1 stool sample was collected for virus studies, and in each of 31 outbreaks, ≥1 stool sample was collected for parasite studies. Enteric microbial pathogens were isolated in samples from ≥1 person in 32% of the 82 outbreaks, and potentially pathogenic parasites were isolated in 6 (19%) of 31 outbreaks (Table 1). A total of 175 samples from 55 outbreaks were obtained for viral detection, of which 47 (27%) from 25 (45%) outbreaks were positive for HuCV by using ≥1 method. HuCV outbreaks affected ≤99 persons with a median of 5 persons (Table 1). In 16 outbreaks, ≥2 persons were positive by using ELISA or RT-PCR; in 9 outbreaks, 1 person was positive by ≥1 method. Overall, 20% of the outbreaks were detected only by ELISA, 24% only by RT-PCR, and 56% by both techniques.

Most HuCV outbreaks occurred in the home, with outbreaks in childcare centers and schools occurring next most frequently; only a small fraction occurred in restaurants. The most commonly implicated food products were seafood, including raw oysters and clams (Table 2). Among a total of 1,137 persons exposed in the 25 HuCV outbreaks, 283 (25%) had typical acute gastroenteritis symptoms. Thirty-nine percent of the cases occurred in children <5 years of age, 28% occurred in children 5–14 years of age, 27% occurred in adolescents and adults 15–60 years of age, and 4% occurred in adults >60 years of age. Most commonly reported symptoms were diarrhea (86%), vomiting (36%), and fever (16%).

HuCV amplicons from 13 outbreaks evaluated belonged to the norovirus genus, including 8 GII, 2 GI, and 3 in a potentially novel genogroup. The 3 new strains differed >40% in nucleotide identity from all prototype strains compared (Figure). Bootstrap analysis based upon 1,000 generated trees yielded a node for the potentially novel genogroup in 100% of the trees. Two of the outbreaks caused by this potentially novel genogroup occurred during the same month, while the third occurred a year later. The distribution of the 8 genogroup II strains fell into 3 genetic clusters. One of the genetic clusters, represented by strain 028/10-2001, was closely related with a distance of 0.11 to Saitama virus (SaiU1, accession no. AB039775), a Japanese strain found in 1998 in a child with acute gastroenteritis. The 2 other genetic clusters are proposed as novel genetic clusters and include strains (i) O55/5-2002, O64/10-2002, O62/9-2002, O71/11-2002, O78/11-2002, and (ii) O77/11-2002, O85/1-2003 (Figure). Both clusters are also most closely related to SaiU1. The first cluster has 2 independent nodes with a distance of 0.19 to 0.28 from SaiU1, the second cluster is represented by 2 strains with a distance of 0.18 and 0.19 from SaiU1, respectively.

HuCVs were associated with almost half of 55 fully evaluated gastroenteritis outbreaks in Santiago, Chile, and

Table 1. Proportion of acute diarrhea outbreaks associated with a bacterial enteropathogens or a human calicivirus (HuCV) and number of persons affected during the HuCV outbreaks

	No. outbreaks positive*/no. tested		No. affected in HuCV outbreaks
Year	Bacteria† (%)	HuCVs (%)	Range (median)
2000	8/13 (61)	4/12 (33)	3–28 (4)
2001	11/32 (34)	6/18 (33)	2–54 (5)
2002	6/34 (18)	14/22 (64)	2–99 (5)
2003‡	1/3 (33)	1/3 (33)	5
Total	26/82 (32)	25/55§ (45)	2–99 (5)

*An outbreak was associated with a given pathogen if ≥1 sample was positive.
†Bacteria isolated included: enteropathogenic *Escherichia coli* (EPEC) (2), enterotoxigenic *E. coli* (ETEC) (3), enterohemorrhagic *E. coli* (3), EPEC + ETEC (1), *Salmonella sp.* (12), *Shigella sp.* (2), *Staphylococcus aureus* (3).
‡January 1–10, 2003.
§In 1 outbreak, ETEC and EPEC and in another, Shiga toxin–-producing *E. coli*, were concomitantly isolated with HuCV. In 1 additional outbreak the only pathogens simultaneously detected in 1 patient were rotavirus and adenovirus by enzyme-linked immunosorbent assay.

Table 2. Human calicivirus outbreak settings and implicated food products by study years

	2000–2001	2002–2003	Total (%)
No. outbreaks	10	15	25
Outbreak settings			
Home	6	11	17 (68)
Childcare center or school	2	3	5 (20)
Restaurant	1	1	2 (8)
Picnic	1	0	1 (4)
Food products implicated			
Seafood	3	11	14 (56)
Meat products	2	3	5 (20)
Prepared cooked food	2	1	3 (12)
Other	3*	0	3 (12)

*Goat cheese, mayonnaise, celery.

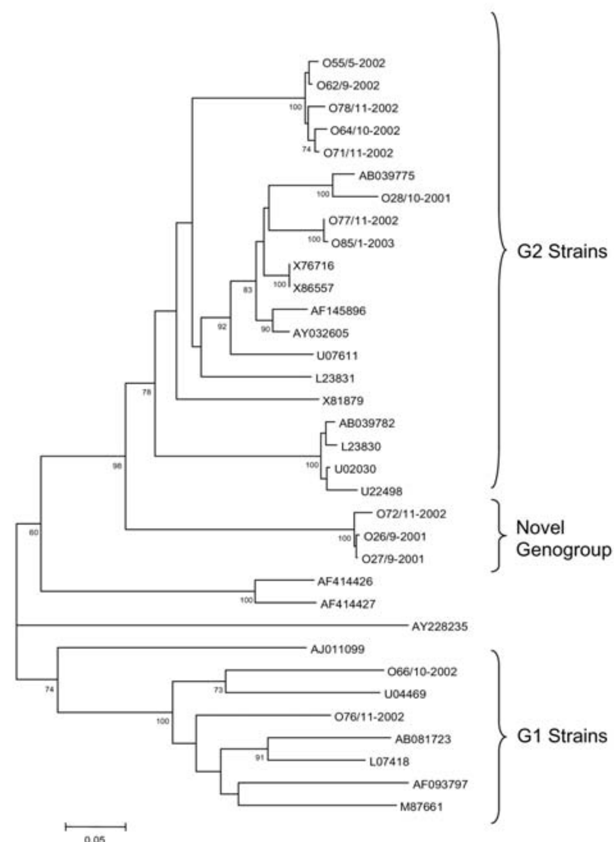

Figure. Phylogenetic tree of noroviruses based on the 327-base region of the 3´ end of the open reading frame 1 using 13 novel sequences designated according to outbreak number/month-year (example: O55/5-2002), and 21 sequences of Norwalk-like virus strains representative of the currently identified genogroups, designated according to GenBank accession number. Comparative strains include: Norwalk virus (M87661), SaitamaU1 (AB039775), Saitama U201 (AB039782), WUG1 (AB081723), Schreier (AF093797), Camberwell (AF145896), Fort Lauderdale (AF414426), Saint Cloud (AF414427), Jena (AJ011099), Maryland (AY032605), Murine NV (AY228235), Southampton (L07418), OTH25 (L23830), Snow Mountain (L23831), Toronto (U02030), Desert Shield virus (U04469), Hawaii (U07611), Mexico (U22498), Bristol (X76716), Melksham (X81879), and Lorsdale (X86557). Bootstrap values based on 1,000 generated trees are displayed at the nodes (values) >60% are shown).

were more common than outbreak-associated enteric bacterial pathogens such as *Salmonella* sp. and diarrheogenic *E. coli*. To our knowledge, this is the first prospective, active surveillance for gastroenteritis outbreaks in Latin America that included a thorough search for HuCVs. Publications from the region have described high seroprevalence for these viruses (3,16) and have reported isolated outbreaks affecting children and adults (3,8).

HuCV-associated outbreaks mostly affected children that ate seafood in homes; other implicated sources included meat products and vegetables. Estimated attack rates were ≈25%. The reported outbreaks in this study reflect the tip of the iceberg; only 10% of all reported outbreaks could be studied because of capacity and resources for prompt reporting and investigation. This study should stimulate efforts for appropriate outbreak investigation in developing regions where food products safety is important for the health of the population, tourism, and international commerce.

Genogroup II strains dominated, as in other studies (3–6), but only 1 of these strains fell into the same genetic cluster of a previously described strain, Saitama virus; in contrast, most strains grouped into 2 closely related new clusters. In addition, 3 strains, 2 temporally related, likely belong to a new genogroup. The circulation of genetically diverse strains indicates the need for further studies to understand the clinical and epidemiologic importance of such diversity.

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Dr. Vidal is an assistant professor in the Microbiology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile. His main research interests are enteric infectious diseases and environmental health.

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Address for correspondence: Miguel L. O'Ryan, Microbiology Program, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, Universidad de Chile, Independencia 1027 Santiago, Chile; fax: 56-2735-5855; email: moryan@med.uchile.cl

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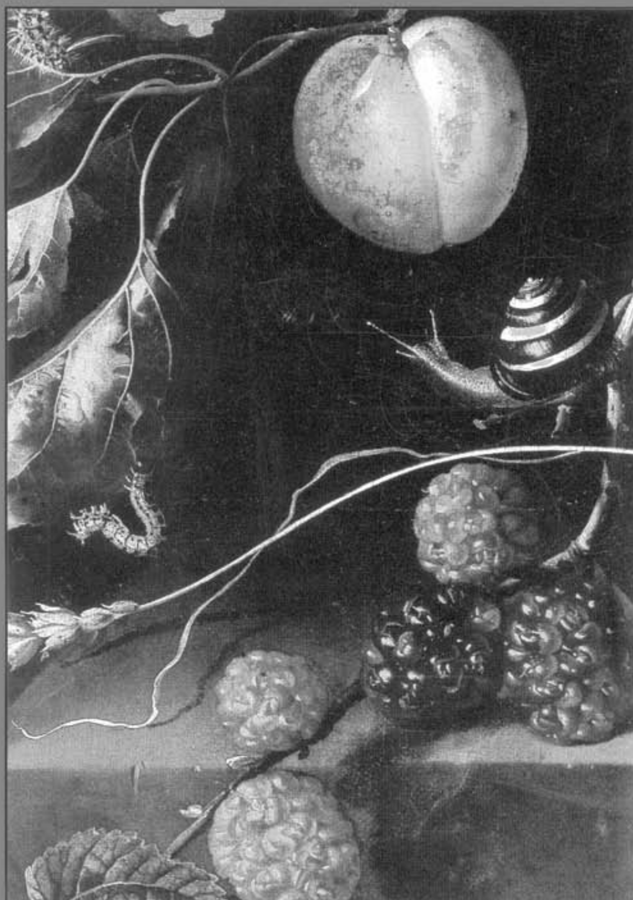

Conference on Emerging Foodborne Pathogens

Pathogen Emergence

Host Susceptibility

Chronic Sequelae

Risk Assessment

Consumer Concern

Beliefs about Appropriate Antibacterial Therapy, California

Kate C. Cummings,* Jon Rosenberg,* and Duc J. Vugia*

To our knowledge, previous population-based surveys have not assessed misconceptions about antibacterial drug use over time. We documented a 26.3% decline in a key misconception in California women in 2003 compared to 2000; declines varied significantly by education level. Educational campaigns specifically designed to influence important subpopulations are needed.

Antibacterial drug–resistant bacteria pose a substantial challenge to public health. Inappropriate use of antibacterial drugs, such as using them to treat viral respiratory infections, accelerates the emergence of resistant organisms (1,2). Nonclinical factors, such as patient expectation and demand, contribute to inappropriate antibacterial prescribing (3–6). To address patient misconceptions and physician prescribing behavior, the Centers for Disease Control and Prevention, along with states and private health organizations, initiated educational programs and campaigns to encourage the judicious use of antimicrobial drugs (7). In 1999, the California Medical Association Foundation initiated the Alliance Working for Antibiotic Resistance Education (AWARE) project. AWARE, a coalition of public and private organizations, was launched to educate physicians and consumers about appropriate antibacterial drug use. In 2003, AWARE worked with a coalition of minority physicians, including Asian physicians, to reduce inappropriate antibacterial use in minority communities (Elissa Maas, pers. comm.).

To date, few studies assess the effect of these educational campaigns over time, especially among underserved populations. We surveyed a population-based sample of California women to identify the magnitude of and characteristics independently associated with the misconception that a cold or flu should usually be treated with an antibacterial drug in 2000 and 2003. We assessed temporal trends in the prevalence of this misconception by comparing the prevalence reported in 2003 with that reported in 2000 for the entire study sample and by specific risk characteristics.

*California Department of Health Services, Berkeley, California, USA

The Study

The California Women's Health Survey is an ongoing, monthly telephone survey that collects information on health-related behavior and attitudes from randomly selected adult women (8,9). Trained staff interviewed participants in 2000 (n = 4,012) and 2003 (n = 4,004) in English (n = 7,058) or Spanish (n = 958). Participants reported race, ethnicity, country of birth, year of entrance into the United States, and current age, county of residence, number and age(s) of children, educational attainment, household income, and access to healthcare coverage. We defined the key misconception as an affirmative answer to the following dichotomized question: "Antibiotics, such as penicillin, doxycycline, or amoxicillin, are used to treat a variety of medical conditions; do you believe that a cold or flu should usually be treated with antibiotics?"

We calculated the crude proportion of women who held this misconception by year with weighted proportions and Mantel-Haenszel chi-square statistics that adjusted each sample to the age and race distribution of the 1990 California population (8,9). We examined the relationship between the misconception and the characteristics listed in Table 1 by year of study by using prevalence proportions and unconditional logistic regression. To produce comparable results, multivariate models for 2000 and 2003 included all variables that demonstrated a significant association with the misconception in either year in univariate analyses. We developed a third multivariate model that combined data from both years to assess the independent association between the misconception and year of study. While we used logistic regression to assess for confounding, we present unadjusted prevalence ratios and calculate relative prevalence reductions to measure changes in the misconception over time. We did so because the unadjusted and adjusted results by logistic regression were equivalent (which suggests an absence of confounding) and because the odds ratio did not reasonably approximate the prevalence ratio for women without a high school education (10). We considered p values ≤ 0.01 to be significant, but we present 95% confidence intervals (CIs) to facilitate comparing our results to those from other studies. We restricted all models to participants who gave usable responses to all variables (n = 7,430) and assessed models for potential 2-way interactions, goodness of fit, and potential colinearity.

In California, 21.0% of women surveyed in 2003 believed a cold or flu should usually be treated with an antibacterial drug. Table 1 lists factors independently associated with this key misconception among California women. In both 2000 and 2003, the unadjusted prevalence of the misconception was greatest among women who were Hispanic, 18–34 years of age, had an annual house-

Table 1. Factors associated with the misconception that cold or flu should be treated with antibacterial drugs, California, 2000 and 2003*

Characteristic	% in 2000† (n = 3,703) (OR‡, 95% CI)	% in 2003† (n = 3,727) (OR‡, 95% CI)
Race/ethnicity		
Black, non-Hispanic	40.6 (2.57, 1.88–3.52)	32.5 (2.82, 1.98–4.08)
Hispanic	46.2 (2.00, 1.58–2.52)	39.5 (1.96, 1.51–2.53)
Asian/other	37.1 (2.67, 1.94–3.67)	19.7 (1.78, 1.21–2.62)
White, non-Hispanic	19.4 (referent)	13.8 (referent)
Age (y)		
18–34	36.1 (2.13, 1.74–2.61)	26.7 (1.55, 1.23–1.94)
35–54	18.9 (referent)	14.5 (referent)
≥55	28.5 (1.59, 1.27–1.97)	19.7 (1.24, 0.97–1.58)
Annual household income ($US)		
≤14,999	46.9 (2.48, 1.91–3.22)	41.9 (3.10, 2.34–4.10)
15,000–24,999	36.8 (2.03, 1.56–2.64)	29.3 (1.94, 1.44–2.62)
25,000–49,999	25.9 (1.62, 1.30–2.03)	20.3 (1.70, 1.32–2.19)
≥50,000	12.9 (referent)	8.5 (referent)
Education		
<12 y	52.2 (3.03, 2.23–4.10)	43.1 (3.16, 2.26–4.44)
High school graduate/GED	31.8 (1.91, 1.50–2.44)	29.3 (3.08, 2.34–4.07)
Some college	20.5 (1.33, 1.04–1.69)	15.7 (1.79, 1.35–2.36)
College graduate	13.9 (referent)	7.3 (referent)
Region of California§		
Central	28.2 (1.34, 1.04–1.74)	24.8 (1.65, 1.25–2.17)
Southern	29.9 (1.40, 1.16–1.70)	21.4 (1.38, 1.10–1.71)
Northern	18.7 (referent)	13.1 (referent)
Years in United States		
≤5	57.1 (2.03, 0.86–4.79)	42.7 (1.58, 0.97–2.58)
6–10	50.8 (1.52, 0.98–2.35)	34.6 (1.24, 0.79–1.94)
>10	36.6 (0.98, 0.77–1.23)	29.6 (1.11, 0.85–1.44)
US-born	21.9 (referent)	15.3 (referent)
Children <6 y in household		
Yes	28.9 (0.61, 0.49–0.76)	24.9 (0.86, 0.68–1.09)
No	25.0 (referent)	17.5 (referent)
Access to health plan		
No	44.1 (1.06, 0.84–1.35)	37.2 (1.30, 1.02–1.65)
Yes	22.9 (referent)	16.4 (referent)

*OR, odds ratio; CI, confidence interval; GED, general equivalency diploma.
†Percent represents number of respondents with misconception divided by total number of respondents. Percentages for race/ethnicity and age are weighted to the 1990 California population; all other percentages are unweighted.
‡Adjusted for all characteristics listed in the table.
§Central = Fresno, Kern, Kings, Madera, Merced, Monterey, San Joaquin, San Luis Obispo, Santa Barbara, Santa Cruz, Stanislaus, and Tulare Counties; southern = Imperial, Los Angeles, Orange, Riverside, San Bernardino, San Diego, and Ventura counties; northern = all remaining counties.

hold income ≤$14,999, had <12 years of education, resided in central or southern California, had lived in the United States for ≤5 years, had children <6 years of age in the household, or had no access to a healthcare plan.

We observed a 26.3% decline in the prevalence of the misconception in all women surveyed in 2003 compared with those surveyed in 2000 (21.0% vs. 28.5%, $p<0.001$). Table 1 shows consistent declines across risk characteristics, with several notable exceptions. First, we detected a strong statistical interaction between education and year ($p = 0.007$); women with a college diploma were 47.0% less likely and women without a high school diploma were 17.0% less likely to report the misconception in 2003 than those surveyed in 2000 (Table 2). Women with a high school diploma were as likely to report the misconception in 2000 and 2003. Second, although not significant, after adjusting for other associated factors, Asian women were 55.7% less likely to report the misconception in 2003 compared with those surveyed in 2000. Other women were, on average, 24.0% less likely to report the misconception in 2003 than in 2000. Among women ≥55 years of age, African American women were as likely to report the misconception in 2003 as 2000, and their adjusted odds ratio for the misconception increased from 3.1 (95% CI 1.70–5.67) in 2000 to 5.3 (95% CI 2.67–10.52) in 2003 (detailed data on race stratified by age and year not shown).

Table 2. Decline in the misconception, by education level, that cold or flu should be treated with antibacterial drugs, California, 2000 and 2003*

Education level	%† (PR, 95% CI)	% decrease‡
<12 y		
2003 study	43.1 (0.83, 0.72–0.95)	17.0
2000 study	52.2 (referent)	
High school graduate/GED		
2003	29.3 (0.92, 0.80–1.06)	8.0
2000	31.8 (referent)	
Some college		
2003	15.7 (0.77, 0.64–0.92)	23.0
2000	20.5 (referent)	
College graduate		
2003	7.3 (0.53, 0.42–0.67)	47.0
2000	13.9 (referent)	

*PR, unadjusted prevalence ratio; CI, confidence interval, GED, general equivalency diploma.
†Number respondents with misconception divided by total number respondents.
‡Percent decrease from 2000 to 2003.

Conclusions

Our survey results show a 26.3% decline from 2000 to 2003 in the misconception among California women that a cold or flu should usually be treated with an antibacterial drug. This finding suggests that general educational campaigns before and during this period may have contributed to moderate reductions in the prevalence of misconceptions about antibacterial drug use. Although this study was not designed to evaluate specific interventions, our study period and population-based sample can provide an ecologic assessment of changing beliefs.

The decline in the misconception was greatest among women who were non-Hispanic white or Asian, had at least some college education, or had higher household incomes. Among levels of education, why women who graduated from high school had the smallest decline is unclear. This finding may reflect a true unexplained finding, an artifact of the sample (selection or information bias), or chance. The inverse relationship between the misconception and the presence of children <6 years of age in the household in 2000 may indicate success in initial educational campaigns focused on pediatricians and mothers with young children; that this relationship becomes less significant in 2003 may indicate that other women are "catching up."

Our findings, drawn from a large, ethnically diverse, population-based sample, show that 21.0% of California women in 2003 still held the misconception, particularly women who were younger, black, or Hispanic; had lower educational attainment or household income; resided in central and southern California; or had no healthcare coverage. Although not restricted to women, other studies identified similar demographic and socioeconomic risk groups (11,12). Social services intended to reach persons with these characteristics may be important venues for education on appropriate antibacterial drug use.

Cultural and social perspectives of disease, including differences in antibacterial drug regulations that affect the availability and cost of drugs in immigrants' countries of birth, can play an important role in misconceptions about antibacterial drugs (13,14). We documented racial and ethnic variation in California women with the misconception, despite adjusting for selected socioeconomic factors; the remaining variation likely reflects unmeasured socioeconomic or cultural factors. We note, in particular, the persistent prevalence of the misconception in African American women and the decreased prevalence of it in Asian women. While this study could not evaluate specific interventions, some California AWARE activities were focused on Asian physicians during 2003. Other research has documented racial and ethnic variation in parental expectations of antibacterial drugs (15). New intervention studies and campaigns aimed at educating and influencing women in specific minority, education, and income subgroups about appropriate antibacterial drug use are needed.

Our study has several limitations. First, although women may be more likely to be the health decisionmakers for young children and for the household, we do not know if our results can be generalized to men. Second, we did not have sufficient sample size to explore specific subgroups within the Asian race category. Third, we only included women in households with telephones who spoke either Spanish or English and chose to participate. Fourth, we were only able to interview women in Spanish or English, which may limit comparability between immigrant Hispanic and Asian women. Finally, this misconception may not equivocate to antibacterial drug expectation or demand.

In conclusion, our surveys document a decline in a key misconception in California women about appropriate antibacterial drug use and support continuation of educational campaigns to address this public health problem. Further refining and focusing of these educational efforts are needed to reach minority women and women with lower educational attainment and lower household incomes.

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Ms. Cummings is an infectious disease epidemiologist at the Division of Communicable Disease Control, California Department of Health Services. Her research interests include the epidemiology of emerging infections, drug-resistant organisms, and application of evidence-based control measures.

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Address for correspondence: Kate C. Cummings, California Department of Health Services, Immunization Branch, Division of Communicable Disease Control, 2151 Berkeley Way, Room 715, Berkeley, CA 94704, USA; fax: 510-883-6015; kcumming@dhs.ca.gov

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Asymptomatic SARS Coronavirus Infection among Healthcare Workers, Singapore

Annelies Wilder-Smith,* Monica D. Teleman,* Bee H. Heng,† Arul Earnest,* Ai E. Ling,‡ and Yee S. Leo*

We conducted a study among healthcare workers (HCWs) exposed to patients with severe acute respiratory syndrome (SARS) before infection control measures were instituted. Of all exposed HCWs, 7.5% had asymptomatic SARS-positive cases. Asymptomatic SARS was associated with lower SARS antibody titers and higher use of masks when compared to pneumonic SARS.

The patterns of spread of severe acute respiratory syndrome (SARS) suggest droplet and contact transmission (1,2). Close proximity of persons and handling of human secretions (respiratory secretions, feces, and the like) enhance the risk for transmission. These facts, together with the fact that transmission is more likely in more severely ill people (who end up in hospitals), have made the hospital setting particularly vulnerable to the rapid amplification of SARS (1–4).

Singapore was one of the countries most affected in the worldwide outbreak of SARS, with a total of 238 cases (available from www.who.int/csr/sars/country/table2003_09_23/en/); 76% of infections were acquired in a healthcare facility. The clinical spectrum of SARS has a strong predominance towards more severe disease associated with pneumonia (5); reports on the incidence of asymptomatic or mild infections attributable to SARS-associated coronavirus (CoV) were conflicting (5–8).

We conducted a seroepidemiologic cohort study among healthcare workers (HCWs) exposed to SARS patients in the first month of the nosocomial SARS outbreak at Tan Tock Seng Hospital in Singapore. Our study goal was to investigate the incidence of and factors associated with asymptomatic SARS-CoV infection.

The Study

Three patients with SARS were admitted to 3 wards of the hospital, in early March 2003, at a time when SARS was not recognized and no infection control measures were in place. Patient 1, who had imported SARS from Hong Kong, was admitted on March 1 and isolated after 5 days. Patient 2, a nurse who had looked after patient 1, was initially misdiagnosed as having dengue and was isolated 3 days after her admission when SARS was suspected. Patient 3 was admitted for other reasons (septicemia, ischemic heart disease, diabetes) but shared a cubicle with patient 2, became infected, and was not isolated until 8 days later, since initially the diagnosis of SARS was not considered (3). From March 6 onwards, HCWs were using N95 masks, gowns, and gloves for personal protection when nursing patient 1 and any persons suspected of having SARS. This meant that when providing nursing care for patients 2 and 3, HCWs did not use personal protective measures until SARS was suspected and the suspected patients were isolated. By March 22, N95 masks, gowns, and gloves were mandatory for all HCWs for any patient contact in the hospital.

Information on staff working on these 3 wards during March 1–22 was retrieved from the outbreak investigation team at the hospital and Human Resources. Only HCWs with exposure to any of these 3 patients were included. Exposure was defined as contact with any of these 3 patients in the same room or cubicle. Telephone interviews were conducted in April 2003, using a closed questionnaire by staff experienced in epidemiologic investigations from the hospital's Department of Clinical Epidemiology. Information collected included demographic data (age, sex, and ethnic group), occupation, history of medical conditions, and history of performing procedures with transmission risk (date, place, type, duration, and frequency). Contact time was defined as the total time in the same room with 1 of the 3 patients. Study participants were surveyed on their use of personal protection, i.e., wearing of N95 masks, gloves, and gown, and consistent handwashing. To verify exposure, names of source patients were included in the questionnaire, and respondents were asked if they had cared for these patients or been close to them (within the same room). Those without direct exposure were excluded from the study. Venous serum samples were taken in May and June 2003, 8–10 weeks after exposure, after informed written consent was given. Serum samples were tested serologically for SARS-CoV total antibodies by enzyme-linked immunosorbent assay (ELISA) using SARS-CoV–infected Vero E6 cell lysate and uninfected Vero E6 cell lysate supplied by the Centers for Disease Control and Prevention (9). The conjugate used was goat antihuman immunoglobulin (Ig)A, IgG, and IgM conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). Samples positive for SARS were repeated again and then confirmed by use of an indirect immunofluorescence assay. The

*Tan Tock Seng Hospital, Singapore; †National Healthcare Group, Singapore; and ‡Singapore General Hospital, Singapore

specificity of our ELISA was 100%, as tested in 50 serum samples from patients admitted to a non-SARS hospital for illnesses other than respiratory problems: we tested both IgG and IgG; all were negative. Samples from all initially positive patients during the SARS outbreak were sent to the National Environment Agency, Singapore, for confirmation with a neutralization test, and we found a good correlation (data not shown). Laboratory personnel were blinded to the clinical data.

Patients with a positive SARS serologic result, fever, respiratory symptoms, and radiologic changes consistent with pneumonia were defined as having pneumonic SARS. SARS-CoV–positive patients with fever and respiratory symptoms without radiologic changes were defined as having subclinical (nonpneumonic) SARS. SARS-CoV–positive patients without fever or respiratory symptoms were defined as having asymptomatic SARS-CoV infection. The study was approved by the Ethics Committee of Tan Tock Seng Hospital.

A total of 105 HCWs were identified by the outbreak team; 98 (93%) consented to answer the questionnaires, and 80 of these 98 (82%) also consented to have SARS serologic tests performed. Those who had SARS serologic tests did not differ from those who did not have these tests in terms of age, sex, job, or contact time.

The median age of the 80 study participants was 28 years (range 19–64), and 73 (91%) were female. Eight were doctors, 62 were nursing staff (staff nurses, assistant nurses, and healthcare assistants), and 10 had other occupations (cleaners, radiology technicians, physiotherapists). All reported to have had contact with 1 of the 3 index SARS patients. Distance to the source patient was <1 m in 73 cases (91%) and >1 m in 7 cases (9%). All 3 index cases resulted in a similar number of secondary cases (range 10–18 secondary cases).

Of these 80 hospital staff, 45 (56%) were positive by SARS serology. Of the 45 SARS-CoV–positive study participants, 37 (82%) were classified as having pneumonic SARS, 2 (4%) as having subclinical SARS, and 6 (13%) as having asymptomatic SARS-CoV infection (Table 1). Four staff members had fever and cough but negative SARS serologic test results; none of them was diagnosed as having suspected SARS by the hospital's SARS outbreak team. The overall incidence of asymptomatic SARS-CoV infection was 6 (7.5%) of 80. The incidence of SARS-CoV–positive cases among all asymptomatic HCWs was 6 (16%) of 37. The median titer of SARS antibodies was 1:6,400 (range 1:1,600–1:6,400) for pneumonic SARS, 1:4,000 (range 1:1,600–1:6,400) for subclinical SARS cases, and 1:4,000 (range 1:400–1:6,400) for asymptomatic cases (Table 1). The antibody titer for the asymptomatic cases was significantly lower than that for the pneumonic SARS cases (Mann-Whitney test; p = 0.0128).

Table 1. Clinical spectrum of SARS-CoV–positive cases and SARS antibody titers*

Classification	No. (%)	Median titer (range)
Pneumonic SARS	37 (82.2)	1:6,400 (1:1,600–1:6,400)
Subclinical (nonpneumonic) SARS	2 (4.4)	1:4,000 (1:1,600–1:6,400)
Asymptomatic SARS	6 (13.3)	1:4,000 (1:400–1:6,400)

*SARS, severe acute respiratory syndrome. CoV, coronavirus.

On univariate analysis, sex, age, use of gloves, handwashing, contact with l of the initial 3 patients, distance to the patient, and contact time were not associated with asymptomatic SARS. However, a higher proportion of those who had asymptomatic SARS (50%) had used masks compared to those in whom pneumonic SARS developed (8%) (p = 0.025) (Table 2).

Conclusions

We found a substantial number of cases with asymptomatic SARS-CoV infection and subclinical (nonpneumonic) SARS during the initial outbreak of SARS at Tan Tock Seng Hospital in Singapore: the incidence of asymptomatic cases among all exposed HCWs was 7.5%, and the proportion of asymptomatic cases out of all SARS-CoV–positive cases was 13%. Our findings regarding asymptomatic or subclinical SARS-CoV–positive HCWs contradict results from some previous studies, which reported an absence of asymptomatic SARS cases (5–7), but agree with results from other studies (8,9). Our incidence rate of 7.5% was higher (although not significantly) than that of 3% and 2.3% reported in asymptomatic HCWs who cared for SARS patients in Hong Kong (8,10). This difference is most likely due to the greater extent of exposure: a large proportion of our cohort was in close, unprotected contact to SARS patients before infection control measures were in place. However, direct comparison is not possible as the exposure is not described in the Hong Kong cohort. The extent of exposure in our cohort also contributed to the high attack rate that we observed (57%). False positivity may have also played a role but is unlikely or minimal given the high specificity of our essay and reports of high specificity from other centers (5,8,11). Overall, a rate of 13% for asymptomatic SARS cases among all SARS-positive cases is lower than the rate of asymptomatic cases of many other viral respiratory diseases. This difference may be explained by the novelty of this emerging pathogen. Because of its minimal genetic relatedness to other coronaviruses of humans and animals, lack of cross-protective immunity may be associated with development of overt disease (5). However, 1 study reports that subclinical SARS-CoV infections may be more common than SARS-CoV pneumonia, when a sensitive ELISA for SARS-CoV is used (8).

We investigated differences between asymptomatic SARS-CoV infection and pneumonic SARS. We found no

Table 2. Univariate analysis of risk factors associated with asymptomatic versus pneumonic SARS*†

Variable	Asymptomatic SARS	Pneumonic SARS	p value‡	Controls	p value§
Median antibody titer (range)	1: 4,000 (1:400–1:6,400)	1:6,400 (1:1,600–1:6,400)	0.013¶	NA	NA
Mean age (SD)	26.5 (4.3)	29.6 (9.2)	0.706¶	33.7 (11.5)	0.098#
Females (%)	6 (100)	32 (86)	>0.999	49 (94)	0.321
No. who used masks (%)	3 (50)	3 (8)	0.025	21 (40)	0.002
No. who used gloves (%)	1 (17)	10 (26)	>0.999	24 (46)	0.090
No. who washed hands (%)	4 (67)	29 (76)	0.63	47 (90)	0.110
No. who were close to a SARS patient (≤3 ft), %	5 (83)	35 (92)	0.456	48 (92)	0.747
Median contact time in minutes (range)	67.5 (10–360)	60 (10–480)	0.863¶	30 (10–960)	0.879#

*SARS, severe acute respiratory syndrome; NA, not available.
†SARS serology–negative asymptomatic controls added for comparison. All p values from Fisher exact test or chi-square test, unless otherwise stated.
‡p value for comparing asymptomatic versus pneumonic SARS.
§p value for comparing any 2 pairs in the 3 groups. For multiple comparisons, level of significance was set at 0.017 using the Bonferroni method.
¶p values from Mann-Whitney test.
#p values from Kruskal-Wallis test.

difference between pneumonic SARS patients and asymptomatic SARS-CoV–positive patients in relation to age, duration and distance of exposure to source patients, handwashing, and use of gloves. These findings indicate that HCWs who are exposed to SARS can be infected with SARS, regardless of the intensity of exposure. However, mask use was significantly more common in asymptomatic SARS-positive versus pneumonic SARS-positive patients. Antibody titers against SARS-CoV were significantly lower in those who remained asymptomatic, consistent with reports from Hong Kong (12). The person with the lowest SARS antibody titer in our cohort was the only one who had only indirect contact with 1 of the 3 initial patients, and she has remained asymptomatic. These observations suggest that the extent of exposure to SARS in persons who remained asymptomatic may have been lower, possibly resulting in a lower viral load of SARS-CoV, associated with less severe symptoms. A correlation with viral load and disease severity has been suggested (13); however, this hypothesis remains controversial as the development of severe respiratory distress is also thought to be due to an overwhelming immunologic response (1,4). Higher viral loads have been associated with increased severity in some but not all viral diseases. Any association between the infecting dose of SARS-CoV and severity of disease needs to be confirmed with animal studies. The low antibody levels observed in asymptomatic SARS-positive cases could also be because asymptomatic patients do not mount as much of an antibody response. It is also possible that cross-reactive antibodies were measured, although this is unlikely given the high specificity of our assay. The existence of asymptomatic or subclinical cases has public health implications, as they may either serve as a reservoir or as an unknown source of transmission. If asymptomatic persons contribute substantially to transmission but are not readily identified as having SARS, control measures will be hampered since they depend on the ready identification of persons who have been exposed to definite cases (14). Based on our data in Singapore, transmission from asymptomatic patients appears to play no or only a minor role, as all but 1 of the pneumonic cases of SARS had a definitive epidemiologic link to another pneumonic SARS contact. Lack of transmission from asymptomatic patients was also observed in other countries with SARS outbreaks (1; http://www.who.int/csr/sars/en/WHOconsen-sus.pdf, 2003). As the survival ability of SARS-CoV in human specimens and in environments seems to be relatively strong (15), determining whether asymptomatic patients excrete SARS-CoV is important. We were unable to determine this in our cohort, since these cases all occurred at a time when even the causal agent of SARS was not yet known and no diagnostic tests were available.

We documented a substantial incidence of asymptomatic SARS-CoV infection in exposed healthcare workers before full infection control was in place. Asymptomatic SARS-CoV infection was associated with lower SARS antibody titers and better protective measures (masks) compared to pneumonic SARS.

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AWS was responsible for blood-taking, data analysis and interpretation and writing the paper. MDT and BHH were responsible for the questionnaires and data entry, AEL for SARS serology, AE for data analysis, and YSL for initiating and leading this study. All authors critically reviewed the final paper.

Dr. Wilder-Smith is a resident physician in infectious diseases with a special interest in travel and international health. Her research interests include meningococcal disease, travel-related problems, dengue, and SARS.

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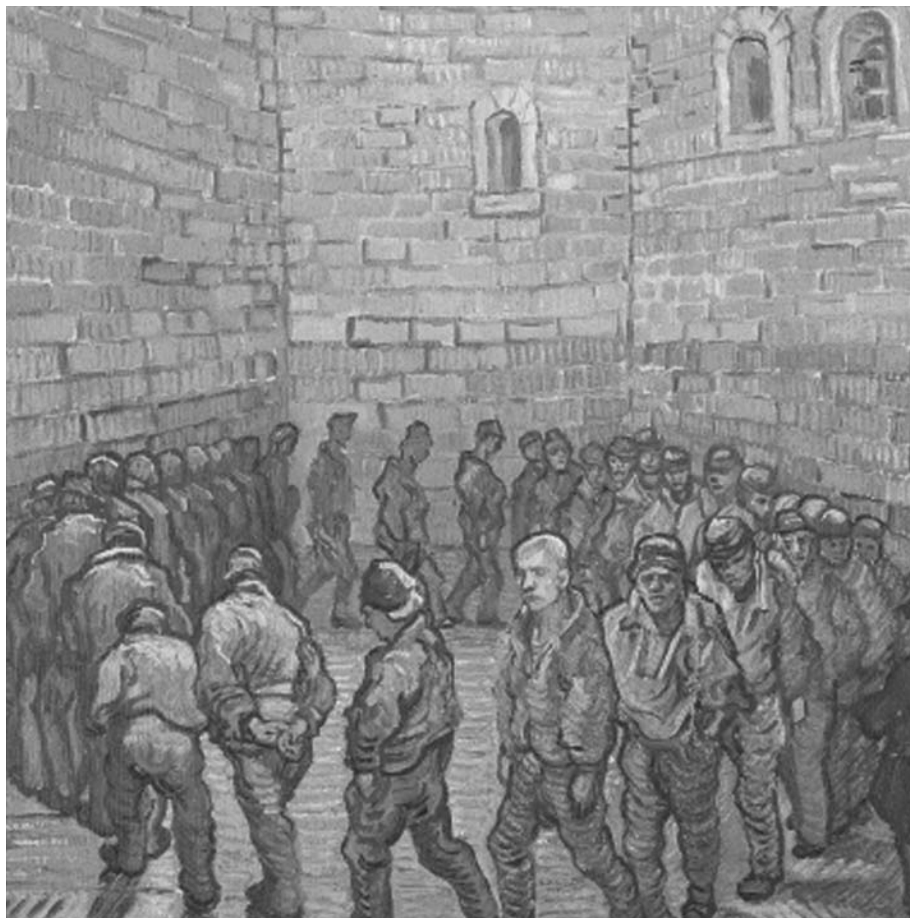
Address for correspondence: Annelies Wilder-Smith, Department of Infectious Diseases, Tan Tock Seng Hospital, 11 Jalan Tan Tock Seng, Singapore 308433; fax: 65-6252-4056; email: epvws@pacific.net.sg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Hedgehog Zoonoses

To the Editor: The article on hedgehog zoonoses (1) reviews diseases transmitted from African and European hedgehogs to humans but does not compare their infectious potential to that of other animals and people. For example, cats and Yorkshire terriers are well-known vectors of ringworm (2), but this has not been highlighted in Emerging Infectious Diseases. Also, the reports of herpesvirus (including human herpes simplex) hepatitis described in the article occurred as fatal hepatitis in hedgehogs, whereas their owners apparently escaped unscathed. These cases appear to be "reverse zoonoses" that are dangerous for the pet but not its human contacts. Perhaps the misleading table in the article should be revised so that busy medical doctors don't jump to conclusions, and hedgehogs don't end up on the euthanasia list at shelters.

Melissa Behr*

*New York State Department of Health, Albany, New York, USA

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Address for correspondence: Melissa Behr, New York State Department of Health, Wadsworth Center, PO Box 22002, Albany, New York 12201-2002, USA; fax: 518-474-2155; email: behr@wadsworth.org

In response: We thank Dr. Behr for her comment (1). The intent of our manuscript was to report, from a literature review, information on zoonotic infections related to hedgehogs. Of course, we are mainly concerned with infections or infestations that hedgehogs can transmit to humans, but we also noted that the inverse can be true, and humans can be a source of infection in pet hedgehogs. This manuscript was intended to inform not only physicians but also veterinarians and wildlife rescuers who may not be familiar with zoonotic diseases borne by or transmitted to hedgehogs. We also would like to take advantage of this letter to clarify a few points from our manuscript. First of all, pet hedgehogs are mainly African pygmy hedgehogs, and no reliable data are available regarding the number of European hedgehogs that are kept as pets either in Europe, the United States, or other parts of the world. In many European countries, native hedgehogs are protected by law and cannot be kept as pets (F. Moutou, pers. comm.). Furthermore, our comment on plague and "hedgehogs" in Madagascar was meant to be informative, as these animals are found only on that island. They are not true hedgehogs (belonging to the family *Tenrecidae* and not *Erinaceidae*) and are unlikely to be kept as pets (2; F. Moutou, pers. comm.). In our literature review from PubMed, we found no report of human leptospirosis infection from hedgehogs. However, the European hedgehog is considered the main host of *Leptospira bratislava* in the Netherlands and Denmark and the main host of *L. canicola* in Israel (2). Finally, if hedgehogs can be infected by lungworms of the genus *Capillaria*, no report of a human infection transmitted by hedgehogs has been published to our knowledge.

Bruno B. Chomel* and Patricia Y. Riley*

*University of California–Davis, Davis, California, USA

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Address for correspondence: Bruno B. Chomel, Department of Population Health and Reproduction–School of Veterinary Medicine, University of California–Davis, Davis, CA 95616, USA; fax: 530-752-2377; email: bbchomel@ucdavis.edu

Bartonella henselae and Domestic Cats, Jamaica

To the Editor: *Bartonella henselae* has been isolated from domestic cats in most countries where it has been investigated (1), with the exception of some countries at northern latitudes, such as Norway (2). The prevalence of both bacteremia and seropositivity in cats is usually highest in warm and humid tropical countries. The worldwide distribution of cat scratch disease (CSD), a zoonotic disease caused mainly by the scratch of a *B. henselae*–infected cat, follows a similar pattern. Limited information is available about CSD in either humans or the feline reservoir in the Caribbean region.

In 1955, 3 febrile children (siblings) admitted to a hospital in Havana, Cuba, were diagnosed with CSD based on their symptoms and the positive results of intradermal tests using the Foshay antigen (3). The results of the bacteriologic examination, however, were negative. All 3 siblings had previous contact with a female cat and her 4 kittens. In 2003, Alvarez et al. (4) reported the case of a 13-year-old Cuban boy who was treated for symptoms compatible with CSD. However, no other information could be found in the scientific literature regarding the isolation of this bacterium from domestic cats in the

Caribbean or seropositivity for *B. henselae* in humans or animals living in that region.

In the summer of 2003, an employee at a veterinary clinic in Kingston, Jamaica, was scratched and bitten on the hand by a cat. Fever and an enlarged axillary lymph node developed in the employee, and CSD was suspected. To confirm the clinical suspicion, and with the employee's permission, a serum sample was taken 7 weeks after the incident. Whole blood from the 62 remaining cats in the cattery was also collected into EDTA-containing tubes and stored at 4°C before being shipped to California for testing. The cat involved in the incident was not available for testing. The age of 63% of the cats ranged from 1 month to >5 years. Forty percent of the cats were formerly owned and put up for adoption and 16% of the cats were strays. The cat's origin was not recorded for the remaining 44% of the cats.

Upon reception at the laboratory, all cat blood samples were frozen at –70°C. They were subsequently thawed, and aliquot plated onto 5% rabbit blood–enriched agar and incubated at 37°C in 5% CO_2 for ≤4 weeks. The EDTA tube supernatant was serogically tested for *B. henselae* (mixed type I and type II antigens) by using a standard indirect immunofluorescence assay (5). The 62 blood samples were cultured and 12 (19.3%) cats were bacteremic for *B. henselae*. None of the cultures yielded *B. clarridgeiae* or *B. koehlerae*. Of the 12 bacteremic cats, 5 (42%) had positive cultures for *B. henselae* type Houston I, and 7 (58%) had positive cultures for *B. henselae* type Marseille, based on restriction fragment length polymorphism profile of the 16S rDNA, by using *DdeI* enzyme (6). The median number of CFUs was 385/mL (range 147–25,300). For the 5 cats infected with *B. henselae* type Houston I, the median was 259 (range 147–513) CFU/mL; for the 7 cats infected with *B. henselae* type Marseille, the median was 534 (range 174–25,300) CFU/mL. Of the 5 cats that were bacteremic for *B. henselae* Houston I, 2 were seronegative. Similarly, 2 of the 7 *B. henselae* type Marseille–bacteremic cats were seronegative. These 4 seronegative cats, 4–10 weeks old, were most likely in the early phase of bacteremia. None of the cats were co-infected with both subtypes. When a titer of ≥1:64 was used, 37 (60%) cats were seropositive for *B. henselae*. Their age ranged from a few weeks to >5 years old (median 11 months), including 7 cats that were <6 months old. The employee's *B. henselae* titer was 1:64.

These results constitute the first report originating from the Caribbean region of *B. henselae* isolation from domestic cats, as well as confirming seropositivity in a human, despite a low titer. Because we were not able to obtain a blood sample from the suspect animal, we cannot prove that this cat was the source of the employee's infection. Nevertheless, this study confirms the existence of both *B. henselae* types I and II in Jamaica, even if no specific conclusions can be drawn with regard to their relative prevalence.

The Caribbean has the highest incidence of HIV/AIDS outside of sub-Saharan Africa, with Jamaica having a HIV prevalence of 1.2% (range 0.6%–2.2%) for persons 15–49 years of age (7). As *B. henselae* is known to cause bacillary angiomatosis and bacillary peliosis in immunocompromised persons, knowledge of its presence in the Jamaican cat population is important for primary prevention. Unfortunately, diagnostic tests for *B. henselae* are not currently available on the island.

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Locksley L. McV. Messam,* Rickie W. Kasten,* Megan J. Ritchie,† and Bruno B. Chomel*

*University of California, Davis, California, USA; and †Jamaica Society for Prevention of Cruelty to Animals, Kingston, Jamaica

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Address for correspondence: Bruno B. Chomel, Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, CA 95616, USA; fax: 530-752-2377; email: bbchomel@ucdavis.edu

Pandemic *Vibrio parahaemolyticus* O3:K6 Spread, France

To the Editor: *Vibrio parahaemolyticus* is a halophilic bacterium that occurs naturally in aquatic environments worldwide. It causes one of the most severe forms of gastroenteritis and is the leading cause of seafood-associated bacterial gastroenteritis in the world, often associated with the consumption of raw or undercooked seafood. Since 1996, the incidence of *V. parahaemolyticus* infections has increased dramatically. *V. parahaemolyticus* strains previously associated with only sporadic cases of gastroenteritis have caused large-scale outbreaks in North America and epidemics in India, Southeast Asia, and Japan (1). This increase in incidence appears to be related to the emergence of a new clone, belonging to the O3:K6 serovar, which has pandemic potential. This clone was named the new O3:K6 clone to distinguish it from strains belonging to this serovar isolated before 1996, which are less pathogenic. We report the first evidence for the presence in France and suggest the presence and persistence in French coastal areas of this pandemic O3:K6 serovar, which is indistinguishable from the O3:K6 clone isolated in Bangladesh in 1996.

We analyzed 13 clinical isolates of *V. parahaemolyticus* collected in France from 1997 to 2004 and sent to the National Reference Center (Table). All isolates were characterized by polymerase chain reaction (PCR) to detect the genes encoding the virulence-associated hemolysins, thermostable-direct hemolysin, and thermostatable-related hemolysin (2), and 2 other genetic markers, *toxRS* and *orf8* (1,3). We also carried out molecular typing by various methods, including ribotyping, pulsed-field gel electrophoresis (PFGE), and arbitrarily primed PCR. Strains were initially identified by biochemical and cultural methods. Strain identities were confirmed by species-specific R72H PCR (4). The slide agglutination test was performed to determine whether the isolates belonged to the O3:K6 serovar. Two pandemic strains of the new O3:K6 clone and 1 strain of the old O3:K6 clone were included as external controls.

Five strains were identified as *V. parahaemolyticus* O3:K6 by slide agglutination test. With the exception of the strain referred to as old O3:K6 clone, all O3:K6 strains studied, whether isolated in France or included as controls, were positive for *tdh*, *toxRS,* and *orf*8 genes. Likewise, all strains, except the old O3:K6 clone, produced *Bgl*I rRNA gene restriction patterns identical to those of a major R4 ribotype previously described (5) and were genetically indistinguishable by arbitrarily primed PCR analysis. With the exception of the strain belonging to the old O3:K6 clone, the PFGE-typeable O3:K6 strains, despite having slightly different *Not*I patterns that reflect genetic rearrangement, clearly belonged to a single clone.

Until recently, *V. parahaemolyticus* caused only sporadic diarrhea and was never associated with a pandemic. The epidemiology of this organism changed abruptly after the new O3:K6 strains appeared in 1996. The spread of this serotype signaled the beginning of the first *V. parahaemolyticus* pandemic. Because the pathogenic *V. parahaemolyticus* O3:K6 isolates in France are derived from the new O3:K6 clone initially described in Bangladesh, this population likely was transported here in the same manner as *V. parahaemolyticus* O3:K6

Table. Characteristics of the *Vibrio parahaemolyticus* O3:K6 strains studied*

Strain no. CNRVC (source no.)	Origin	Date of isolation	Source of transmission	Detection of gene or phage sequences by PCR					Ribotype profile	PFGE profile	AP-PCR profile
				R72H	*tdh*	*trh*	*toxRS*	*orf8*			
970136	France (Atlantic coast)	Oct 1997	Local oysters	+	+	-	+	+	R4†	P-1a	AP-a
980402	France (southwest)	Sep 1998	Shellfish	+	+	-	+	+	R4	P-1c	AP-a
990346	France (Mediterranean coast)	Aug 1999	–	+	+	-	+	+	R4	P-1c	AP-a
030478	France (Atlantic coast)	Aug 2003	Local shellfish	+	+	-	+	+	R4	UT‡	AP-a
030479	France (Atlantic coast)	Aug 2003	–	+	+	-	+	+	R4	UT	AP-a
020468 (AN7410)	Bangladesh	1998		+	+	-	+	+	R4	P-1a	AP-a
020469 (AO1851)	Bangladesh	1999		+	+	-	+	+	R4	P-1b	AP-a
030085 (AQ4037)	Maldives	1985		+	-	+	-	-	Rb-2§	P-2	AP-b

*CNRVC, Centre National de Référence des Vibrions et du Cholera; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; AP-PCR, arbitrarily primed PCR.
†R4 ribotype pattern as described previously (5).
‡UT, untypeable: DNA was degraded before PFGE, presumably by DNases.
§According to our pattern designation.

was introduced into US coastal waters (6) and *V. cholerae* serogroup O1 was introduced into Gulf Coast waters in 1991 (7).

Epidemiologic information was collected from all patients with a standardized questionnaire concerning clinical history, symptoms, and seafood consumption. Responses indicated that some persons affected by *V. parahaemolyticus* O3:K6 had eaten local seafood harvested in uncontrolled areas. Furthermore, some had eaten seafood harvested in the same place several years apart. This provides evidence that pathogenic *V. parahaemolyticus* is present and suggests that it can persist in the French coastal environment.

The consumption of raw and lightly cooked seafood is increasing, as is the number of susceptible persons, which causes concern that the incidence of *V. parahaemolyticus* infections in Europe will increase. Monitoring this foodborne illness is difficult because only cases involving severe gastroenteritis are reported. Estimates are that only 1 in 20 cases of bloody diarrhea and only 1 in 38 cases of nonbloody diarrhea are reported in the United States (8). In France, the official surveillance authority estimated that the number of cases reported by the National Reference Center was representative of severe *Vibrio* infections. Making *Vibrio* isolations and infections reportable could help us estimate the true incidence of the disease and could improve the surveillance of *V. parahaemolyticus* infections.

Detecting the pathogenic *V. parahaemolyticus* O3:K6 in France, and previous results showing that pathogenic *V. parahaemolyticus* strains are present in French coastal areas at a higher frequency than was usually reported (9), may provide an early warning. Much effort is required to develop *V. parahaemolyticus* prevention strategies. Educating consumers about basic principles of food safety, particularly storage conditions, is an important component of prevention. Lack of continuous refrigeration from harvest to consumption may have contributed to these infections. The number of bacteria in seafood contaminated with only a small number of *V. parahaemolyticus* organisms can reach the infectious dose, thought to be $\geq 10^5$ CFU per gram according to the Centers for Disease Control and Prevention (10), within a few hours when left in a warm place. Another component of prevention is the improvement of microbial surveillance by systematic testing for pathogenic *V. parahaemolyticus* isolates in the environment and in locally produced and imported seafood.

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Marie-Laure Quilici,*
Annick Robert-Pillot,*
Jessica Picart,*
and Jean-Michel Fournier*

*Institut Pasteur, Paris, France

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Address for correspondence: Marie-Laure Quilici, Unité du Choléra et des Vibrions, Centre National de Référence des Vibrions et du Choléra, Institut Pasteur, 25 rue du Dr Roux, 75 724 Paris Cedex 15, France; fax: 33-1-45-68-82-23; email:quilici@pasteur.fr

Third *Borrelia* Species in White-footed Mice

To the Editor: The white-footed mouse, *Peromyscus leucopus*, is a natural reservoir host of several pathogens, including *Borrelia burgdorferi*, an agent of Lyme borreliosis (LB) (1). *B. burgdorferi* spirochetes are transmitted in the mouse population by *Ixodes scapularis* ticks. This tick vector also bears *B. miyamotoi*, a sister species to the relapsing fever group of spirochetes (2,3). *B. miyamotoi* infects *P. leucopus* in the laboratory (2), but the role of this mouse as a reservoir was not known. Here we report that *P. leucopus* is a reservoir for *B. miyamotoi* in nature and, in addition, that this mouse is host for a third, hitherto unknown, species of *Borrelia*.

In a recent study of a 9-hectare site in a mixed hardwood forest in eastern Connecticut, we found that ≈35% of *I. scapularis* nymphs were infected with *B. burgdorferi* and ≈6% were infected with *B. miyamotoi* (4). For that study of a field vaccine we also collected blood from *P. leucopus* mice captured from June to early September of 2001. DNA was extracted from the blood and then subjected to quantitative polymerase chain reaction (PCR) assay for the presence of *B. burgdorferi* as described (4). In the present study, we analyzed the extracts of 556 blood samples from 298 mice from the non-vaccine control grids by a multiplex, quantitative real-time PCR for 16S rDNA that discriminated between *B. burgdorferi* and *B. miyamotoi* at the site (4). Sixty-nine (12%) of the samples were positive for *B. burgdorferi* and 36 (6%) were positive for *B. miyamotoi*; 5 (0.9%) of the samples were positive for both species. In infected mice, the mean number of *B. miyamotoi* cells per milliliter of blood was 251 (95% confidence limits of 126–631), 5-fold greater than that of *B. burgdorferi* at 50 cells/mL (40–63).

A standard PCR assay of the blood samples with primers for the 16S–23S rDNA intergenic spacer (IGS) was performed as described by Bunikis et al. (5); results suggested the presence of a third species of *Borrelia* among the blood samples of the mice. A uniquely sized amplicon of ≈350 bp was observed in the reactions of 6 of 100 samples that were positive for *B. burgdorferi* and or *B. miyamotoi* by 16S PCR, and of 2 of 31 randomly selected samples that were negative for both *B. burgdorferi* and *B. miyamotoi* ($p = 0.3$ by 2-sided exact chi-square test).

Samples with the 350-bp amplicon were further investigated by PCR assay with *Borrelia* genus-specific primers for the 16S rRNA gene (rDNA), as described by Barbour et al. (6). The resultant ≈830-bp PCR product from these samples was directly sequenced on a Beckman 3000CEQ automated sequencer (5). The 788-bp sequence was aligned with sequences of other *Borrelia* species representing the LB and relapsing fever clades, and phylogenetic analysis was conducted. The accompanying Figure shows that the new species clusters with the monophyletic relapsing fever group of species rather than with the LB group

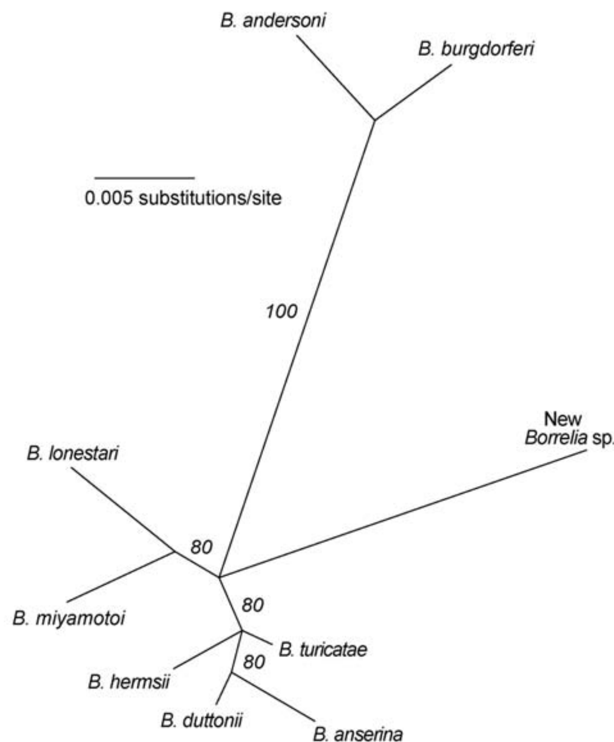

Figure. Unrooted maximum-likelihood phylogram for partial 16S rRNA gene sequences of selected *Borrelia* species, including a novel *Borrelia* organism, and representing Lyme borreliosis and relapsing fever groups. Sequence alignment corresponded to positions 1138 to 1924 of *B. burgdorferi* rRNA gene cluster (GenBank accession no. U03396). Maximum likelihood settings for version 4.10b of PAUP* (http://paup.csit.fsu.edu) for equally weighted characters corresponded to Hasegawa-Kishino-Yano model with an empirical estimate of transition/transversion ratio = 7. Support for clades was evaluated by 25 bootstrap replications by using branch-and-bound search, and values >50% are indicated along branches. Sequences (with GenBank accession nos.) used in the analysis were the following: *B. andersoni* (L46688), *B. miyamotoi* (D45192), *B. lonestari* (U23211), *B. hermsii* (U42292), *B. turicatae* (U42299), *B. duttonii* (U28503), *B. anserina* (U42284), and new *Borrelia* species (AY536513).

species. However, the new spirochete is distinct from all other known *Borrelia* spp. with an available 16S rDNA sequence in the GenBank database. Its partial 16S rDNA sequence differed by 3.3% to 4.2% from 9 LB group species and 2.4% to 3.4% from 15 relapsing fever group species. For comparison, intragroup sequence differences were ≤1.9%. On this basis, as well as the finding of partial IGS sequences (GenBank accession nos. AY668955 and AY668956) that were unique among all *Borrelia* spp. studied to date (3,5), we propose that this is a new species of *Borrelia*, provisionally named *Borrelia davisii* in honor of Gordon E. Davis for his contributions to *Borrelia* research and taxonomy.

While the new species was detected in 8 of 131 *P. leucopus* blood samples by using PCR for the IGS, the assays for this organism in the DNA extracts of 282 *I. scapularis* nymphs (4) from the same geographic site were uniformly negative ($p = 0.0003$, 2-sided Fisher exact test). This finding suggests that the new spirochete has another vector. The only other documented tick species that has been found feeding in small numbers on *P. leucopus* in Connecticut is *Dermacentor variabilis* (7). Holden et al. reported the presence of *Borrelia* in *D. variabilis* ticks in California by using PCR with genus-specific primers, but the species in these ticks was not identified by sequencing (8).

Although how *B. miyamotoi* and *B. davisii* affect the health of humans and other animals remain to be determined, our finding of 3 *Borrelia* species with overlapping life cycles in the same host in the same area shows that the ecology of *Borrelia* is more complex than was imagined. The presence of species other than *B. burgdorferi* in a major reservoir will have to be considered in future surveys and interventions.

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Jonas Bunikis*
and Alan G. Barbour*

*University of California Irvine, Irvine, California, USA

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Address for correspondence: Jonas Bunikis, Department of Microbiology and Molecular Genetics, B240 Medical Sciences I, University of California Irvine, Irvine, CA 926974025, USA; fax: 949-824-6452; email: jbunikis@uci.edu

Comparing Diagnostic Coding and Laboratory Results

To the Editor: The global Military Health System maintains electronic inpatient (Standard Inpatient Data Record, SIDR) and outpatient (Standard Ambulatory Data Record, SADR) clinical diagnostic coded data generated by the Department of Defense Composite Health Care System (CHCS), an electronic system that tracks and stores administrative and other patient encounter data. Because these records are readily available, widespread monitoring of these data as a means of medical surveillance has been suggested (1,2). Only 1 study in the literature assessed electronic coding reliability of these data (3); those authors found SIDRs to be a reliable source of billing data for common diagnoses, not including notifiable infectious diseases. We compared SADR and SIDR infectious disease diagnostic codes to laboratory data to assess the usefulness of these datasets in notifiable disease surveillance.

We identified SADRs and SIDRs coded for malaria, syphilis, acute hepatitis B, and Lyme disease in sailors, marines, and their family members, who were beneficiaries for medical care in a large metropolitan area. Medical encounters from January 1, 2001, to June 30, 2002, were studied. All records for the same patient with the same diagnostic code(s) were considered as 1 encounter. Records were

selected on the basis of International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes (4) as defined by the Department of Defense (5). Laboratory data were not part of SIDRs and SADRs but were part of CHCS.

For records with diagnostic codes relating to any of the 4 diseases of interest, laboratory records were searched to determine: 1) whether the provider ordered an appropriate test or tests and 2) if these were ordered, were the test results confirmatory (positive). Appropriate and confirmatory test results were determined by using published references (5–7) and local laboratory practices. For malaria, a blood smear was considered an appropriate test with a positive blood smear accepted as confirmatory (5,6). We considered both nontreponemal and treponemal tests to be appropriate for syphilis but only a positive treponemal test as confirmatory (5,6). For acute hepatitis B, we considered hepatitis B surface antigen or immunoglobulin (Ig) M anti-hepatitis B core (anti-HBc) to be an appropriate test, but only a positive IgM anti-HBc was accepted as confirmatory (5,6). We considered enzyme immunoassay total antibody screens or Western blot (WB) IgG or IgM tests to be appropriate for Lyme disease and accepted any positive test as confirmatory (5–7). χ^2 calculations were conducted ($\alpha = 0.05$).

Twenty-one SIDRs and 155 SADRs met the selection criteria (Table). While 61.9% of SIDRs studied had appropriate laboratory tests ordered, only 19.0% had associated confirmatory results in CHCS. For outpatient records, 64.5% had appropriate tests ordered, and 15.5% had confirmatory results. Among the SADRs, the proportions of appropriate laboratory tests for the diseases studied differed significantly (summary χ^2 =11.5, p = 0.01). These results suggest that tracking electronic SADR and SIDR datasets for the selected reportable diseases could produce a high number of false-positive reports; in this study, 81.0% of inpatient and 84.5% of outpatient reports would lack a confirmatory laboratory test result.

This initial evaluation is limited but supports the need to evaluate electronic datasets before using them for medical surveillance. We examined only ICD-9-CM coded records of selected diseases from 1 geographic area, with resulting small samples. Therefore, our results may not be generalizable. This study was restricted to laboratory, inpatient, and outpatient data recorded within 1 coordinated military system. Laboratory testing or clinical visits may have occurred outside of this network and may not have been captured in this study. Laboratory data were not recorded or stored in a standardized format in CHCS, increasing the likelihood of misclassification. We did not evaluate all related sources of data, including the hard-copy clinical records, so we do not know the completeness of the ICD-9-CM codes or the extent of ICD-9-CM code misclassification. Additionally, local clinical practices in terms of both ordering laboratory tests and coding diagnoses for the diseases studied were not defined.

Future studies would benefit from comparing reported medical events, paper medical records, and electronic datasets to include determination of sensitivity as well as positive predictive value (2,8,9). Discordance in these data sources should be investigated for miscoding, incomplete data, and unexpected clinical practices.

Efforts to improve medical record coding at military medical treatment facilities are under way (10). Additionally, standardization of CHCS laboratory test files, including adoption of the Logical Observation Identifiers Names and Codes system for standardized reporting of test names, is under way (available from http://www.ha.osd.mil/policies/2003/03-023.pdf). However, a documented, complete, reliable, and closely monitored single source of data for medical surveillance and disease reporting does not currently exist. Therefore, surveillance programs for infectious diseases in the US military should include monitoring of multiple, related sources of data and information (e.g., electronic inpatient and

Table. Clinical records with associated laboratory test results*

	Inpatient records (SIDR)			Outpatient records (SADR)		
Disease†	No. records selected	No. tests ordered (%)	No. confirmatory results (%)‡	No. records selected	No. tests ordered (%)§	No. confirmatory results (%)‡¶
Malaria	3	3 (100.0)	1 (33.3)	17	8 (47.1)	1 (5.9)
Syphilis	1	1 (100.0)	1 (100.0)	44	31 (70.4)	12 (27.3)
Acute hepatitis B	16	8 (50.0)	1 (6.3)	39	32 (82.1)	5 (12.8)
Lyme disease	1	1 (100.0)	1 (100.0)	55	29 (52.7)	6 (10.9)
Total	21	13 (61.9)	4 (19.0)	155	100 (64.5)	24 (15.5)

*This table presents Standard Inpatient Data Records (SIDR) and Standard Ambulatory Data Records (SADR) studied and percentages with appropriate laboratory tests ordered and confirmatory laboratory test results.
†International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) codes: malaria, 084.0–084.6; syphilis, 090, 091, 095, 096; acute hepatitis B, 070.30, 070.31; Lyme disease, 088.81.
‡Percentages reported reflect the proportion of records that had positive confirmatory laboratory results.
§Summary χ^2 = 11.5; p = 0.01.
¶Summary χ^2 = 7.0; p = 0.07.

outpatient encounters, laboratory results, and pharmacy data). All of these sources should be evaluated for completeness and accuracy.

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Asha J. Riegodedios,*
Anuli Ajene,* Mark A. Malakooti,*
Joel C. Gaydos,†
Victor H. MacIntosh,†
and Bruce K. Bohnker*[1]

*Navy Environmental Health Center, Portsmouth, Virginia, USA; and †Department of Defense Global Emerging Infections Surveillance and Response System, Silver Spring, Maryland, USA

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[1]Preliminary findings were presented at the US Army Force Health Protection Conference, August 2003, Albuquerque NM, the International Conference on Emerging Infectious Diseases, February 2004, Atlanta, GA, and the Navy Occupational Health and Preventive Medicine Workshop, March 2004, Norfolk, VA.

Address for correspondence: Asha J. Riegodedios, Navy Environmental Health Center, c/o Naval Dosimetry Center, 8901 Wisconsin Ave, Bethesda, Maryland 20889, USA; fax: 301-295-5981; email: riegodediosa@nehc.med.navy.mil

Concurrent Dengue and Malaria

To the Editor: A 37-year-old woman, a logistics director for a nongovernment organization, returned to France in March 2004 from an 18-day trip to Guinea, Senegal, and Sierra Leone. Fever, chills, and myalgia developed in the woman 3 days before she returned to France, and she treated herself with aspirin and paracetamol (acetaminophen). Malaria prophylaxis was taken neither during nor after the trip.

The day after returning to France, the woman's condition progressively worsened; diarrhea and extreme weakness that led to the inability to walk developed. Ten days after her return, she was admitted to the local hospital and treated with intravenous quinine and oral doxycycline (2 g per day) after thick and thin blood films showed 3% parasitemia with *Plasmodium falciparum*. Three days later, she was still febrile and had conjunctival jaundice, vomiting, insomnia, and moderate hemorrhagic manifestations (epistaxis, blood in urine and feces). Three days after initial hospitalization, the patient was transferred to the Infectious Diseases Unit in Marseille; fever (39.5°C) continued, and hepatosplenomegaly developed. Biologic analyses showed disseminated intravascular coagulation with platelet count of 22,000/µL, an elevated prothrombin time (54% higher than the control value), a longer activated clotting time (51 seconds versus a control value of 34 seconds), a fibrinogen level of 0.9 g/dL, exaggerated plasma fibrin formation and degradation, and hepatic cytolysis with both aspartate aminotransferase and alanine aminotransferase levels of 80 U/L.

Although acute malaria had been diagnosed, viral serologic tests were performed because the patient had returned from a tropical country with a fever. Persons in these circumstances are systematically administered a series of tests to determine the cause of their fever. Serologic tests for dengue performed on the acute-phase serum (collected 13 days after onset of symptoms) and convalescent-phase serum (collected 23 days after onset of symptoms) showed the presence of immunoglobulin (Ig) M (titers 1:800 and 1:3,200, respectively) and IgG (titers 1:400 and 1:3,200, respectively), which suggested that the patient had dengue fever and malaria concurrently. These results were obtained by using the Dengue Duo IgM-capture and IgG-indirect enzyme-linked immunosorbent assay (Biotrin,

PanBio Pty. Ltd., Brisbane, Australia). The same acute-phase serum was tested for flavivirus RNA by seminested reverse transcription–polymerase chain reaction (RT-PCR) by using flavivirus consensus primers PF1S and PF2R as previously described (1) in conjunction with the sense primer PF3S (GCIATHTGGTAYATGTGGYT). Attempts to isolate viruses by using C6/36 and Vero cells were unsuccessful, which might be expected given the delay between the onset of symptoms and specimen collection.

Sequence analysis of the 163-bp (primers excluded) PCR product (GenBank accession no. AY862501) showed 89%–99.4% range of homology with 34 dengue 3 virus strains by using the BLAST nucleotide program. Similarities obtained with sequences of dengue virus 1, 2, and 4 were ≤87%. Phylogenetic analysis performed with the patient sequence together with homologous sequences from dengue viruses and other flaviviruses showed that it corresponded to dengue 3 virus species. RT-PCR amplification on the convalescent-phase serum was negative. Based on World Health Organization criteria, the patient was diagnosed with dengue fever (2). The patient's interview showed a previous dengue fever episode in Haiti in 1995 and a previous malaria episode in Burundi in 2002, but biologic confirmation was not available, and serum was not collected before this episode. Therefore, we could not determine definitively whether this patient experienced primary or secondary dengue. In light of virologic tests results, the diagnosis of secondary dengue infection was more likely (3).

A PubMed search using the keywords dengue, mixed infections, dual infections, simultaneous infections, and concurrent infections retrieved 14 references published since 1958. In most cases, concurrent infection was with 2 dengue virus strains from 2 different serotypes in a single patient (4,5). Only 6 published studies reported concurrent infection with dengue virus and a bacterium (*Salmonella typhi*, *Shigella sonnei*, *Leptospira* spp.) (6–8) or with a virus such as Chikungunya virus (9).

To our knowledge, this is the first report of mixed dengue–parasite infection, dengue virus with *P. falciparum*. The authors previously questioned the accuracy of a serologic test to diagnosis dengue fever in patients experiencing malaria because reactivity was nonspecific on certain rapid serologic assays (10); however, serologic tests used in this study have demonstrated good specificity (10), and molecular tests are not prone to such specificity problems. Classifying this case as dengue hemorrhagic fever is questionable since some of the hemorrhagic signs may have been caused by acute malaria. In cases of concurrent infections involving a dengue virus, questions related to the influence of mixed infection on severity and prognosis are, therefore, impossible to address because of lack of information. Further investigations are required because this situation likely occurs frequently in nature, despite scant available data.

Remi N. Charrel,*
Philippe Brouqui,†
Cedric Foucault,†
and Xavier de Lamballerie*

*Université de la Méditerranée, Marseille, France; and †AP-HM Hôpital Nord, Marseille, France

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Address for correspondence: Remi N. Charrel, Unite des Virus Emergents, Université de la Méditerranée, 27 bd J Moulin, Marseille, 13005, France; fax: 33-491324495; email: rncvicophdm@gulliver.fr

West Nile Virus Detection and Commercial Assays

To the Editor: Roehrig and colleagues described the long-term persistence of immunoglobulin (Ig) M antibody in patients with West Nile virus (WNV) infection, as tested using an in-house Centers for Disease Control and Prevention (CDC) enzyme immunoassay (EIA) (1). This result suggests that interpreting WNV IgM results in subsequent years would be difficult. With the commercial availability and widespread use of US Food and Drug Administration–

approved WNV IgM tests, we were concerned that this phenomenon might also occur with new tests. Thus in 2004, we initiated a follow-up study of patients infected during the inaugural (2003) WNV season in Alberta, Canada.

Fifty patients who were WNV IgM positive by 2 commercial IgM kits (West Nile virus capture EIAs, Focus Technologies, Cypress, CA, USA, and Panbio, Windsor, Queensland, Australia) during the fall of 2003 were contacted. Sera were recollected and tested for IgM and IgG antibodies to WNV with current kits from these 2 companies. Sera were also tested for hemagglutination-inhibiting (HI) antibodies to WNV (2).

Of 39 serum samples from 38 patients, 28 were positive, 5 were indeterminate, and 6 were negative with the Focus IgM kit. Twenty-one were positive, 3 were indeterminate, and 14 were negative with the Panbio IgM kit. All had WNV IgG antibodies detected by Focus and Panbio IgG kits. We detected HI antibodies to WNV in all patients, and titers in 12 were ≥320. The time course for IgM index values for the Focus IgM kit used in 2003 and 2004 is shown in the Figure.

These data show that when tests are conducted with newly available kits, as with the CDC in-house test, IgM antibody to WNV persists for ≥8 months in most patients. A single high HI titer is not helpful in identifying recent infection. In addition, the IgM test cannot differentiate between recent and past infections. Interpreting a positive IgM result in WNV-endemic areas will be complex because a positive WNV IgM result could indicate a current acute infection or a previous WNV infection even in a person with a different acute illness.

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Peter A.G. Tilley,* Gail A. Zachary,† Roberta Walle,* and Paul F. Schnee†

*Provincial Laboratory for Public Health, Calgary, Alberta, Canada; and †Palliser Health Region, Medicine Hat, Alberta, Canada

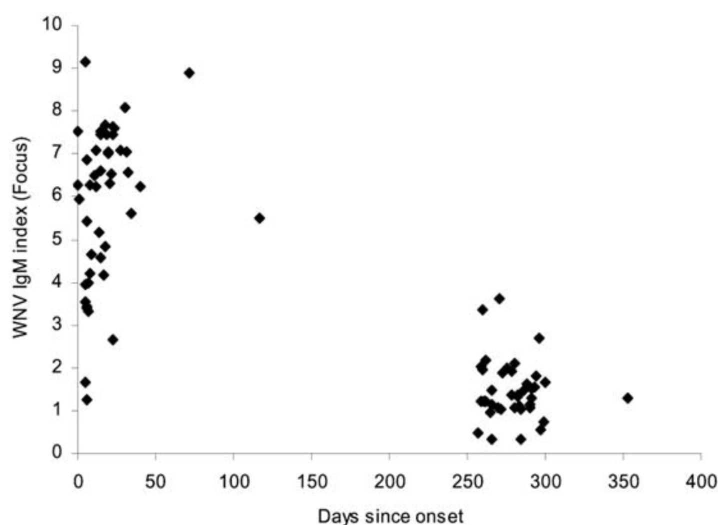

Figure. West Nile virus (WNV) immunoglobulin (Ig)M index values in serum specimens from 38 WNV case-patients detected in the fall of 2003. The assay was performed by using the Focus Technologies kit, as per the manufacturer's instructions. An index >1.1 indicates a positive result and an index <0.9 indicates a negative result.

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Address for correspondence: Peter A.G. Tilley, Provincial Laboratory for Public Health, 3030 Hospital Drive, Calgary, Alberta, Canada T2N 4W4; fax: 403-283-0142; email: p.tilley@provlab.ab.ca

Hepatitis A, Italy

To the Editor: Hepatitis A virus (HAV) infection rates are very low in industrialized countries. A noticeable fall in the prevalence of HAV antibodies (anti-HAV) has been reported in southern European and Mediterranean countries such as Spain (1) and Greece (2), reflecting improvements in hygiene standards in the last decades.

An HAV prevalence of 66.3% in 1981 (3) and 29.4% in 1990 (4) was shown in studies conducted in military recruits from all Italian regions. In both studies, subjects from southern regions had a higher HAV prevalence than those from north-central regions. In 2003, we conducted a study of recruits to show changes in HAV infection prevalence in younger Italian generations.

Military service was compulsory in Italy at that time; all men 18–26 years of age were included. From September to December 2003, 323 recruits 18–26 years of age (mean age 20 years), representing all Italian regions, who had been accepted for Air Force military service were tested

for anti-HAV in the recruitment center at Viterbo. This recruitment center, used in the 1990 study, was chosen again because it is located near Rome and adherence to protocol was easier to control.

A standard, precoded questionnaire was designed to collect information in the same sequence as questions asked by military personnel during the examination. The same information was collected as in the previous studies: date of birth, residence, father's years of education, and family size. After informed consent was obtained, blood samples were collected and stored at –30°C until tested. No person was vaccinated against HAV. Anti-HAV assay was performed by using commercial immunoenzymatic method (Abbot Laboratories, North Chicago, IL, USA). The methods used in the 1981 and 1990 studies have similar sensitivity and specificity between them and in relation to that used in the current study, and are detailed elsewhere (3,4).

The prevalence of anti-HAV declined from 66.3% in 1981 to 5.3% in 2003 ($p<0.01$, χ^2 test). In 2003, the prevalence was 2.1 % in the north-central region and 7.9% in southern regions (Figure). However, southern residents were more likely to have been exposed to HAV than north-central residents ($p<0.02$, χ^2 test). No statistical difference relative to father's years of education or family size was shown. Basic requirements for Navy (1981 study) and Air Force (1990 and 2003 studies) enrollment were similar. Thus, the 3 studies are comparable and a valid estimation of epidemiologic changes over time.

The anti-HAV prevalence shown in this study (5.3%) indicates that Italy has very low endemicity of HAV infection, at least in the young male population. The decline of HAV infection for >20 years is a consequence of good sanitation and hygienic conditions (vaccination against HAV is rarely performed in Italy) and has generated an increasing proportion of adults who are susceptible to this virus at an age characterized by the likely occurrence of a more severe clinical illness (5). This situation will likely necessitate costly interventions, such as vaccinating risk groups (e.g., military personnel, healthcare workers), to prevent HAV infection. Thus, HAV vaccination has been included in the compulsory vaccination schedule of the Italian military personnel since 1998 (6).

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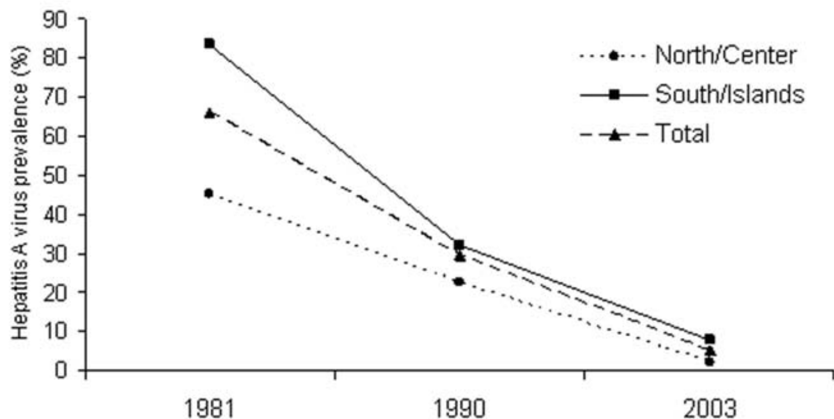

Figure. Anti-hepatitis A virus prevalence, 1981–2003

Raffaele D'Amelio,*† Alfonso Mele,‡ Andrea Mariano,‡ Luisa Romanò,§ Roberto Biselli,¶ Florigio Lista,# Alessandro Zanetti,§ and Tommaso Stroffolini‡**

*Direzione Generale della Sanità Militare, Rome, Italy; †Università degli Studi "La Sapienza" II Facoltà di Medicina e Chirurgia, Rome, Italy; ‡Istituto Superiore di Sanità, Rome, Italy; §Istituto di Virologia, Università degli Studi, Milano, Italy; ¶Centro Sperimentale di Volo, Pratica di Mare, Italy; #Centro Studi e Ricerche di Sanità e Veterinaria, Rome, Italy; and **Ospedale S. Giacomo, Rome, Italy

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Address for correspondence: Raffaele D'Amelio, Università "La Sapienza" II Facoltà di Medicina e Chirurgia, Ospedale S. Andrea Via di Grottarossa 1039 00189, Rome, Italy; fax: 39-06-8034-5427; email: raffaele.damelio@ospedalesantandrea.it

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Stenotrophomonas maltophilia in Salad

To the Editor: *Stenotrophomonas maltophilia* has emerged as an important nosocomial pathogen, especially in debilitated and immunocompromised persons (1). However, comparatively little is known of the epidemiology of this bacterium, and sources and routes of transmission of *S. maltophilia* are not well understood. The bacterium is widely distributed in the environment, including in plant rhizospheres (2). Although environmental sources such as ice-making machines have been implicated in outbreaks of nosocomial *S. maltophilia* sepsis, the source of infection in other outbreaks and in sporadic cases often remains unidentified. Food as a source of the bacterium has not been investigated; given the association of the bacterium with plants, we investigated the prevalence of the bacterium in salad vegetables.

Salads were purchased from reputable supermarkets and transported immediately to the laboratory. Ten grams of salad was homogenized in 90-mL sterile saline in a stomacher for 1 to 2 min. Aliquots (200 μL) of decimal dilutions of the homogenate were plated onto vancomycin-imipenem-amphotericin B (VIA) agar (3) and incubated for 24 to 48 h at 30°C. Occasional imipenem-resistant environmental bacteria, such as *Janthinobacterium lividum* or vancomycin-resistant *Enterococcus faecium*, may grow on VIA, but the medium contains a mannitol/bromothymol blue indicator system, allowing these bacteria, which produce acid from mannitol, to be distinguished from *S. maltophilia*. Putative *S. maltophilia* colonies were further identified by the API 20NE system (bioMérieux, Marcy l'Etoile, France).

Susceptibilities of 9 confirmed isolates to ceftazidime, chloramphenicol, colistin sulfate, gentamicin, minocycline, piperacillin/tazobactam, and trimethoprim-sulfamethoxazole were determined by using a disk diffusion method. *Pseudomonas aeruginosa* (NCTC10662) was used as a control. Because disk diffusion is not a reliable method for determining the susceptibility of *S. maltophilia* to quinolone antimicrobial agents (1), the Etest was used.

S. maltophilia was cultured from 14 (78%) of 18 salads. Numbers ranged from 1.50×10^2 to 1.96×10^5 CFU/g (mean 1.75×10^5 CFU/g, median 7.05×10^3 CFU/g). All isolates were susceptible to ciprofloxacin, colistin sulfate, minocycline, and trimethoprim-sulfamethoxazole. Eight (89%) were resistant to chloramphenicol; 7 (78%) to piperacillin/tazobactam; 5 (56%) to ceftazidime, and 2 (22%) to gentamicin.

All products examined were labeled "washed and ready to eat," and, thus, consumers would be unlikely to wash these products before consumption. The growth characteristics of *S. maltophilia* in products of this type, especially if subject to temperature abuse, are unknown, but in the domestic setting, numbers of the bacterium may increase before use.

All of the 9 isolates examined exhibited resistance to >2 of the 8 agents tested, with 2 isolates resistant to 4 compounds and thus had resistance phenotypes similar to those of strains associated with human infection. These findings are in agreement with those of Berg (2), who reported multiple resistances among isolates of *S. maltophilia* associated with oilseed rape. However, as with most strains of clinical origin, all the isolates tested here remained susceptible to trimethoprim-sulfamethoxazole and minocycline.

Although other investigators have examined salad products for the prevalence of pathogenic bacteria (4), they did not attempt to isolate *S. maltophilia.* Another study may have underestimated the prevalence of the bacterium in these products since a selective medium was not used (5). We have shown in a clinical setting that the use of a medium selective for *S. maltophilia* improves the recovery of this bacterium (6). Furthermore, the optimal growth temperature of *S. maltophilia* is 30°C, and incubation at higher temperatures (7) may reduce the likelihood of recovering the bacterium from food products. We recommend that future studies of *S. maltophilia* in food products use a medium such as VIA and that cultures be incubated at 30°C.

Prepackaged, ready-to-eat salads, such as those examined in this study, are washed in chlorinated water before sale. This measure is clearly insufficient to remove *S. maltophilia* from these items, possibly because the bacterium may exist in biofilms in some of the components of these products. *S. maltophilia* is capable of forming biofilms on a number of materials (8). Alternatively, products may become contaminated from environmental sources in production plants after washing.

The importance of *S. maltophilia* in ready-to-eat salads, which are marketed in a manner that assumes the product does not need washing before consumption, is unknown; nevertheless the presence of the bacterium in these products serves to highlight recommendations that these items should be avoided by severely immunocompromised persons, especially those with neutropenia (9). Recently, Apisarnthanarak et al. (10), in a prospective study of hospitalized oncology patients, identified intestinal colonization with *S. maltophilia* in 4 (9.5%) of 41 patients, which emphasizes that foodstuffs may be a potential source of this bacterium for some patients. This is a preliminary study, however, and further studies are needed, in particular, molecular typing of food and human-associated isolates,

to investigate the hypothesis that intestinal carriage of *S. maltophilia* may follow consumption of contaminated foodstuffs.

Andleeb Qureshi,* Louise Mooney,* Miles Denton,† and Kevin G. Kerr‡§

*University of Leeds, Leeds, United Kingdom; †General Infirmary at Leeds, Leeds, United Kingdom; ‡Harrogate and District National Health Service Foundation Trust, Harrogate, United Kingdom; and §Hull York Medical School, York Campus, Heslington, United Kingdom

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Address for correspondence: Kevin G. Kerr, Department of Microbiology, Harrogate and District NHS Foundation Trust, Harrogate HG2 7SX, UK; fax: +44-1423-553077; email: kevin.kerr@hdft.nhs.uk

Avian Influenza H5N1 and Healthcare Workers

To the Editor: Since January 2004, 35 human cases of avian influenza A virus H5N1 have been reported in Vietnam. Human-to-human transmission of H5N1 is a major concern, particularly because of reported family clustering (1). Two probable cases of human-to-human transmission were recently reported from Thailand (2), and evidence for human-to-human transmission was found in the 1997 Hong Kong outbreak (3). We evaluated healthcare workers exposed to 2 patients (patients 5 and 6 [1], referred to as patients A and B, respectively, in this article) with H5N1 infection, confirmed by polymerase chain reaction (PCR), to determine the potential risk for nosocomial human-to-human transmission of H5N1.

Patient A was admitted to a general ward of a pediatric hospital in Ho Chi Minh City on January 15, 2004, on day 8 of illness; no infection control measures were taken at that time. On January 18, 2004, she was transferred to the intensive care unit (ICU). Eight hours after ICU admission, limited infection control measures were implemented: the patient was transferred to a single room, and healthcare workers were required to use disposable surgical masks and gloves and wear nondisposable gowns. However, because resources were limited, each healthcare worker wore only 1 glove. On January 23, patient A was transferred to another hospital.

Patient B was admitted to the infectious diseases ward of the pediatric hospital on January 19, 2004, on day 6 of illness; he was transferred to the ICU after 4 hours and stayed there until he died on January 23. Infection control measures were implemented 2 days after ICU admission; these measures were similar to those taken for patient A except that no single room was available.

From January 25 to 27, 2004, a nasal swab specimen and baseline serum sample were collected from healthcare workers at the hospital; each worker also completed a questionnaire. On February 9 and 10, follow-up serum samples were collected. Nasal swab samples were tested by reverse transcription (RT)-PCR to detect the H5 gene (1). Paired serum samples were subjected to enzyme-linked immunosorbent assay (ELISA) (Virion/Serion, Würzburg, Germany) to detect immunoglobulin G against the nucleoprotein of influenza A; samples were also subjected to an H5-specific microneutralization assay (4).

Of 62 healthcare workers involved in caring for patient A, patient B, or both, 60 (97%) provided both samples and questionnaires: 16 who cared for patient A on the general ward, 33 who cared for patients A and B in the ICU, and 11 who cared for patient B on the infectious diseases ward or who were consulted for diagnostic or clinical procedures involving either patient. Characteristics of the workers and their exposures are shown in the Table.

The median time between last exposure and collection of the nasal swab and the baseline serum samples was 7 days (range 2–12 days). The median time between last exposure and collection of the follow-up serum sample was 21 days (range 17–26 days). All 60 nasal swab samples were negative by RT-PCR. Paired serum samples were available from 46

Table. Characteristics of 60 healthcare workers exposed to avian influenza patient A, patient B, or both

Characteristic*	No. (%)
Median age, y (n = 60)	33 (range 22–54)
Male/female (n = 60)	14/46
Occupation (n = 60)	
Nurse	28 (46.7)
Physician	10 (16.7)
Cleaner	9 (15.0)
Technician (laboratory/radiology)	9 (15.0)
Other	4 (6.7)
Flulike illness in preceding 2 weeks (n = 49)	6 (12.0)
Contact with poultry or birds (healthy or sick) (n = 59)	2 (3.4)
Recent travel to Mekong Delta (n = 59)	5 (8.4)
Duration of exposure (n = 59)	
<12 h	30 (50.8)
12–36 h	18 (30.5)
>36 h	11 (18.6)
Contact with secretions (n = 59)	
Yes	15 (25.4)
No	15 (25.4)
Don't know	29 (49.2)

*n indicates number of healthcare workers for which data were available.

healthcare workers, and 42 were negative in the influenza A–specific ELISA, 2 reacted with a negative-to-borderline response, 1 had a borderline-to-positive response, and 1 had 2 positive responses. A positive response indicates recent infection. All paired serum samples, 12 additional baseline samples, and 2 additional follow-up samples were negative in the H5-specific microneutralization assay. None of the paired samples from 4 healthcare workers that were reactive in the ELISA showed 4-fold or greater changes in titer in H1- and H3-specific hemagglutination inhibition and microneutralization assays, which indicates they had not recently been infected with human influenza. None of these 4 healthcare workers reported any illness or potential exposure to H5N1 other than to patient A or B. The ELISA results were considered nonspecific. Paired serum samples from patient A showed clear seroconversion in both ELISA and H5 microneutralization. Serum specimens were not available from patient B.

We found no transmission of H5N1 to healthcare workers, despite the lack of infection control measures, which suggests inefficient human-to-human H5N1 transmission; similar results were found in Hanoi (5). Droplet and contact transmission are considered the most effective means of transmitting influenza A in hospitals, and the clinical importance of airborne transmission has not been fully elucidated (6). Diarrhea in H5N1-infected patients potentially contains viable virus (1,7) and may affect the H5N1 transmission route. While these results appear reassuring, the limited options that were available to prevent nosocomial infection are worrisome. If reassortment between avian and human influenza A virus were to occur, resulting in a virus with pandemic potential, nosocomial transmission would be a concern. Infection control measures are crucial in all cases of avian influenza, and resources to prevent nosocomial infection must be made available in affected countries.

Constance Schultsz,*
Vo Cong Dong,†
Nguyen Van Vinh Chau,‡
Nguyen Thi Hanh Le,† Wilina Lim,§
Tran Tan Thanh,*
Christiane Dolecek,*
Menno D. de Jong,*
Tran Tinh Hien,‡
and Jeremy Farrar*

*Oxford University Clinical Research Unit at The Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam; †Pediatric Hospital Number 2, Ho Chi Minh City, Vietnam; ‡The Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam; and §Department of Health, Hong Kong Special Administrative Region, People's Republic of China

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Address for correspondence: Constance Schultsz, Oxford University Clinical Research Unit at the Hospital for Tropical Diseases, 190 Ben Ham Tu, Quan 5, Ho Chi Minh City, Vietnam; fax: 84-8-9238-904; email: schultsz@hcm.vnn.vn

Cell Phones and *Acinetobacter* Transmission

To the Editor: Nosocomial *Acinetobacter baumannii* is commonly acquired through cross-transmission because of its propensity to survive in the hospital environment and persistently contaminate fomites. Since cell phones are used increasingly by health personnel worldwide, we sought to determine their role in nosocomial transmission of multidrug-resistant (MDR) *A. baumannii*.

The study was conducted in a tertiary-care hospital in Israel, where MDR *Acinetobacter* spp. is endemic. Cell phones are used by personnel both for private communication and instead of traditional pagers. During 2002, 124 personnel (71 physicians, 54 nurses) were screened randomly for *Acinetobacter* spp. in a point-prevalence study; samples from hands of 119 personnel and 124 cell phones were cultured simultaneously for 2 months. Swabs from the back and sides of the cell phones were cultured. Cultures of hand samples were done by using the broth-bag technique (1).

To assess cross-transmission between hands, cell phones, and patients, we studied 2 additional *Acinetobacter* spp. culture cohorts, nosocomial blood isolates from 2000 to 2002, and axilla and groin *Acinetobacter* spp. skin colonization in an intensive care unit (ICU) during 2002. Cohorts represent wards in which 73% of study personnel worked.

Isolates were identified by the ID20NE system (bioMerieux, Marcy l'Etoile, France) without differentiation between *A. baumannii* and species 3 and 13TU. Antimicrobial susceptibility was determined for aminoglycosides, penicillins, cephalosporins, carbapenems, fluoroquinolones, tetracyclines, polymyxin E, and ampicillin/sulbactam by using disk diffusion according to Clinical and Laboratory Standards Institute guidelines (2). MDR was defined as resistance $\geq$3 different classes.

Genotypic analysis of isolates from all cohorts was performed using pulsed-field gel electrophoresis. Chromosomal DNA was digested with *ApaI* and analyzed by using a CHEF-DRIII apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Strain relatedness was interpreted according to consensus (3). Isolates showing an identical banding pattern were considered indistinguishable, and those showing differences of 3 bands were considered closely related.

Study personnel were assigned to medical (22%), surgical (44%), pediatric (23%), and ICU (11%) wards. The respective contamination rate with *Acinetobacter* spp. was 27%, 7.4%, 7.4%, and 0% for cell phones and 24%, 22%, 14%, and 41% for personnel hands. Of 30 hand and 15 cell phone cultures positive for *Acinetobacter* spp., 17% and 20%, respectively, were MDR.

Both hand and cell phone cultures of 3 personnel were positive (unrelated strains). Cell phone and hand isolates exhibited substantial clonal diversity. *Acinetobacter* spp. transmission (including MDR strains) was documented between hands, as well as between cell phones and hands, of different persons (Figure, panel A). One clone, recovered from cell phones and hands of ICU personnel, was also involved in skin colonization of ICU patients (Figure, panel B) but was unrelated to blood isolates.

We found that a significant percentage of cell phones and hands were contaminated with MDR *Acinetobacter spp*. and that cross-contamination between hands, cell phones, and patients occurred. Co-contamination of hands and cell phones was found in only 10% of

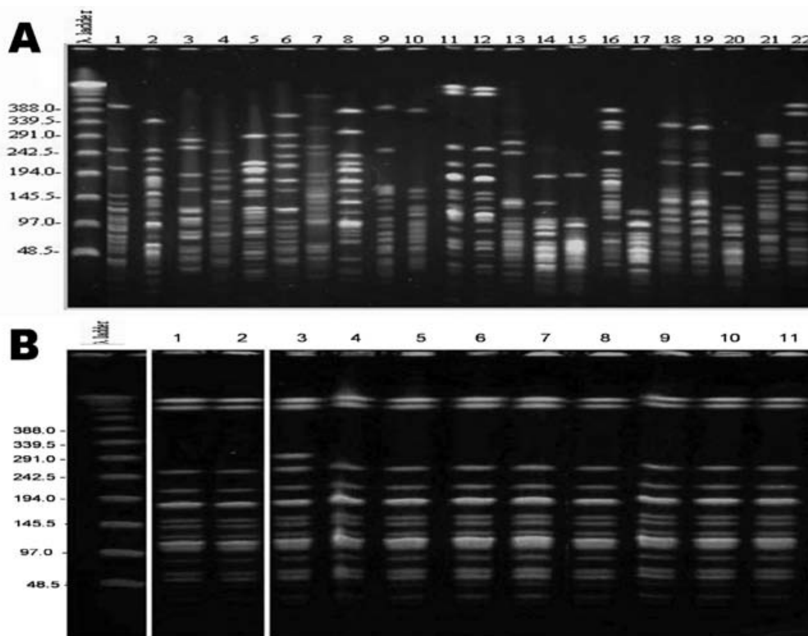

Figure. Pulsed-field gel electrophoresis of representative *Acinetobacter* strains. Twenty different clones (panel A) were recovered from cell phones (lanes no. 1, 3, 8–11) and hands of personnel (remaining lanes). Indistinguishable isolates were recovered from cell-phones and hand cultures (lanes 11 and 12), and 2 hand cultures (lanes 18 and 19). Both pairs were obtained from different persons. Panel B shows a multidrug-resistant *Acinetobacter* spp. strain recovered from cell phones (lane 1), personnel hand cultures (lane 2), and patients with skin colonization (lanes 3–11). All isolates are indistinguishable except for no. 3, which is a closely related strain (demonstrating a 1-band difference). The λ ladder was used as a molecular weight marker.

cases and may be explained by small sample size and that personnel were sampled only once. Higher co-contamination would likely have been found with repeated sampling.

The ability of *Acinetobacter* spp. to contaminate cell phones is not unexpected; it has been isolated from numerous sources in hospital environments in outbreak and nonoutbreak settings. Contamination and nosocomial transmission of pathogens by other electronic devices also has been demonstrated; a contaminated personal computer has been implicated in transmission of methicillin-resistant *Staphylococcus aureus* to a nurse. Computer keyboards have been contaminated with staphylococci and *Pseudomonas* spp. (4). Keyboards also have been implicated in nosocomial *A. baumannii* infection in burn units and ICUs (5) and have been contaminated with enterococci and *Enterobacter* spp. with a genetically identical methicillin-resistant *S. aureus* strain (6).

Stationary phones may also harbor pathogens; stationary phones in a day-care facility were contaminated with rotavirus (7), and home phones were contaminated with enteroviral DNA (8). In the hospital, $\leq$47% of stationary phones were contaminated with pathogenic microbes (9). Hand-to-mouth transfer of microbes was documented after contaminated fomites were handled during casual activities, with the highest transfer efficiency noted with stationary phone receivers (10).

Thus, cell phones may have a notable role in the nosocomial transmission of MDR microbes to patients. Cell phones are particularly problematic compared to stationary devices and may facilitate intra- and inter-ward (and perhaps inter-hospital) transmission. Additionally, the potential for nosocomial transmission of MDR pathogens by other electronic devices, such as handheld computers or personal digital assistants, with bedside applications, should be recognized.

Since restriction or even prohibition of such devices may prove impractical, strategies for preventing nosocomial transmission in this context are needed, especially given the risk of continuing contamination through repeated hand-cell phone contact. Such strategies should target behavioral controls of personnel (enforcing infection control precautions), environmental disinfection, and ultimately, optimal disinfection methods that will prevent contamination without damaging these sensitive electronic devices.

This work has been presented in part at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, USA, September 2003.

Abraham Borer,* Jacob Gilad,* Rozalia Smolyakov,* Seada Eskira,* Nechama Peled,* Nurith Porat,* Eytan Hyam,* Ronit Trefler,* Klaris Riesenberg,* and Francisc Schlaeffer*

*Soroka University Medical Center and Ben-Gurion University of the Negev, Beer-Sheva, Israel

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Address for correspondence: Jacob Gilad, Department of Internal Medicine E, Soroka University Medical Center, PO Box 151, Beer-Sheva, 84101, Israel; fax: 972-8-640-3366; email: giladko@bgumail.bgu.ac.il

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Beasts of the Earth: Animals, Humans, and Disease

E. Fuller Torrey and Robert H. Yolken

Rutgers University Press, New Brunswick, NJ, USA, 2005
ISBN: 0-81-353571-9
Pages: 191, Price US$23.95

In their new book Beasts of the Earth: Animals, Humans, and Disease, Torrey and Yolken provide us with a thoroughly researched and well-written account of the animal origins of many human diseases. With interesting insight into this very timely topic, the authors describe the impact of animals and their diseases on the rise and subsequent decline of ancient civilizations. The book traces the history of humans' relationship with animals as humans evolved from hunters to villagers, traders, and, more recently, pet owners and international consumers, an evolutionary process that has given microbes unlimited passports to new populations and geographic areas. As testament to the adaptability of these pathogens, the book describes the increasing challenges to global health presented by zoonotic infections recently emerging from parts of Asia and Africa.

Fortunately, Torrey and Yolken also have plenty of advice for how the scientific community can address these challenges. The authors strongly emphasize the need for closer coordination and communication between the medical and veterinary sectors at national and international levels. Stressing that it is illogical for the animal and human health worlds to conduct zoonotic disease research separately, they state that interdisciplinary zoonotic disease research centers should be the model research platforms for the future.

Those who have read the 2003 Institute of Medicine Report Microbial Threats to Health: Emergence Detection and Response (Institute of Medicine, 2003) may find many of the themes in this book familiar. However, as the authors chillingly state, "We live with these pathogens in a negotiated peace, but what happens when biological circumstances change?" Torrey and Yolken do an excellent job of reminding us of the interconnectedness between human and animal hosts and their pathogens and environments.

Nina Marano*
*Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Address for correspondence: Nina Marano, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd, Mailstop C12, Atlanta, GA 30333, USA: fax: 404-639-3059; email: nmarano@cdc.gov

Viral Haemorrhagic Fevers, Perspectives in Medical Virology, Volume 11

Colin R. Howard

Elsevier, Amsterdam, the Netherlands; 2005
ISBN: 0-444-50660-8
Pages: 205, Price US$130

Viral Haemorrhagic Fevers is a compact and highly readable monograph written by Collin Howard, an authoritative and veteran virologist with hands-on practical experience in this field. This volume is highly satisfying on a variety of levels. Self-contained chapters deal with each of the 4 taxonomic viral families (*Flaviviridae*, *Arenaviridae*, *Bunyaviridae*, and *Filoviridae*), which make up the category of viral hemorrhagic fever agents. The properties of each virus family are presented in terms of molecular virology and replication strategy, followed by the epidemiology, clinical presentation, and treatment options. Cross-referencing to other chapters is kept to a minimum, enhancing the readability of the text. The information is reasonably current, with the exception of the current Marburg outbreak in Angola. For each virus, the author offers his candid assessment of the available treatment options. My only quibble is that I do not share his pessimism that effective vaccines will not be developed and distributed in the near future.

The author made a conscious decision to avoid encyclopedic referencing to enhance readability. On occasion, this results in bold statements that the specialist might wish had been referenced. One example is the discussion of Whitewater Arroyo virus and its probable (but contentious) role in 3 fatal cases of hemorrhagic fever from 1999 to 2000. Another is a statement that infection of endothelial cells is a critical event in the pathogenesis of Ebola virus, which, to my knowledge, has never been adequately documented.

The text is enhanced by electron micrographs of representative agents, photographs of rodent reservoirs in their native habitat, maps of geographic distributions, and schematic representations of genome organization and replication strategies. The legend to Figure 2 in the Filovirus chapter compares Marburg and Ebola viruses, but only 1 image (which I recognize to be Marburg) is displayed.

The author states that this book was directed "primarily at healthcare workers, clinicians, and microbiologists wishing to gain a rapid overview of these widely varying agents." This

book should appeal to that audience, and also to a wider audience of persons interested in the public health ramifications of these serious viral diseases in their geographic niches as well as their potential to cause disease in industrialized nations, either when imported by returning travelers or deliberately released in an act of terrorism. These aspects of viral hemorrhagic fever agents are discussed in both the introductory and concluding chapters of the book. A nice touch was the inclusion of a short annotated bibliography of books for the lay audience dealing with the history of these agents, plus a second list of specialized textbooks of more interest to the practicing virologist. Other appendices list useful Web sites and a capsule explanation of how biocontainment facilities are designed to deal with these fearsome pathogens. There is something for everyone in this book; I will keep my copy handy, next to my desk.

Peter B. Jahrling*

*National Institutes of Health, Frederick, Maryland, USA

Address for correspondence: Peter B. Jahrling, NIAID/NIH-Integrated Research Facility, 6700 A Rockledge Dr, Bethesda, MD 20892, USA; fax: 301-480-2319; email: jahrlingp@niaid.nih.gov

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The Painted Gallery (detail)
(17,000 BC)
Ceiling, Lascaux Caves,
Perigord, Dordogne, France
Image courtesy of CNP -
Ministère de la Culture, France

Paleolithic Murals and the Global Wildlife Trade

Polyxeni Potter*

"Antiquities are history defaced," argued Renaissance philosopher Francis Bacon in The Advancement of Learning; they are "…remnants of history which…escaped the shipwreck of time" (1). These remnants, battered by the elements and scattered around the globe, are all we have to piece together human heritage. In recent years, radiocarbon dating techniques (e.g., accelerator mass spectrometry) have allowed us to date samples of pigment from cave paintings and not rely solely on evidence from surrounding artifacts. These techniques shed light on the chronology and evolution of prehistoric art and show that cave painting began much earlier than believed, as early as the Upper Paleolithic period (2).

The Paleolithic period (Old Stone Age), the earliest stretch of human history 2 million years ago, saw the development of the human species. Nomadic hunters and gatherers, who lived in caves and crafted tools out of stone, progressed during the last ice age to communal hunting, constructed shelters, and belief systems. The Upper Paleolithic (end of Old Stone Age) marks humanity's cognitive and cultural, as well as artistic, beginnings (3).

When, why, and how precisely humans moved from rote tool-making to symbolic self-expression is not known. Evidence gathered in the past 200 years indicates that graphic activity (figurative and nonfigurative marks) began as early as 40,000 years ago, preceding the development of agriculture. It coincided with human migration around the globe, the production of implements from multiple materials, and the building of simple machines. Images carved, etched, or painted on stone, clay, bone, horn, ivory, or antler were found in Africa, Australia, the Middle East, and Europe, and portable sculptures of animal and human figures were more common and widespread than cave paintings (4).

The first discoveries of Paleolithic painting (40,000 BC-12,000 BC), which were greeted with skepticism and disbelief, were made around 1835 in France and Switzerland (5) and later in Spain, Australia, South Africa, and other places. The western edges of the Massif Central region of France and the northern slopes of the Pyrenees are dotted with more than a hundred Paleolithic caves, among them renowned Lascaux. Naturally blocked for thousands of years, these deep caves maintained sufficiently stable temperature and humidity to preserve, untouched, not only paintings but also footprints and handprints of their inhabitants. After the discovery of Lascaux in 1940, environmental conditions changed. A fungus (*Fusarium*) began to grow inside the cave, and algae spread on the floor, walls, and ceiling, threatening the integrity of the paintings. The threat was contained, but access to the public was curtailed (6).

The cavern "gave onto a steep slope, slippery and slimy…with flakes of worked flint of poor quality, some fragments of reindeer horns and many pieces of conifer charcoal…" recalled speleologist Abbé Breuil about the initial opening to Lascaux (7). The ground was different 17,000 years ago. The cave had sunk and was difficult to reach, the entrance obscured by millennia of erosion and sediment.

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA

The dark sanctuary contained a network of passages, caverns, and shafts, later named Great Hall of the Bulls, Painted Gallery, Chamber of Engravings, Chamber of Felines, Shaft of the Dead Man (8). Uneven wall and ceiling surfaces sported enigmatic scratches, smudges, combinations of dots and grids, as well as drawings and painted figures whose narrative meaning had been rendered unfathomable by the passage of time.

Mineral pigments (ochre, charcoal, iron oxide, hematite, manganese) were ground into animal fat and applied with brushes or were blown through hollow sticks. The artists, working in confined, possibly dangerous space, under unsteady or flickering light from a torch or oil lamp, incorporated distortions and shadows in the design, along with soot.

As if suddenly awakened from extended hibernation, herds of aurochs, horses, chamois, ibexes, bison, stags, rhinoceroses rush en masse on the cave walls. Large wild beasts, drawn not from life but from the imagination, populate free-flowing compositions in coordinated movement with each other. Their bodies, intently outlined and punctuated with color, at once realistic and stylized, conform to the contours of the cave, the cracks and imperfections of which are often incorporated in the drawing. Anatomical details betray familiarity with the animals, as well as observational and artistic skill. Proportions are mostly accurate, except for the heads, which tend to be small, and the horns, which are sometimes exaggerated. Unrestricted and unbound, the beasts frolic on the dark walls, at times overlapping as they gallop toward or away from each other.

A strong “occluding contour,” an essential silhouette, is etched or painted on the hard surfaces. The silhouette, often the only graphic, boasts a prominent cervicodorsal line, in profile. And this line is at times the only line, as if part of the animal is drawn to suggest the whole. Even though figures are presented in profile, distinctive details (horns or hooves) have an independent, “twisted” orientation (9).

Large animals are the protagonists. Humans, if present at all, are stick figures, masked or headless, crudely drawn, stiff and nonexpressive, their puzzling presence possibly symbolic and secondary. We do not know if these murals represent early social interactions (the hunt, sacred rites, tribal ceremonies); hallucinogenic imagination inflamed by fumes in unventilated caves; unknown primitive rituals; or simply artistic compulsion. Yet, this preferential and exuberant treatment of animals suggests on the part of our ancestors inexplicable fascination with wildlife.

As far back as the Paleolithic age, humans have lived in close proximity with animals, associating not only with those they could domesticate but also with wild and dangerous beasts. Encounters contained an element of risk, for humans were injured or killed as much as nourished or entertained. The enigmatic portrayal of large, wild beasts on the walls and ceiling at Lascaux suggests a complex early relationship that went beyond the necessities of food or fiber. In our time, interaction with animals continues to encompass cohabitation at all levels, including the microbial. Encounters, compounded by increased travel and trade, still involve risks as well as benefits. And even though we are less likely to be injured or killed by animals, the exotic pathogens living and traveling with them counterbalance amusement and companionship with illness and death (10).

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Address for correspondence: Polyxeni Potter, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D61, Atlanta, GA 30333, USA; fax: 404-371-5449; email: PMP1@cdc.gov

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