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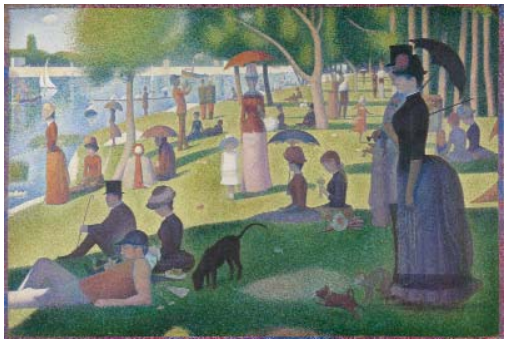

On the Cover

George Seurat (1859–1891)
Sunday Afternoon on the Island
of La Grande Jatte (1884–86)
Oil on canvas (2.08 m × 3.08 m)
The Art Institute of Chicago, Helen Birch Bartlett
Memorial Collection

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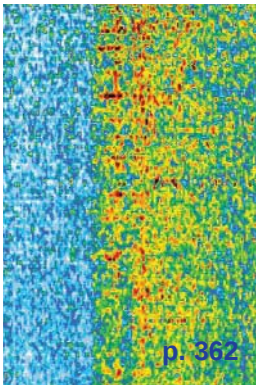

Perspective

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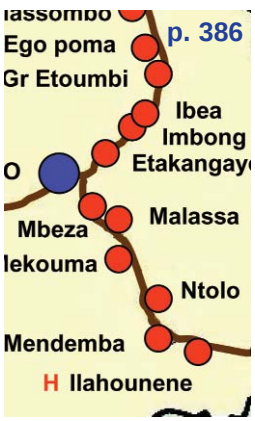

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Policy Review

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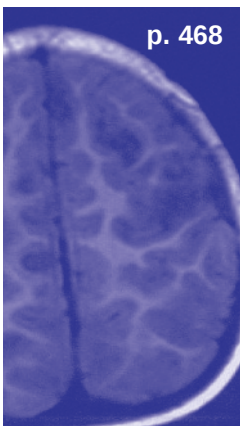

Another Dimension

Commentary

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News & Notes

Fly Transmission of *Campylobacter*

Gordon L. Nichols*

An annual increase in *Campylobacter* infection in England and Wales begins in May and reaches a maximum in early June. This increase occurs in all age groups and is seen in all geographic areas. Examination of risk factors that might explain this seasonal increase identifies flies as a potential source of infection. The observed pattern of infection is hypothesized to reflect an annual epidemic caused by direct or indirect contamination of people by small quantities of infected material carried by flies that have been in contact with feces. The local pattern of human illness appears random, while having a defined geographic and temporal distribution that is a function of the growth kinetics of one or more fly species. The hypothesis provides an explanation for the seasonal distribution of *Campylobacter* infections seen around the world.

Campylobacter spp. are the most common bacterial causes of diarrhea in England and Wales (1). The epidemiologic features of *Campylobacter* infection have proved difficult to discover, and extensive strain typing has failed to clarify the main transmission routes. Testable hypotheses must be established to explain available evidence, particularly the reason for the observed seasonality. Relatively few outbreaks of *Campylobacter* gastroenteritis occur (2), and most cases are sporadic. In case-control and case-case studies of sporadic *Campylobacter* infections, most cases remain unexplained by recognized risk factors (3,4).

The annual increase in *Campylobacter* infections in England and Wales begins at approximately day 130 (May 9) and reaches a maximum at approximately day 160 (June 8) (Figure 1). Although this seasonal rise is seen in all ages, it is more marked in children (5). Cases in towns and cities across England and Wales show broadly similar seasonal changes in distribution (Figure 2). The relative geographic uniformity of the increase seen in May of most years has the temporal appearance of an annual national epidemic. Because person-to-person infection within the community is uncommon, it is likely that the epidemic is caused by a single main driver for human *Campylobacter* infection. The possible seasonal drivers were examined, and only vector transmission by flies appears to provide a convincing explanation for the observed seasonal trends (Table).

The seasonal increase in *Campylobacter* infections in May and June in England and Wales is hypothesized to reflect an annual epidemic caused by direct or indirect exposure of humans to contaminated material carried by several fly species that have been in contact with human, bird, or animal feces or contaminated raw foods. Flies have been shown to carry *Campylobacter* and can infect both humans and animals (6–8). Intervention studies have demonstrated diarrheal disease reduction linked to control of flies (9–11), and deaths from diarrheal diseases have been linked to measurements of fly abundance (12). The local pattern of human *Campylobacter* infection appears random, while having a defined geographic and temporal distribution. This distribution is predicted to be linked to the growth kinetics of 1 or more fly species and their access to environmental sources of *Campylobacter* in feces or food. The seasonal increase in fly populations results from rainy weather and an increase in temperature that causes the development from egg to fly to occur in days rather than months. Individual flies can lay hundreds

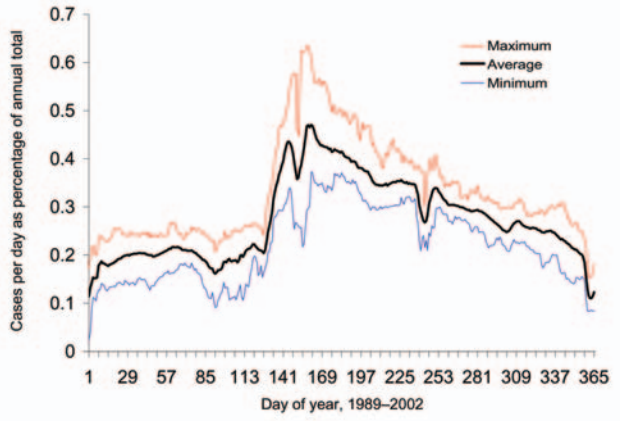

Figure 1. Distribution of *Campylobacter* cases per day. When averaged for 1989 to 2002, the epidemic begins at approximately day 130, peaks at approximately day 160, and gradually declines through the rest of the year.

*Health Protection Agency Centre for Infections, London, United Kingdom

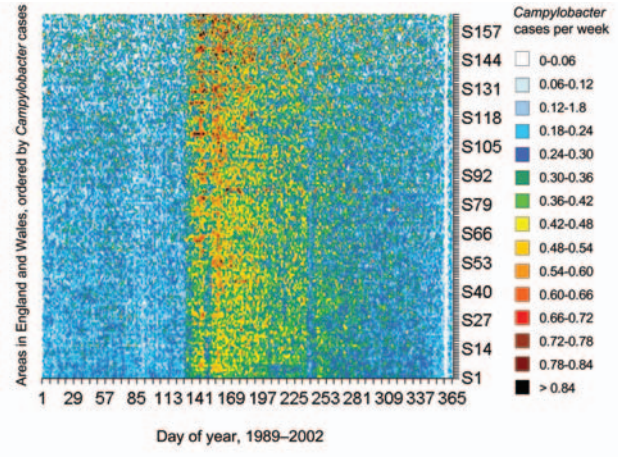

Figure 2. Cases of *Campylobacter* infection in England and Wales based on the patient specimen date. Figure shows broadly similar changes in patterns of infection across the country as measured by laboratory reporting per town or city (cases as a percentage of the annual total) by day of year. Laboratories were ordered by the total number of cases reported over the 14-year period (online Appendix available from http://www.cdc.gov/ncidod/EID/vol11no03/04-0460_app.htm).

of eggs, which can result in a large increase in fly numbers in a short period. Fly numbers fluctuate through the summer and decline in October, but the decline is less dramatic and defined than the spring increase.

Disease transmission is hypothesized to occur through small quantities of contaminated material carried on the feet, proboscis, legs, and body hairs or from material regurgitated or defecated by flies. The variety, numbers, virulence and viability of organisms in the contaminated material will differ, and some contamination will include *Campylobacter* while others will not. Contamination will be distributed over a variety of food types. Contamination of food by flies could occur at any stage of the food supply chain, but *Campylobacter* counts within the contaminated material on foods will decrease over time; consequently, most infection will result from contamination close to consumption (e.g., in the domestic or catering environment). Because whether a fly has visited contaminated feces is unknown and how a person becomes infected is uncertain, epidemiologic investigation is difficult.

A number of synanthropic fly species could be involved, including houseflies (e.g., *Musca* spp., *Fannia* spp.), blowflies (e.g., *Calliphora* spp., *Lucilia* spp.), and other dung-related flies (e.g., *Sarcophaga* spp., *Drosophila* spp.) (13). These flies have individual behavioral patterns, ecology, physiology, and temporal and geographic distributions that will influence the likelihood of their being in kitchens, on human or animal feces, and on food. Although *Musca domestica* is the species most likely to be involved because it is commonly found in houses and food-processing establishments, larger flies (e.g.,

Table. Risk factors that might affect *Campylobacter* seasonality*

Risk factor	Outbreaks	Evidence of seasonality	Credibility as the main seasonal driver
Barbecuing	Yes	Medium	Low
Birds	Yes	Strong	Low
Bottled water	No	None	Low
Chicken	Yes	Medium	Medium
Cross-contamination	Yes	None	None
Domestic catering	No	None	None
Farm visit	Yes	None	None
Farm animals	Yes	Weak	Low
Flies	No	Strong	High
Food handlers	Yes	None	None
Food packaging	No	None	None
Immunologic response	No	Weak	None
Mains supply drinking water	Yes	None	None
Nosocomial	Yes	None	None
Pets	No	Weak	Low
Pools, lakes, streams	No	None	None
Private drinking water supplies	Yes	Weak	None
Protozoa	No	None	Low
Salads and fruit	Yes	Weak	Low
Stir-fried food	Yes	None	None
The countryside	No	Weak	Medium
Transmission in families	Yes	None	None
Travel abroad	No	None	None
Unpasteurized milk	Yes	Weak	None
Weather/climate	No	Medium	Medium

*Evidence base provided in online Appendix (available from http://www.cdc.gov/ncidod/EID/vol11no03/04-0460_app.htm).

Calliphora spp.) may be able to transmit larger numbers of *Campylobacter*.

Flies contaminated through fecal contact will carry heterogeneous mixtures of organisms, including any pathogens that are present within the feces, and may be able to cause a variety of human infections, including infection by different *Campylobacter* species and types. This fact partially explains the lack of a clear epidemiologic picture arising from *Campylobacter* typing work. Gastrointestinal disease caused by flies is more likely to involve pathogens with a low infectious dose (e.g., *Shigella*, *Campylobacter*, *Cryptosporidium*, *Giardia*, *Cyclospora*, *Escherichia coli* O157), and some of these could have a seasonal component related to flies. Where high fly populations and poor hygiene conditions prevail, as in disasters or famines, or where pathogens can grow within fly-contaminated food, the potential exists for transmitting pathogens with a high infectious dose (e.g., *Vibrio cholerae*, *Salmonella* spp.). The access that flies have to human and animal feces will influence the degree to which they are contaminated with different enteric pathogens.

Contamination of a range of foods by flies will result in a pattern of infection that will not be amenable to identifying specific vehicles through standard case-control, case-case, or cohort studies, unless specific objective or subjective assessments of fly numbers can be obtained. Fly monitoring will need to be undertaken. An alternative approach could use estimates of fly population numbers based on climatic conditions to compare with data on human *Campylobacter* infections. This approach has the advantage of being able to use historical climatic and disease surveillance data. The broad relationship between *Campylobacter* cases and ambient temperature has not been explained in terms of disease causation. The time taken for the larvae of *M. domestica* to develop (13) was applied to temperature data for England and Wales and has been used to show a strong relationship between *Campylobacter* cases per week and *M. domestica* larval development time for 1989 to 1999 (Figure 3). Periods when *Campylobacter* cases exceed a 7-day average of 170 cases per day occurred when *M. domestica* larval development time was <3 weeks.

The hypothesis predicts that the *Campylobacter* infection rates will be higher in persons living close to animal production and lower in urban settings because fly numbers will be lower. Some evidence from the United Kingdom (1,14) and Norway (15) supports this hypothesis. Seasonal changes in *Campylobacter* incidence that are seen around the world may result from changes in fly populations and flies' access to human and animal feces. Much emphasis on foodborne disease reduction has rightly been on kitchen hygiene, since the low infectious dose of *Campylobacter* makes cross-transmission from raw meats

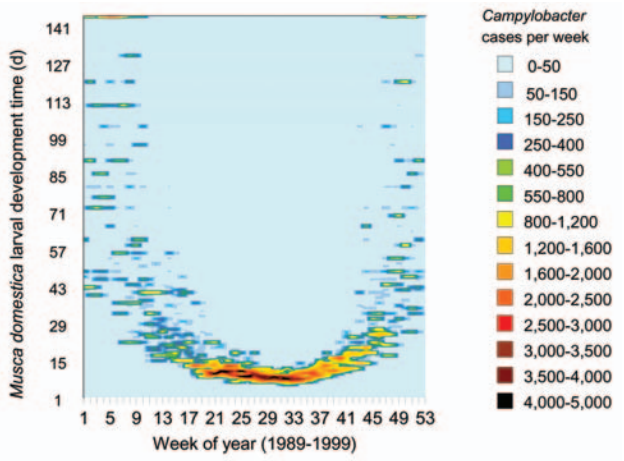

Figure 3. *Campylobacter* cases by week and *Musca domestica* larval growth times. *Campylobacter* cases per day are plotted against the minimum *M. domestica* growth times for the 14 days before the date for weeks from January 1989 to December 1999. The time taken for *M. domestica* larvae to develop was based on understood growth temperatures (145 days divided by the number of degrees above 12°C, up to an optimum of 36°C) (8). The temperatures were based on a maximum temperature in 47 temperature sampling sites across England and Wales in the 2 weeks before (online Appendix available from http://www.cdc.gov/ncidod/EID/vol11no03/04-0460_app.htm).

to ready-to-eat foods a substantial risk in domestic and catering environments. Fly transmission may be the most important source of infection in kitchen transmission routes, and establishments that sell ready-to-eat foods may be sources of *Campylobacter*, if effective fly control is not in operation. Flies may also be important in transmitting *Campylobacter* in poultry flocks (16) and between other agricultural animals.

While flies are regarded as important mechanical vectors of diarrheal disease in developing countries, control has largely concentrated on improving drinking water and sewage disposal. In the industrialized world, flies are thought to play a minor role in the transmission of human diarrheal diseases. Immediately intervening in the transmission of *Campylobacter* gastroenteritis should be possible through increased public awareness and more effective fly control.

Acknowledgments

This hypothesis arose after a lecture by Professor Sandy Cairncross at the Centers for Disease Control and Prevention, Atlanta, in the spring of 2002. I thank Fay Burgess, Radha Patel, Chris Lane, Douglas Harding, and Erol Yousef for help in preparing the data; Jim McLauchlin, Barry Evans, Chris Little, and John Edmonds for critically commenting on versions of the paper; and André Charlett for statistical support.

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Disease Risks from Foods, England and Wales, 1996–2000

Goutam K. Adak,* Sallyanne M. Meakins,* Hopi Yip,* Benjamin A. Lopman,* and Sarah J. O'Brien*

Data from population-based studies and national surveillance systems were collated and analyzed to estimate the impact of disease and risks associated with eating different foods in England and Wales. From 1996 to 2000, an estimated 1,724,315 cases of indigenous foodborne disease per year resulted in 21,997 hospitalizations and 687 deaths. The greatest impact on the healthcare sector arose from foodborne *Campylobacter* infection (160,788 primary care visits and 15,918 hospitalizations), while salmonellosis caused the most deaths (209). The most important cause of indigenous foodborne disease was contaminated chicken (398,420 cases, risk [cases/million servings] = 111, case-fatality rate [deaths/100,000 cases] = 35, deaths = 141). Red meat (beef, lamb, and pork) contributed heavily to deaths, despite lower levels of risk (287,485 cases, risk = 24, case-fatality rate = 57, deaths = 164). Reducing the impact of indigenous foodborne disease is mainly dependent on controlling the contamination of chicken.

Foodborne infection is a major cause of illness and death worldwide (1–4). Recognizing this, the World Health Organization (WHO) developed its Global Strategy for Food Safety (1). In the developing world, foodborne infection leads to the death of many children (2), and the resulting diarrheal disease can have long-term effects on children's growth as well as on their physical and cognitive development (5,6). In the industrialized world, foodborne infection causes considerable illness, heavily affecting healthcare systems (3,4).

The WHO Global Strategy for Food Safety acknowledges, "Effective control of foodborne disease must be based on evaluated information about foodborne hazards and the incidence of foodborne disease." Estimates of the contributions of specific pathogens to the overall extent of foodborne infection at a national level are available (3,4). We refined the techniques used to estimate the acute health effects and the risks associated with consuming different foods. Our analyses should inform evidence-based control strategies for foodborne infection.

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Methods

Indigenous Foodborne Disease

Indigenous foodborne disease is defined as food-related infectious gastroenteritis acquired and occurring in England and Wales. We derived pathogen-specific estimates for indigenous foodborne disease (Table 1) by using the method of Adak et al. (4) for the following 5 disease parameters: all disease, case-patients seen at a primary care setting (by general practitioners), hospitalizations, hospital occupancy, and deaths (online Appendix 1, stages A–C; available from http://www.cdc.gov/ncidod/EID/vol11no3/04-0191_app1.htm).

Foods Causing Indigenous Foodborne Disease

Outbreaks reported as foodborne, involving a single vehicle of infection and identified by epidemiologic or microbiologic investigations (N = 766, online Appendix 2; available from http://www.cdc.gov/ncidod/EID/vollll-no3/04-0191_app2.htm), were extracted from the National Surveillance Database for General Outbreaks of Infectious Intestinal Disease (GSURV) (7). Reported outbreaks in which investigators implicated either no (n = 612) or >1 (n = 234) vehicle of infection were excluded from these analyses. We also excluded outbreaks in which no pathogen was confirmed by laboratory testing (n = 113), although most of these outbreaks were suspected to be due to norovirus and were also linked to the same range of vehicles of infection. Foods were classified into broad food groups, such as poultry, and more specific food types, e.g., chicken (Table 2). A "complex foods" group was created to accommodate dishes consisting of ingredients of various food types in which the precise source of infection was not verified.

We calculated the percentage of outbreaks due to each food type for each pathogen. For disease of unknown origin, we used the percentages as determined above for disease due to all known pathogens. These percentages were applied to the pathogen-specific estimates for the mean values for all disease, visits to general practitioners, hospitalizations, hospital occupancy, and deaths for the years

Table 1. Estimated annual impact of indigenous foodborne disease by etiologic agent, England and Wales

Pathogen	Cases	General practitioner cases	Hospital		Deaths
			Cases	Days	
Bacteria					
Aeromonas spp.	0	0	0	0	0
Bacillus spp.	10,717	4,287	26	67	0
Campylobacter spp.	337,655	160,788	15,918	58,897	80
Clostridium perfringens	168,436	88,651	709	10,496	177
C. difficile cytotoxin	0	0	0	0	0
Escherichia coli O157:H7	1,026	1,026	389	2,216	23
Non–O157:H7 STEC*	114	114	43	246	3
Other *E. coli*	62,050	13,850	319	1561	6
Listeria monocytogenes	221	221	221	3,959	78
Nontyphoidal salmonellae	73,193	52,280	2,666	15,465	209
Salmonella Typhi	86	86	35	239	0
S. Paratyphi	91	91	29	181	0
Shigella spp.	308	308	7	37	0
Staphylococcus aureus	9,196	3,678	232	278	0
Vibrio cholerae O1 and O139	0	0	0	0	0
V. cholerae, other serotypes	194	97	8	30	0
Other vibrio species	291	146	4	16	2
Yersinia spp.	129,338	11,054	619	5,448	3
Parasites					
Cryptosporidium parvum	1,699	894	32	119	3
Cyclospora cayatenensis	1,026	540	3	10	0
Giardia lamblia	1,999	1,052	6	22	0
Viruses					
Adenovirus 40/41	0	0	0	0	0
Astrovirus	17,741	4,032	12	47	4
Norovirus	61,584	9,775	39	152	10
Rotavirus	8,205	1,368	42	110	4
Sapovirus	0	0	0	0	0
Unknown	839,144	106,221	637	1,785	85
Total†	1,724,315	460,560	21,997	101,382	687

*STEC, Shiga toxin–producing *Escherichia coli*.
†Totals are calculated on the basis of rounding to whole numbers.

1996–2000 to produce pathogen-specific totals by food type for each of the 5 disease parameters used to describe the annual disease impact (Tables 2 and 3, Online Appendix 1, stage D). We then calculated food-specific totals for all disease, visits to general practitioners, hospitalizations, hospital occupancy, and deaths by adding together the appropriate food-specific totals for each pathogen (Online Appendix 1, stage E).

Food-Specific Risk

The U.K. Government National Food Survey (8) collects population-based food consumption data. These data were used to calculate the number of servings of each food type consumed per resident for the period 1996–2000. These denominators were used to calculate food-specific risks, expressed as cases per million servings for all disease and hospitalizations per billion servings (Table 4, Online Appendix 1, stage F).

Quality of Evidence

Each of the above steps was classified according to whether the pathogen-specific data elements used were direct measures, extrapolations, or inferences (Table 5). This classification system permitted us to evaluate the effects of potential biases on the final estimates produced.

Results

Causes of Disease

Unknown agents accounted for 49% of all cases but only 23% of all visits to general practitioners, 3% of all hospitalizations, 2% of hospital occupancy, and 12% of all deaths (Table l). *Campylobacter* spp. had the greatest effect on healthcare provision, according to all of the parameters examined. Nontyphoidal salmonellae and *Clostridium perfringens* caused most deaths. *Listeria monocytogenes* and *Escherichia coli* O157:H7 together accounted for 15% of all deaths but <0.1% of all cases.

Disease Impact According to Food

Of the 1,724,315 estimated cases of indigenous foodborne disease in England and Wales, 67,157 (4%) were

Table 2. Estimated annual impact of indigenous foodborne disease, by food group and type, England and Wales

Food group/type	Cases (%)	Deaths (%)	Case-fatality rate*
Poultry	502,634 (29)	191 (28)	38
Chicken	398,420 (23)	141 (21)	35
Turkey	87,798 (5)	45 (7)	52
Mixed/unspecified	16,416 (1)	4 (1)	27
Eggs	103,740 (6)	46 (7)	44
Red meat	287,485 (17)	164 (24)	57
Beef	115,929 (7)	67 (10)	58
Pork	46,539 (3)	24 (4)	53
Bacon/ham	17,450 (1)	9 (1)	53
Lamb	46,239 (3)	27 (4)	59
Mixed/unspecified	61,329 (4)	36 (5)	59
Seafood	116,603 (7)	30 (4)	26
Fish	22,311 (1)	10 (2)	47
Shellfish	77,019 (4)	16 (2)	21
Mixed/unspecified	17,273 (1)	4 (1)	24
Milk	108,043 (6)	37 (5)	34
Other dairy products	8,794 (0)	5 (0)	55
Vegetable/fruit	49,642 (3)	14 (2)	29
Salad vegetables	37,496 (2)	11 (2)	28
Cooked vegetables	6,870 (0)	2 (0)	35
Fruit	5,275 (0)	1 (0)	25
Rice	26,981 (2)	5 (1)	20
Complex foods	453,237 (26)	181 (26)	40
Infected food handler	67,157 (4)	14 (2)	20
Total†	1,724,315	687	40

*Deaths/100,000 cases.
†Totals given are calculated on the basis of rounding to whole numbers.

cases in which humans were considered to be the source of infection (foods contaminated by infected food handlers; Tables 2 and 3). Subtracting these cases left 1,657,158 cases in which contaminated food was the likely source. Within this subset, most illness was attributed to eating poultry (502,634, 30%), complex foods (453,237, 27%), and red meat (287,485, 17%). Only 76,623 (5%) patients were infected by eating plant-based foods, i.e., vegetables, fruit, and rice.

Chicken consumption accounted for more disease, deaths, and healthcare usage than any other food type. Milk also exerted a considerable impact on healthcare provision. No other single food type accounted for >8% for any of the healthcare use measures. In general, the healthcare impact arising from plant-based foods was low.

The lowest case-fatality rates were associated with plant-based foods. By contrast, foods of bovine origin tended to have the highest case-fatality rates. Shellfish had the lowest case-fatality rate of all of the foods of animal origin.

Illness and Risk

Analysis by food group (Table 4) shows that vegetables and fruit had the lowest disease and hospitalization risks and poultry had the highest. Red meat accounted for more illness than seafood but was associated with a lower risk for disease (24 cases/million servings compared with 41 cases/million servings).

The lowest disease risk for a single food type was for cooked vegetables, at 0.11 cases/million servings. This risk was used to calculate disease risk ratios for the other food types. Disease risk ratios ranged from 2 for fruit to 5,869 for shellfish. Within individual food groups, large variations in disease risk ratios occurred. A disease risk ratio was not calculated for the vegetable and fruit food group because cooked vegetables contribute to the overall risk for the group.

The lowest hospitalization risk for a single food type was for cooked vegetables, 0.45 hospitalizations/billion servings. This risk was used to calculate hospitalization risk ratios for the other food types. While salad vegetables had a disease risk ratio of 53, the hospitalization risk ratio was 229. Chicken had the highest hospitalization risk ratio, 5,595. This figure is >4 times the value estimated for turkey and more than double the estimate for shellfish, both of which had higher disease risk ratios than chicken.

Discussion

To our knowledge, our study is the first to examine the impact of and risk for indigenous foodborne disease by food type. When all parameters were considered, infection due to chicken was consistently responsible for more disease, while disease linked to plant-based foods had a minor impact on the population.

Table 3. Estimated annual healthcare impact of indigenous foodborne disease, by food group and type, England and Wales

Food group/type	General practitioner cases (%)	Hospital cases (%)	Hospital days (%)
Poultry	159,433 (35)	9,952 (45)	41,645 (41)
Chicken	129,271 (28)	9,005 (41)	36,425 (36)
Turkey	23,679 (5)	360 (2)	3,001 (3)
Mixed/unspecified	6,483 (1)	587 (3)	2,219 (2)
Eggs	19,554 (4)	552 (3)	3,410 (3)
Red meat	80,805 (18)	1,231 (6)	10,935 (11)
Beef	34,981 (8)	429 (2)	4,284 (4)
Pork	11,923 (3)	219 (1)	1,685 (2)
Bacon/ham	4,470 (0)	82 (0)	632 (0)
Lamb	14,283 (3)	157 (1)	1,721 (2)
Mixed/unspecified	15,148 (3)	343 (2)	2,613 (3)
Seafood	23,998 (5)	828 (4)	3,690 (4)
Fish	4,603 (1)	112 (1)	748 (1)
Shellfish	12,861 (3)	134 (1)	752 (1)
Mixed/unspecified	6,534 (1)	582 (3)	2,190 (2)
Milk	40,755 (9)	3,681 (17)	14,176 (14)
Other dairy products	1,561 (0)	67 (0)	402 (0)
Vegetable/fruit	11,912 (3)	702 (3)	2,932 (3)
Salad vegetables	9,874 (2)	660 (3)	2,671 (3)
Cooked vegetables	1,184 (0)	27 (0)	168 (0)
Fruit	853 (0)	15 (0)	93 (0)
Rice	5,127 (1)	73 (0)	432 (0)
Complex foods	103,409 (22)	4,175 (19)	20,646 (20)
Infected food handler	14,007 (3)	736 (3)	3,113 (3)
Total*	460,560	21,997	101,382

*Totals given are calculated on the basis of rounding to whole numbers.

Our methods build on approaches to estimate the impact of foodborne diseases in the United States (3) and England and Wales (4). To minimize bias, we avoided using assumptions whenever possible. We concluded that the effects of bias on the etiologic data (Table 1) were moderate (Table 5) because we were able to estimate the incidence of disease for each agent by taking national laboratory surveillance data and applying pathogen-specific multiplication factors that had been determined through a large population-based study (9). We were also able to use direct measurements from special studies and national surveillance systems to estimate the impact of foreign travel. We avoided using expert opinion (Table 5). Techniques such as Delphi (10) are available to assimilate the judgments of expert panels to produce consensus data. However, the Delphi estimate for the incidence of salmonellosis due to the consumption of products made from chicken and eggs (10) in the United Kingdom was >3 times the incidence for all salmonellosis calculated from a national population-based incidence study (9).

The use of data from published outbreak investigations also presents difficulties. Comparing outbreak surveillance data with those from published reports demonstrates a bias that favors the publication of novel findings and exceptional events (11). Therefore, we only used contemporary data drawn from locally based surveillance systems, population-based studies, and surveys (Table 5) (4) in these analyses. Nevertheless, certain reservations apply when using outbreak surveillance data to estimate the proportion of disease due to each food type for each pathogen. Ideally, a full account should be taken of the relative pathogen-specific contributions of each food type to both sporadic and outbreak-associated disease. However, determining the proportion of cases that fall into these 2 categories for any pathogen is problematic.

For sound epidemiologic reasons, case-control studies of sporadic disease test specific hypotheses that might explain disease transmission (12–15). Sample sizes are determined to detect associations for major risk factors. Population-attributable fractions are calculable for only a small number of foods for the small number of pathogens studied with these methods. Each study delivers a snapshot of the epidemiology of disease at a point in time for a particular population. While some of the findings from these studies are generalizable, population-attributable fractions for individual foods are not because food production patterns and consumer preferences change from country to country and with time (8,16,17). Corroborative evidence to support identified associations between disease and food consumption for studies of sporadic disease is usually lacking. However, in outbreak investigations, microbiologic findings, production records, and the like lend weight to the inferences drawn from analytic epidemiology (18–20). We believe that the true impact of outbreak-associated disease has likely been greatly underestimated (21,22).

Table 4. Estimated risks associated with food groups and types, England and Wales

Food group/type	Disease risk*	Risk ratio	Hospitalization risk†	Risk ratio
Poultry	104	947	2,063	4,584
Chicken	111	1,013	2,518	5,595
Turkey	157	1,429	645	1,433
Mixed/unspecified	24	217	852	1,893
Eggs	49	448	262	583
Red meat	24	217	102	227
Beef	41	375	153	339
Pork	20	180	93	208
Bacon/ham	8	75	39	86
Lamb	38	343	128	285
Mixed/unspecified	17	157	96	214
Seafood	41	374	293	650
Fish	8	75	41	92
Shellfish	646	5,869	1,121	2,490
Mixed/unspecified	NA‡	NA	NA	NA
Milk	4	35	133	295
Other dairy products	2	17	14	32
Vegetable/fruit	1	NA	8	NA
Salad vegetables	6	53	103	229
Cooked vegetables	0	1	0	1
Fruit	0	2	1	1
Rice	11	101	30	67

*Cases/1 million servings.
†Hospitalizations/1 billion servings.
‡NA, not applicable.

Accounting for disease caused by intermittent or unpredictable food processing failures is important. For example, an estimated 224,000 people throughout the United States were infected with *Salmonella enterica* serotype Enteritidis after eating ice cream that had become contaminated as a result of a processing failure (20). However, outbreak cases were only formally recognized in Minnesota. The scale of the outbreak emerged because of an unusually detailed epidemiologic investigation. Therefore, under normal circumstances, most of those affected would have been classified as sporadic cases. This outbreak alone would have accounted for 17% of the 1.3 million cases of foodborne salmonellosis in the United States for 1994 (3). The 1996/7 FoodNet case-control study did not find an association between pasteurized ice cream and sporadic salmonellosis (12) because the study was not conducted during the narrow timeframe when the implicated product was on the market. This example is not isolated; milk-processing failures have resulted in hundreds of outbreak cases of *Campylobacter* and *E. coli* O157:H7 infections in the United Kingdom (18). While outbreaks of this type continue to be identified through routine surveillance, others likely go undetected. However, testing for associations between apparently sporadic disease and consumption of contaminated "pasteurized" milk using case-control studies is difficult for several reasons: study participants are unaware of the process history of the milk that they drink; pasteurized milk is very commonly drunk and identifying differences in exposure rates would involve extremely large sample sizes; and since the geographic and temporal distribution of cases would be expected to be heterogeneous, studies would have to extend over long periods and large areas. For these reasons, recent case-control studies of sporadic *Campylobacter* and *E. coli* O157:H7 infections in the United Kingdom failed to show associations between disease and consumption of milk (13,14,23). Similar arguments apply for the role of fruit juice or sprouts in the transmission of *E. coli* O157:H7 (24,25) or salad vegetables and *Salmonella* serotypes (26). While all of these foods have made considerable, if intermittent, contributions to the overall impact of disease in the population, their role in sporadic disease is hard to test and has seldom been demonstrated. Thus, published case-control studies of sporadic infection provide insufficient applicable data for our purposes.

By contrast, GSURV is large, comprehensive, and provides contemporary locally defined evidence-based data that takes into account the contribution of a much broader range of foods. For example, the foods most frequently associated with disease in published studies of sporadic *Campylobacter* infection (15,23), i.e., chicken, pork, red meat, and unpasteurized milk, also feature most prominently in GSURV, but GSURV also takes into account the more minor contributions of foods such as salad vegetables, fruit, and seafood. However, for certain pathogens the amount of outbreak data available is limited. The food distribution percentages for *Campylobacter* were based on 28

Table 5. Quality of evidence

Stage	Data sources	Evidence	Principal assumptions	Potential effects of bias on final estimates
All infectious intestinal disease	Population studies	Measured	Representivity of data	Moderate
Etiology	Population studies	Measured for most; inferred rarely	Accuracy and sensitivity of diagnostic methods	Moderate
Indigenous infection	National laboratory report surveillance; special studies	Measured	Completeness of reporting	Negligible
Foodborne transmission	National outbreak surveillance (GSURV)*	Measured for most; inferred rarely	Representivity of data	Major
Food attribution	GSURV	Measured	Representivity of data	Major
Presentations to primary care	Population studies	Measured	Representivity of data	Moderate
Hospitalizations	GSURV; special studies	Measured	Representivity of data	Moderate
Hospital occupancy	Hospital episode statistics	Measured	Representivity of data	Moderate
Deaths	GSURV	Measured	Representivity of data	Negligible
Food specific risks	National food survey	Measured	Representivity of data	Major

*GSURV, National Surveillance Database for General Outbreaks of Infectious Intestinal Disease.

outbreaks (Online Appendix 2). Therefore, we have exercised considerable caution in interpreting these data and have identified this area as one in which the effects of bias on the final estimates are likely to be most profound (Table 5). Nevertheless, the results are also plausible. In our analyses, chicken emerges as the most important contributor to *Campylobacter* infection. This finding is consistent with data from food and veterinary studies (27,28), evaluations of the interventions enforced after the Belgian dioxin crisis (29), and observations on the relationships between human infection and poultry operations in Iceland (30). Our estimates for impact and risk for disease linked to shell eggs is consistent with a U.S. Department of Agriculture risk assessment on *Salmonella* Enteritidis in shell eggs and egg products (31). Therefore, after taking all of these factors into account, we concluded that GSURV was the most suitable source of pathogen-specific risk exposure data.

Our analyses were based on data drawn from 766 outbreaks in which a single vehicle of infection was identified. The 612 outbreaks that were reported as foodborne but had no identified vehicle of infection were excluded from analysis. In effect, we have made the tacit assumption that distribution of foods in the subset of outbreaks in which a vehicle was identified is representative of the complete population of outbreaks. However, certain vehicles may be more likely to be implicated in outbreak investigations than others. This situation might occur if investigators tend to preferentially collect data on the types of food that are perceived as high risk or when laboratory methods vary in sensitivity according to food type. Therefore, a systematic vehicle detection bias could potentially result in our analyses underestimating the contribution and risks attributable to those foods that were rarely implicated in outbreak investigations, e.g., salad items such as sprouts, which are now being recognized as potential sources of infection (25), fruit, or background ingredients such as herbs and spices.

Eggs are used as an ingredient in a wide range of foods such as desserts, sauces, and savories (complex foods). These dishes always include other ingredients so ascribing disease-causing ingredients in the complex foods category is difficult. There are inherent difficulties in demonstrating epidemiologic association beyond the level of vehicle of infection to that of source. However, several factors (being seen by a general practitioner, hospitalization, and case-fatality rates) linked to complex foods are similar to those for eggs. Also, ≈70% of the complex foods associated with illness included eggs as an ingredient. Therefore, we suggest that eggs are probably a major source of infection for disease related to complex foods.

Eating shellfish was associated with the highest disease risk. Shellfish tends to be a luxury food, and consumption levels were low when compared with those of other food types. Although the number of cases attributed to shellfish was of the same order as beef or eggs, the level of risk was much higher. Preharvesting contamination of oysters with norovirus had a major impact in generating cases of disease. This finding presents an additional impact to that arising from the cross-contamination with *Salmonella* of ready-to-eat items such as cocktail shrimp (32).

When severity of illness data are taken into consideration, an elevated risk is associated with eating chicken. Chicken has a lower disease risk ratio than either shellfish or turkey but has a higher hospitalization risk ratio. This finding is explained by the relative prominence of *Campylobacter* and nontyphoidal salmonellae in illness attributable to chicken. Infection with these pathogens is much more likely to result in hospitalization than disease due to norovirus, which accounts for much shellfish-associated illness, or *C. perfringens*, one of the more common turkey-associated infections.

Risks associated with eating vegetables were generally low. However, risks associated with cooked vegetables were much lower than those associated with salad vegetables. This finding is mainly because cooking would normally eliminate the pathogens that can contaminate vegetables in the field, the processing plant, the market, or the kitchen through cross-contamination. However, no parallel control process exists for salad vegetables, which are generally regarded as ready to eat.

While these analyses provide data on the impact of disease attributable to different food types, considerable heterogeneity exists in the origin, production, and handling of each of these types of food. Further research is needed to examine the influence of imported foods, organic production, factory farming, and commercial catering.

We have also attempted to define the contribution of foods by infected food handlers. One of the key reasons for conducting these analyses was to provide an evidence base for developing disease control strategies. Controlling transmission of infection from infected food handlers in commercial and domestic catering requires different strategies than controlling foodborne zoonoses through the food chain. The pathogen most frequently transmitted by infected food handlers was norovirus. Given the ubiquity of norovirus infection (9,33), its extreme infectivity, and the sudden and violent onset of symptoms (34), control of transmission is difficult and more focused strategies are needed.

Our evidence-based analyses demonstrate that the most important priority in reducing the impact of indigenous foodborne disease in England and Wales is controlling infection from contaminated chicken. Chicken was associated with relatively high levels of risk and accounted for more disease, health service usage, and death than any other individual food type. Interventions introduced during the mid-1990s to control *S.* Enteritidis in the Great Britain chicken flock (35) appear to have been successful in reducing the burden of salmonellosis in England and Wales (4). These findings are consistent with analyses from Sweden (36), Denmark (37), and the United States (38), which together demonstrate that foodborne salmonellosis can be substantially reduced by implementing targeted initiatives to control *Salmonella* in domestic livestock.

The greatest challenge to protect the population from foodborne infection is to develop effective programs to control *Campylobacter* through the chicken production chain. This intervention is possible, as witnessed in Iceland, where measures at retail level and in the household were introduced to prevent *Campylobacter* transmission. Parallel declines (>70%) were subsequently observed in the carriage of *Campylobacter* in broiler flocks and in human infections (29). Finally, the data from Europe and the United States show that the largest benefits in reducing *Salmonella* and *Campylobacter* levels have come from implementing controls in farm-to-retail processing rather than in instituting them in domestic kitchens, where the estimated impacts are much smaller in scale (39), although still important.

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Rapid Identification of Emerging Pathogens: Coronavirus

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We describe a new approach for infectious disease surveillance that facilitates rapid identification of known and emerging pathogens. The process uses broad-range polymerase chain reaction (PCR) to amplify nucleic acid targets from large groupings of organisms, electrospray ionization mass spectrometry for accurate mass measurements of PCR products, and base composition signature analysis to identify organisms in a sample. We demonstrate this principle by using 14 isolates of 9 diverse *Coronavirus* spp., including the severe acute respiratory syndrome–associated coronavirus (SARS-CoV). We show that this method could identify and distinguish between SARS and other known CoV, including the human CoV 229E and OC43, individually and in a mixture of all 3 human viruses. The sensitivity of detection, measured by using titered SARS-CoV spiked into human serum, was ≈1 PFU/mL. This approach, applicable to the surveillance of bacterial, viral, fungal, or protozoal pathogens, is capable of automated analysis of >900 PCR reactions per day.

Nucleic acid tests for infectious diseases are primarily based on amplification methods that use primers and probes designed to detect specific organisms. Because prior knowledge of nucleic acid sequence information is required to develop these tests, they are not able to identify unanticipated, newly emergent, or previously unknown infectious organisms. Thus, the discovery of new infectious organisms still relies largely on culture methods and microscopy, which were as important in the recent identification of the severe acute respiratory syndrome–associated coronavirus (SARS-CoV) as they were in the discovery of HIV 2 decades ago (1–4).

Broad-range polymerase chain reaction (PCR) methods provide an alternative to single-agent tests. By amplifying gene targets conserved across groups of organisms, broad-range PCR has the potential to generate amplification products across entire genera, families, or, as with bacteria, an entire domain of life. This strategy has been successfully used with consensus 16S ribosomal RNA primers for determining bacterial diversity, both in environmental samples (5) and in natural human flora (6). Broad-range priming has also been described for detection of several viral families, including CoV (7), enteroviruses (8), retroid viruses (9), and adenoviruses (10). The drawback of this approach for epidemiologic applications is that the analysis of PCR products for mixed amplified samples requires sequencing hundreds of colonies per reaction, which is impractical to perform rapidly or on large numbers of samples. New approaches to the parallel detection of multiple infectious agents include multiplexed PCR methods (11,12) and microarray strategies (13–15). Microarray strategies are promising because undiscovered organisms might be detected by hybridization to probes designed to conserved regions of known families of bacteria and viruses.

We present an alternative approach for rapid, sensitive, and high-throughput detection of infectious organisms. We use broad-range PCR to generate amplification products from the broadest possible grouping of organisms, followed by electrospray ionization mass spectrometry and base composition analysis of the products (16,17). The base compositions of strategically selected regions of the genome are used to identify and distinguish organisms in the sample. Enhanced breadth of priming is achieved through the use of primers and probes containing 5-propynyl deoxycytidine and deoxyuridine nucleotides that offer increased affinity and base pairing selectivity (18,19). Positioning the 5-propynyl primidine-modified nucleotides at highly conserved positions enables priming at short consensus regions and significantly increases the extent to which broad groups of organisms can be amplified.

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Materials and Methods

CoV Isolates and Broad-range PCR Primer Pairs

Table 1 lists all the CoV used in this study. Multiple sequence alignments of all available CoV nucleotide sequences from GenBank were scanned to identify pairs of potential PCR priming loci. Two target regions were selected in CoV ORF-1b (annotations based on Snijder et al. [20]), 1 in RNA-dependent RNA polymerase (RdRp) and the other in Nsp14 (Table 2). 5′ propynyl-modified pyrimidine nucleotides (shown in bold) were positioned at universally conserved positions within these primers to extend the breadth of broad-range priming to allow efficient PCR from all CoV species tested.

For each primer region, a database of expected base compositions (A, G, C, and T base counts) from all known CoV sequences in GenBank was generated (data not shown) and used in the identification and classification of the test isolates. Several of the isolates used in this study did not have a genome sequence record in GenBank. Experimentally measured base compositions from these isolates were independently verified by sequencing ≈500 bp regions that flanked both target regions used in this study (GenBank accession nos. AY874541 and AY878317–AY878324).

RNA Extraction, Reverse Transcription, and PCR

RNA was isolated from 250 μL of CoV-infected cells or culture supernatant spiked with 3 μg of sheared poly-A DNA using Trizol or Trizol LS, respectively (Invitrogen

Table 1. Coronaviruses used in the study and mass spectrometry results*

					RdRp		Nsp14	
Group	CoV species	Strain	Source	Strand	Experiment determined masses (Da)	Calculated base compositions	Experiment determined masses (Da)†	Calculated base compositions
1	Canine	1-71	VR809	S	27486.514	A24 G24 C8 T32	42475.955	A33 G31 C19 T54
				AS	26936.574	A32 G8 C24 T24	42185.117	A54 G19 C31 T33
		CCV-TN449	VR2068	S	27471.510	A24 G24 C9 T31	42474.899	A34 G30 C18 T55
				AS	26952.548	A31 G9 C24 T24	42184.072	A55 G18 C30 T34
	Feline	WSU 79-1683	VR-989	S	27471.517	A24 G24 C9 T31	42490.945	A33 G31 C18 T55
				AS	26952.556	A31 G9 C24 T24	42169.118	A55 G18 C31 T33
		DF2	VR2004	S	27472.497	A23 G25 C10 T30	42450.904	A33 G30 C19 T55
				AS	26953.536	A30 G10 C25 T23	42209.081	A55 G19 C30 T33
	Human 229E	229E	VR740	S	27450.532	A25 G24 C11 T28	42462.994	A36 G30 C20 T51
				AS	26975.545	A28 G11 C24 T25	42198.061	A51 G20 C30 T36
		229E	NHRC‡	S	27450.506	A25 G24 C11 T28	42462.930	A36 G30 C20 T51
				AS	26975.512	A28 G11 C24 T25	42198.040	A51 G20 C30 T36
2	Bovine	Calf diarrheal virus	VR874	S	27358.452	A22 G22 C12 T32	42606.039	A38 G32 C15 T52
				AS	27066.586	A32 G12 C22 T22	42052.897	A52 G15 C32 T38
	Human OC43	OC43	NHRC‡	S	27328.473	A22 G22 C14 T30	42580.959	A38 G31 C15 T53
				AS	27098.562	A30 G14 C22 T22	42076.028	A53 G15 C31 T38
	Murine hepatitis virus	MHV1	VR261	S	27344.491	A21 G23 C14 T30	42602.022	A37 G34 C18 T48
				AS	27083.564	A30 G14 C23 T21	42061.016	A48 G18 C34 T37
		JHM-thermostable	VR1426	S	27344.497	A21 G23 C14 T30	42529.960	A34 G34 C21 T48
				AS	27083.571	A30 G14 C23 T21	42136.047	A48 G21 C34 T34
		MHV-A59	VR764	S	27344.503	A21 G23 C14 T30	42599.989	A34 G35 C18 T50
				AS	27083.572	A30 G14 C23 T21	42064.089	A50 G18 C35 T34
	Rat	8190	VR1410	S	27344.491	A21 G23 C14 T30	42544.967	A34 G34 C20 T49
				AS	27083.567	A30 G14 C23 T21	42120.041	A49 G20 C34 T34
3	Infectious bronchitis virus	Egg-adapted	VR22	S	27396.544	A24 G24 C14 T26	42530.984	A33 G32 C17 T55
				AS	27032.524	A26 G14 C24 T24	42129.100	A55 G17 C32 T33
4	SARS	TOR2	University of Manitoba§	S	27298.518	A27 G19 C14 T28	42519.906	A34 G33 C20 T50
				AS	27125.542	A28 G14 C19 T27	42144.026	A50 G20 C33 T34
		Urbani	CDC¶	S	27298.518	A27 G19 C14 T28	42519.906	A34 G33 C20 T50
				AS	27125.542	A28 G14 C19 T27	42144.026	A50 G20 C33 T34

*CoV, coronavirus; SARS, severe acute respiratory syndrome.
†Exact mass measurements for the sense and antisense strands of the dsDNA amplicon reported. Experimentally observed masses were within ±1ppm of expected masses, based on sequence data for each of the amplified DNA. Sense and antisense strand base compositions reported.
‡Clinical isolate obtained from Kathryn Holmes, University of Colorado, via Kevin Russell, Naval Health Research Center, San Diego.
§Obtained from Heinz Feldmann, University of Manitoba.
¶Obtained from Dean Erdman, Centers for Disease Control and Prevention.

Table 2. PCR primer pairs used in this study*

Primer name	Gene name	Product name	Genome coordinates	Orientation	Product length (bp)	Sequence (5′ to >3′)
RdRp primer	ORF 1b	Nsp12-pp1ab (RdRp)	15146–15164	Sense	88	TAAG**TT**T**T**ATGGCGGCTGG
			15213–15233	Antisense		TTTAGGATAGT**CCC**AACCCAT
Nsp14 primer	ORF 1b	Nsp14-pp1ab (nuclease ExoN homolog)	19113–19138	Sense	137	TGTTTG**TTTT**GGAATTGTAATGTTGA
			19225–19249	Antisense		TGGAATGCATGC**TT**A**TT**AACATACA

*All coordinates are based on SARS TOR2 genome (GenBank accession no. NC_004718.3). 5′ propynyl-modified pyrimidine nucleotides are shown in **bold**. Each primer was designed to include a thymidine (T) nucleotide on the 5′ end to minimize addition of nontemplated adenosine (A) during polymerase chain reaction (PCR) (data not shown). RdRp, RNA-dependent RNA polymerase.

Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription was performed by mixing 10 μL of the purified RNA with 5 μL of water treated with diethyl pyrocarbonate (DEPC, Sigma-Aldrich Co., St. Louis, MO, USA) containing 500 ng random primers, 1 μg of sheared poly-A DNA, and 10 U SUPERaseIn (Ambion Inc., Woodlands, TX, USA). The mixture was heated to 60°C for 5 min and then cooled to 4°C. Following the annealing of the random primers to the RNA, 20 μL of first-strand reaction mix consisting of 2x first-strand buffer (Invitrogen Inc.), 10 mmol/L DTT, 500 μmol/L deoxynucleoside triphosphates (dNTPs), and 7.5 U SuperScript II was added to the RNA primer mixture. The RNA was reversed transcribed for 45 min at 45°C. Various dilutions of the reverse transcription reaction mixes were used directly in the PCR reactions.

All PCR reactions were performed in 50 μL with 96-well microtiter plates and M.J. Dyad thermocyclers (MJ Research, Waltham, MA, USA). The PCR reaction buffer consisted of 4 U Amplitaq Gold (Applied Biosystems, Foster City, CA USA), 1x buffer II (Applied Biosystems, Foster City, CA, USA), 2.0 mmol/L $MgCl_2$, 0.4 mol/L betaine, 800 μmol/L dNTP mix, and 250 nmol/L propyne-containing PCR primers. The following PCR conditions were used to amplify CoV sequences: 95°C for 10 min followed by 50 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s. After PCR, the amplified products were desalted before analysis by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) by methods described previously (21). A small oligonucleotide SH2 (CGTGCATGGCGG, Synthetic Genetics, San Diego, CA, USA) was added as an internal mass standard (22); the final concentration of SH2 was 50 nmol/L.

Mass Spectrometry and Signal Processing

The mass spectrometer used in this work is based on a Bruker Daltonics (Billerica, MA, USA) Apex II 70e ESI-FTICR-MS that used an actively shielded 7 Tesla superconducting magnet. All aspects of pulse sequence control and data acquisition were performed on a 1.1 GHz Pentium II data station running Bruker's Xmass software (Bruker Daltonics). Inputs to the signal processor are the raw mass spectra for each of the parallel PCR reactions used to analyze a single sample. The ICR-2LS software package (23) was used to deconvolute the mass spectra and calculate the mass of the monoisotopic species using an "averagine" fitting routine (24) modified for DNA (Drader et al., unpub. data). Using this approach, monoisotopic molecular weights were calculated. The spectral signals were algorithmically processed to yield base composition data as described previously (25). The amplitudes of the spectra are calibrated to indicate the number of molecules detected in the mass spectrometer versus m/z and the m/z values are corrected by using internal mass standards. The algorithm computes the organism's identity and abundances consistent with observations over all the PCR reactions run on the input sample.

Results and Discussion

Detection of Individual CoV Isolates

For broad-range detection of all CoV, the 2 PCR primer target regions shown in Table 2 were used against each virus listed in Table 1. Resultant products were desalted and analyzed by FTICR-MS by methods described previously (21). The spectral signals were algorithmically processed to yield base composition data. Figure 1 shows a schematic representation of electrospray ionization, strand separation, and the actual charge state distributions of the separated sense and antisense strands of the PCR products from the RdRp primer pair for SARS-CoV. Due to the accuracy of FTICR-MS (mass measurement error ± 1 ppm), all detected masses could be unambiguously converted to the base compositions of sense and antisense strands (25).

One of the limitations of all molecular methods for detecting pathogens, including the one described here, is that unexpected variations in PCR primer target sequences in unknown species can lead to missed detection. To minimize this possibility, the primers designed in this study were selected on the basis of highly conserved regions identified by multiple sequence alignments of all previously known CoV species sequences. Further, we chose 2 amplification targets for redundant detection of the CoV and to have increased resolution to distinguish the

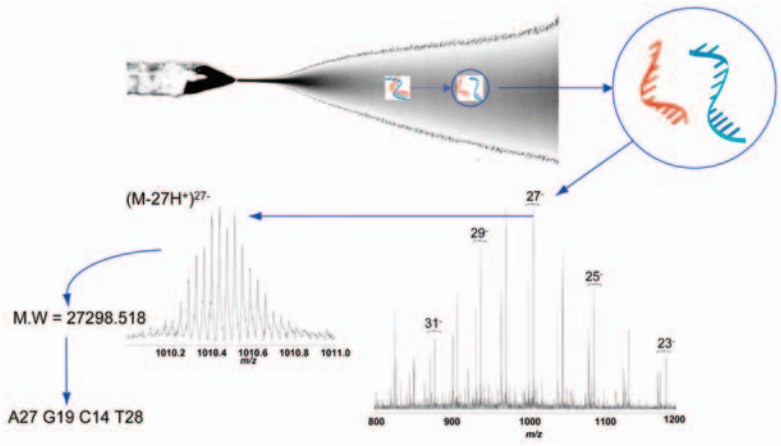

Figure 1. Electrospray ionization Fourier transfer ion cyclotron resonance (ESI-FTICR) mass spectrum from the polymerase chain reaction (PCR) amplicons from the severe acute respiratory syndrome (SARS)-associated coronavirus obtained with the propynylated RNA-dependent RNA polymerase primer pairs. The electrospray ionization conditions separate the sense and antisense strands of the PCR products. Multiple charge states are observed across the m/z range shown. The inset shows an expanded view of the isotope envelope of the $(M-27H+)^{27-}$ species. As enumerated in Table 1, the derived molecular masses for the amplicon strands are 27298.518 (±0.03) Da and 27125.542 (±0.03) Da, corresponding to an unambiguous base composition of A27G19C14T28/A28G14C19T27 for the double-stranded amplicon, the composition expected for the SARS isolate.

different viral species. Both primer pairs were tested against multiple isolates from the 3 previously known CoV species groups and from SARS-CoV isolates.

The results from analysis of 14 CoV isolates are shown in Table 1. For both target regions, the measured signals agreed with compositions expected from the known CoV sequences in GenBank. Several of the isolates used in this study did not have a genome sequence record in GenBank. Nevertheless, we were able to amplify all test viruses and experimentally determine their base compositions. These experimentally determined base compositions were confirmed by sequencing (data not shown). Thus the strategy described here enables identification of organisms without the need for prior knowledge of the sequence, provided that the broad range primers do not fail to amplify the target because of excessive numbers of mismatches.

Multiple CoV Isolates in Mixture

To demonstrate the potential to detect multiple viruses in the same sample, as might occur during a coinfection, we pooled the viral extracts from 3 human CoV, HCoV-229E, HCoV-OC43, and SARS-CoV, and analyzed the mixture. Signals from all 3 viruses were clearly detected and resolved in the mass spectra (Figure 2), which demonstrated that coinfections of >1 CoV species could be identified. We have previously determined that the system can reliably detect multiple species in ratios of ≈1:1,000, while varying input loads from 10 to 10,000 organisms (data not shown).

Sensitivity

To determine sensitivity in a clinical sample, viable, titered SARS-CoV was added to human serum and analyzed in 2 ways. In the first, RNA was isolated from serum containing 2 concentrations of the virus (1.7×10^5 and 170 PFU/mL), reverse transcribed to cDNA with random primers, and serially diluted (10-fold), before PCR amplification with both RdRp and Nsp14 primer sets. By using this approach, the assay was sensitive to $\approx 10^{-2}$ PFU per PCR reaction (≈1.7 PFU/mL serum). We estimated the number of reverse-transcribed SARS genomes by competitive, quantitative PCR with a nucleic acid internal

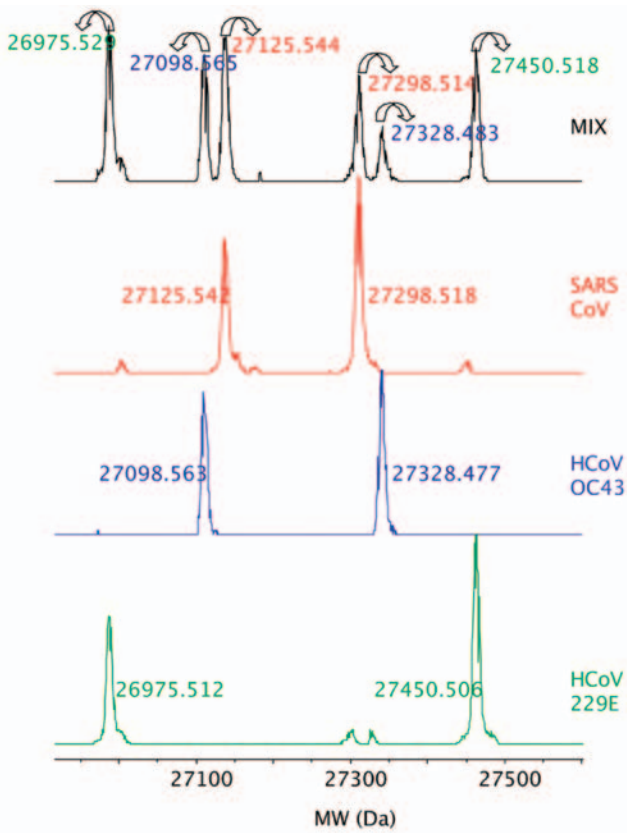

Figure 2. Detection of 3 human coronaviruses (CoV) in a mixture. The deconvoluted (neutral mass) mass spectra obtained for the RNA-dependent RNA polymerase primer for the 3 human CoV, HCoV-229E, HCoV-OC43, and severe acute respiratory syndrome-associated CoV, which were tested individually and in a mixture, are shown. Forward and reverse amplicons are shown with the measured monoisotopic masses for each strand. Colors of the monoisotopic masses for the mixed spectra correspond to the individual viral species.

standard (data not shown). Analysis of ratios of mass spectral peak heights of titrations of the internal standard and the SARS cDNA showed that ≈300 reverse-transcribed viral genomes were present per PFU, similar to the ratio of viral genome copies per PFU previously reported for RNA viruses (26). By using this estimate, PCR primers were sensitive to 3 genome equivalents per PCR reaction, which is consistent with previously reported detection limits for optimized SARS-specific primers (27,28). In the second method, we spiked 10-fold dilutions of the SARS virus into serum before RT-PCR and could reliably detect 1 PFU (≈300 genomes) per PCR reaction or 170 PFU (5.1×10^4 genomes) per mL serum. The discrepancy between the detection sensitivities in the 2 experimental protocols described above suggests that losses were associated with RNA extraction and reverse transcription when very little virus was present (<300 genome copies) in the starting sample in serum. This finding is consistent with results for direct measurement of RNA viruses from patient samples (26). Therefore, in a practical experimental analysis of a tissue sample, the limit of sensitivity was ≈1 PFU per PCR reaction.

RNA Virus Classification with Base Compositions

We have described a novel approach using base composition analysis for viral identification. However, since RNA virus nucleotide sequences mutate over time within the functional constraints allowed by selection pressure (29), the utility of this method to correctly classify RNA viruses depends on the resolution needed for a particular application. We considered 2 specific applications. The first was to distinguish SARS-CoV from other species of CoV that infect humans, namely HCoV-OC43 and HCoV-229E. The second application was the utility of the technique for exploration of animal reservoirs for the discovery of SARS-related CoV species.

To quantitatively analyze the resolving power of base compositions, we mathematically modeled base composition variations using known sequences of multiple isolates of hepatitis C virus (HCV) in GenBank (H. Levene et al., unpub. data). HCV sequence-derived mutation probabilities were used to estimate the extent of base composition variations for CoV species. Figure 3 shows a plot of the base compositions for the RdRp target region for the 3 CoV known to infect humans. Δ_{bc} represents the net changes in composition required for strain variants of 229E or OC43 to be misidentified as SARS, and Δ_m the probability of occurrence of these changes. The cumulative probability of misclassifying either 229E or OC43 as SARS by using base composition measurements from both target regions was low (Δ_m >10), even allowing for unseen variations in those 2 viruses. Thus, for use in human clinical diagnostics, base composition analysis of the 2 target regions described here would provide corroborative information and accurate species identification of CoV infections.

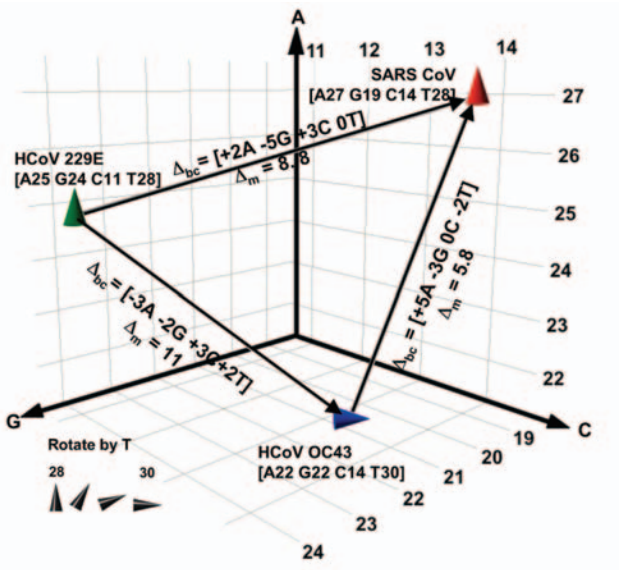

Figure 3. Spatial representation of base compositions for the 3 coronavirus (CoV) species known to infect humans. Severe acute respiratory syndrome (SARS), HCoV-OC43, and HCoV-229E base compositions in the region amplified by RNA-dependent RNA polymerase primers (Table 1) are plotted on the A, G, and C axes. T counts are shown by the tilt of the symbol. Within a species, all known isolates of each virus (37 isolates for SARS, 4 for HCoV-229E, and 2 for OC43) had identical sequences in this region. Δ_{bc} represents the number of changes in the A, G, C, and T bases needed for 1 species to be misidentified as another in the direction of the arrow. Δ_m represents the pairwise mutation distance between 2 species, or the cumulative probability of Δ_{bc} occurring.

To determine the utility of base composition analysis in the search for animal CoV species, we calculated the cumulative mutation distances for both target regions for all known CoV and plotted groups where all members fall within certain probability thresholds, as shown in Figure 4. A series of nested ovals represents subgroupings of species, where the maximal distance between known members of a subgroup is represented by the Δ_m next to the oval. By using the above classification metric, SARS-CoV would be considered the first member of a new group of CoV, not a member of the core group 2 cluster, although it would be placed closest to group 2 (Δ_m < 10.2). These findings are similar to those recently described by Snijder et al., who used sequence data from the replicase genes (5,487 bp) in ORF1b and suggested that the SARS-CoV was most closely related to and possibly an early split-off from group 2 CoV (20). However, substantial space exists around SARS-CoV where as yet undiscovered SARS-CoV could populate a subgroup without being confused with the group 2 or other CoV.

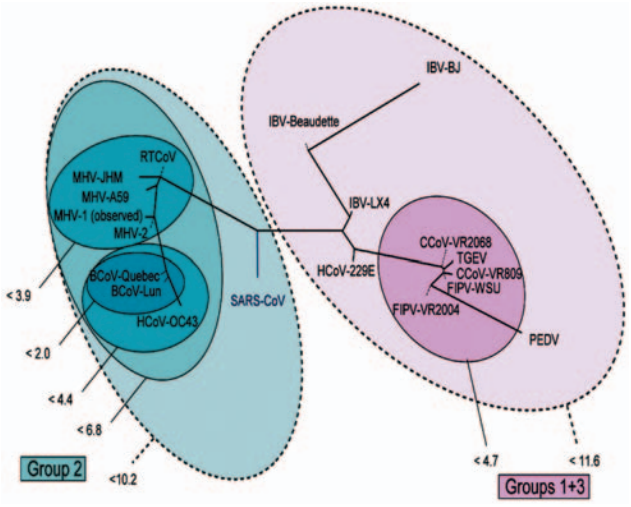

Figure 4. Representation of the mutational distances calculated for the 2 selected primer sets overlaid on the coronavirus phylogenetic tree. Each oval represents grouping of members contained within it; numbers next to the group indicate the maximum distance between any 2 members of the group. Distances are computed as the base 10 logarithm of the geometric average of the pair-wise probabilities for any given pair of base compositions.

Conclusion

The strategy we describe allows rapid identification of new viral species members of previously characterized viral families, without the need for prior knowledge of their sequence, through use of integrated electrospray ionization mass spectrometry and base composition analysis of broad-range PCR products. Broad-range PCR reactions are capable of producing products from groups of organisms, rather than single species, and the information content of each PCR reaction is potentially very high. Further, in many cases, including the SARS-CoV detection described in this article, priming across broadly conserved regions provides adequate species detection and taxonomic resolution. In cases where additional subspecies level classification becomes important, broad primers can be followed up with more species-specific primers that can detect even single nucleotide changes (SNPs) or alternatively, larger regions of the identified species can be analyzed by sequencing. Despite the advances in high throughput sequencing, however, it is impractical as a front-end detector in a routine survey and detection setting. The mass spectrometer is capable of analyzing complex PCR products at a rate of ≈1 minute per sample. Because the process is performed in an automated, microtiter plate format, large numbers of samples can be examined (>900 PCR reactions/day/instrument), which makes this process practical for large-scale analysis of clinical or environmental surveillance samples in public health laboratory settings. The current generations of ESI mass spectrometers used in the detector cost approximately U.S.$150,000 and can be operated 24 hours per day by trained technicians. Tools for analyzing mass spectrometry data are widely available and are described in detail elsewhere (23–25). A comparable alternative to the methods described here are microarrays, which can also provide broad range detection.

This approach can be extended to other viral, bacterial, fungal, or protozoal pathogen groups and is a powerful new paradigm for timely identification of previously unknown organisms that cause disease in humans or animals and for monitoring the progress of epidemics.

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Dr. Sampath is the director of Genomics and Computational Biology at Ibis Therapeutics, a division of Isis Pharmaceuticals, Inc. He leads Ibis' genomics efforts in microbial detection and diagnosis. His research interests include pathogen discovery, epidemiologic surveillance, and clinical infectious diagnostics.

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Antimicrobial Drug Prescribing for Pneumonia in Ambulatory Care

Conan MacDougall,* B. Joseph Guglielmo,* Judy Maselli,† and Ralph Gonzales†

To determine patterns and predictors of antimicrobial drug use for outpatients with community-acquired pneumonia, we examined office visit and pharmacy claims data of 4 large third-party payer organizations from 2000 to 2002. After patients with coexisting conditions were excluded, 4,538 patients were studied. Despite lack of coexisting conditions, fluoroquinolone use was commonly observed and increased significantly ($p < 0.001$) from 2000 to 2002 (24%–39%), while macrolide use decreased (55%–44%). Increased age correlated with increased fluoroquinolone use: 18–44 years (22%), 45–64 years (33%), and >65 years (40%) ($p < 0.001$). Increased use of fluoroquinolones occurred in healthy young and old patients alike, which suggests a lack of selectivity in reserving fluoroquinolones for higher risk patients. Clear and consistent guidelines are needed to address the role of fluoroquinolones in treatment of outpatient community-acquired pneumonia.

Community-acquired pneumonia (CAP) is a leading cause of death due to infection in the United States and a primary indication for antimicrobial drug use in inpatient and outpatient settings. The fluoroquinolone class of antimicrobial agents has become increasingly popular for the management of CAP because of coverage of common CAP pathogens, toleration by patients, and excellent oral absorption (1). "Respiratory" fluoroquinolones, such as levofloxacin, gatifloxacin, moxifloxacin, and gemifloxacin, have activity against most strains of drug-resistant *Streptococcus pneumoniae* (DRSP) (2). Concerns for infection due to DRSP may drive fluoroquinolone use because providers fear that traditional CAP regimens will fail (3,4). However, fluoroquinolone resistance, while generally low, appears to be increasing in *S. pneumoniae* (5–8). Therapeutic failures have been reported in patients infected with fluoroquinolone-resistant organisms treated with levofloxacin (9,10). Although data from community settings are lacking, resistance to fluoroquinolones is also increasing among gram-negative organisms in hospital settings (11,12). Increased use of fluoroquinolones for outpatient respiratory tract infections may lead to increased resistance rates among community-acquired gram-negative organisms. Fluoroquinolones may promote colonization and infection with methicillin-resistant *Staphylococcus aureus* (MRSA) (13–15). Community-acquired MRSA infections, once rare, have increased in frequency (16,17).

Clinicians are faced with the dilemma of attempting to limit broad-spectrum antimicrobial drug use on a population level while trying to maximize therapeutic success in individual patients (18). Routine prescribing of fluoroquinolones for CAP may limit the possibility of therapeutic failure due to drug-resistant organisms but may compromise the future effectiveness of this class of drugs. Practice guidelines available for management of CAP from various professional societies provide mixed messages on the use of fluoroquinolones, particularly for patients eligible for outpatient treatment. The American Thoracic Society recommends reserving fluoroquinolones for outpatients with cardiopulmonary disease or other modifying factors, advocating a macrolide or doxycycline for patients without such coexisting conditions (19). The Drug-Resistant *Streptococcus pneumoniae* Working Group recommends reserving fluoroquinolones for patients whose treatment has failed on other regimens or those with documented infections due to DRSP (20). Previous guidelines of the Infectious Diseases Society of America considered macrolides, doxycycline, or fluoroquinolones as equivalent options for treating outpatients, with the suggestion that older patients and those with underlying disease have a stronger indication for fluoroquinolone therapy (21). A recent update of these guidelines categorizes patients according to whether they recently received antimicrobial drugs and presence of underlying conditions: patients without underlying illnesses and no recent antimicrobial drug therapy should receive a macrolide or doxycycline,

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whereas fluoroquinolones are an option for the other groups (22). Such extensive subclassification and conflict among guidelines may pose difficulties for clinicians practicing in busy outpatient settings (23). Without clear and consistent guidelines, clinicians may base their therapeutic decisions on tradition, the practice of colleagues, or advice from pharmaceutical sales representatives, rather than the best evidence.

We examined a database of office visit and pharmacy claims from 4 large managed-care organizations in Colorado. Our objective was to determine the patterns of antimicrobial drug prescribing, especially fluoroquinolone use, in a group of outpatients with CAP without serious underlying conditions.

Methods

We used administrative claims data from 4 healthcare organizations in Colorado. Identifiable patient information was removed before the information was provided, and patients were assigned a unique identification number for the purpose of data manipulation. Information contained in the database included the patient's date of birth, sex, visit date, health plan, provider identification and specialty, up to 3 International Classification of Diseases, 9th edition, Clinical Modification (ICD-9-CM) diagnostic codes, and drugs prescribed during the visit. Data were available from March 1, 2000, to March 1, 2003. The study received approval from the institutional review board of the University of California, San Francisco.

Our criteria for inclusion in the study were age ≥18 years, primary diagnosis of CAP (based on ICD-9-CM codes 481, 482, 483, 485, and 486), and prescription of an antimicrobial agent associated with the visit. As serious coexisting conditions may justify the use of fluoroquinolones according to some guidelines, we excluded those patients with coexisting conditions to examine prescribing patterns in an otherwise healthy population. Specifically, we excluded patients who had a second or third diagnosis of chronic obstructive pulmonary disease, congestive heart failure, diabetes, lung cancer, renal failure, atrial fibrillation, respiratory failure, pleural effusion, Parkinson disease, multiple sclerosis, and asphyxia. We excluded patients who had sought treatment for an acute respiratory tract infection (bronchitis, pharyngitis, otitis media, sinusitis, and upper respiratory tract infection) or urinary tract infection during the 4 weeks before the visit for pneumonia. Consequently, we excluded results for the first month of the study (March 2000) since data regarding prior visits were unavailable for that group. Finally, we limited the dataset to 1 pneumonia visit per patient to reduce the likelihood of including patients whose previous therapy had been unsuccessful. We categorized patients by age into 3 strata: 18–44 years, 45–64 years, and ≥65 years. We also categorized patients according to health plan (1 through 4). For categorization by year, we used March 1 as the start and end date (e.g., year 2001 was March 1, 2001, to March 1, 2002).

Antimicrobial agents were identified by using National Drug Codes. Antimicrobial drugs were assigned to one of the following categories: tetracyclines (doxycycline, tetracycline), macrolides (azithromycin, clarithromycin, erythromycin), fluoroquinolones (ciprofloxacin, levofloxacin, ofloxacin, moxifloxacin, gatifloxacin), aminopenicillins (amoxicillin, amoxicillin/clavulanate), cephalosporins (primarily cefuroxime and cefprozil), sulfonamides (trimethoprim-sulfamethoxazole), and other. Only 1 antimicrobial agent was recorded per patient visit. We used the visit date to analyze data by year of prescription (2000, 2001, or 2002).

Comparisons between proportions in groups were performed by using the Mantel-Haenszel χ^2 test for trend, with a significance level of 0.05. Logistic regression analysis was performed on the group of patients without coexisting conditions. The outcome was prescription of a fluoroquinolone. Variables included in the model were year of treatment, age (by category), patient sex, and health plan; and interactions between year of treatment with age, patient sex, and health plan were also tested. All analyses were performed by using SAS, version 8.2 (SAS, Cary, NC, USA).

Results

Inclusion criteria were met by 5,001 patients in our database. Of our sample, 463 patients were excluded because of ≥1 conditions. Excluded patients were more likely to be in 1 of the older age categories ($p < 0.001$). Characteristics of the final group of study patients (N = 4,538) without coexisting conditions are presented in Table 1.

Data from the 4,538 patients were analyzed to determine use of antimicrobial agents. Figure 1 shows changes in antimicrobial prescribing from 2000 to 2002 for all patients. Use of fluoroquinolones increased from 25% of all prescriptions in 2000 to 39% in 2002 ($p < 0.001$). Macrolide use decreased from 55% in 2000 to 44% in 2002 ($p < 0.001$). Aminopenicillin use (≈9%) did not change significantly, but cephalosporin use decreased by almost half (7% to 4%). Use of tetracyclines, sulfa drugs, and other antimicrobial agents was minimal. Of note, fluoroquinolone use in the 18- to 44-year age group more than doubled from 2000 to 2002 (14% to 30%)

Figure 2 shows the distribution of antimicrobial prescribing between the 3 age categories for all years in the study. Use of fluoroquinolones differed significantly between groups ($p < 0.001$); patients in the oldest group received more fluoroquinolones (40%) than those in the

Table 1. Pneumonia patients with no serious underlying conditions treated with antimicrobial drugs

Characteristic	No. of patients (%) (N = 4,538)
Age (y)	
18–44	1,581 (35)
45–64	1,610 (35)
≥65	1,347 (30)
Sex	
Male	2,103 (46)
Female	2,435 (54)
Study year	
2000	1,827 (40)
2001	1,328 (30)
2002	1,383 (30)

45- to 64- (33%) and 18- to 44-year groups (22%). Macrolide use was highest in persons 18–44 years (61%), followed by those 44–65 years (49%) and persons >65 years (37%) ($p < 0.001$). Differences in prescribing of aminopenicillins, cephalosporins, and tetracyclines also were observed across age groups. Among fluoroquinolones prescribed over all years of the study, 74% were for levofloxacin; among all macrolides, 72% were for azithromycin.

Results of logistic regression analysis to determine predictors of fluoroquinolone use are presented in Table 2. When the 45- to 64-year age group was used as a referent, patients in the older group were more likely, and those in the younger group less likely, to receive fluoroquinolones. Likelihood of fluoroquinolone use also differed significantly by health plan: 1 health plan was less likely to prescribe fluoroquinolones. The interaction between year of prescription and age category was of marginal significance ($p = 0.0855$), which suggests that increases in fluoroquinolone use were similar across age groups. However, a significant interaction occurred between year of prescription and health plan ($p < 0.0001$), which indicates that changes in fluoroquinolone prescription rates differed by health plan.

Discussion

We found significant changes in the pattern of antimicrobial prescribing for the outpatient management of patients with CAP from 2000 to 2002. Fluoroquinolone use increased by >50%, from 25% to 39% of all prescriptions. This increase was at the expense of the macrolide class of antimicrobial drugs, the use of which declined 20% during the study period. Use of β-lactam drugs and doxycycline was low throughout the study period. Although fluoroquinolones were prescribed for older patients more frequently than for younger patients, the growth in fluoroquinolone use over the study was similar across all age groups.

Few published studies have documented trends in use of fluoroquinolones for the management of respiratory tract infections in the community. No study has specifically addressed the use of fluoroquinolones for pneumonia. In a national sample of U.S. office-based physicians from 1992 to 2000, a 78% increase in the use of fluoroquinolones across all indications was documented (24). Chen et al. noted a 5-fold increase in community fluoroquinolone prescribing for all indications in Canada from 1988 to 1997 (5). In a survey of U.S. community-based prescribing from 1991 to 1999, use of fluoroquinolones for upper respiratory tract infections in adults increased from <1% to 13% of antimicrobial drug prescriptions (25).

A number of factors may contribute to the observed increased fluoroquinolone use, including the convenience of once-daily dosing, reliable spectrum of activity against CAP pathogens, and relatively low toxicity. In addition, changes in health plan formularies, pharmaceutical advertising, and concerns about resistance to standard therapies may have influenced these prescribing trends.

We found age-specific differences among patients for whom fluoroquinolones were prescribed. Patients in the older age groups were more likely to receive fluoroquinolones than those in persons 18–44 years, even after those with underlying conditions were excluded. Age itself may be an important underlying condition as well as a risk factor for drug-resistant *S. pneumoniae* (26). Thus, clinicians may choose to use drugs they perceive to be more potent to reduce the risk for treatment failure in this population. In a survey of fluoroquinolone use from the National Hospital Ambulatory Medical Care Survey from 1993 to 1998, persons ≥65 years of age had the highest use of fluoroquinolones (12.4 prescriptions/100 persons/year, compared to a mean of 4.6/100 persons/year for all groups) (27).

A recent study evaluated outpatient fluoroquinolone use for CAP in 6 emergency departments in Canada (28). The most commonly prescribed antimicrobial drugs were macrolides (53%) and fluoroquinolones (32%; 98% of these prescriptions were for levofloxacin). Similar to our

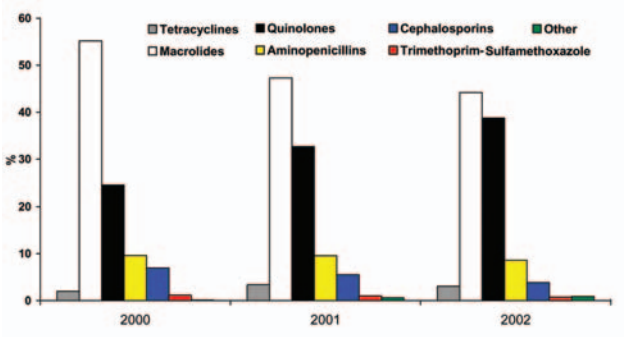

Figure 1. Antimicrobial drug treatment of outpatient pneumonia by year. Percentage of all study patients receiving a particular class of antimicrobial drug for an episode of community-acquired pneumonia for each year of the study, across all age groups.

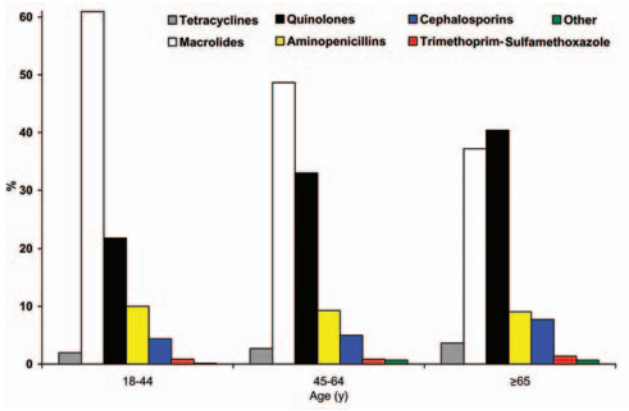

Figure 2. Antimicrobial drug treatment of outpatient pneumonia by age. Percentage of all study patients receiving a particular class of antimicrobial drug for an episode of community-acquired pneumonia by age group, across all study years.

results in this study, increasing age was a significant predictor of levofloxacin use; 25% of patients 16–40 years of age received levofloxacin, compared to 28% of those 41–64 years and 47% of those ≥65 years. We also determined appropriateness of levofloxacin use on the basis of Canadian guidelines. When we used an interpretation of these guidelines in which fluoroquinolone use in all patients with chronic obstructive pulmonary disease or recent antimicrobial drug use was considered appropriate, 51% of levofloxacin prescriptions were considered inappropriate.

We did not have adequate information to fully assess appropriateness of therapy, and the lack of agreement among currently published U.S. guidelines prevents establishing a universal benchmark. However, in the absence of coexisting conditions, the prognosis for patients <45 years of age with CAP is favorable. The American Thoracic Society, as well as CDC, recommends use of either a macrolide or doxycycline in this group (current Infectious Diseases Society of America guidelines would use a fluoroquinolone if the patient had recently used an antimicrobial agent). Although fewer fluoroquinolones were used in the younger age groups, substantial use was noted in all age groups, and increases in the rate of fluoroquinolone use were independent of age group. These findings suggest that those prescribing antimicrobial drugs may be increasingly using fluoroquinolones as a "one-size-fits-all" regimen without accounting for differences due to age and other risk factors. Clarifying the appropriateness of fluoroquinolone use on the basis of patient characteristics should be a goal of future joint guidelines for CAP. We also observed that patterns of fluoroquinolone prescribing varied across health plans. Although this finding probably reflects differences in each health plan's pharmacy benefits management programs, our study did not collect information to help further characterize this finding. Further investigation of the differences between these programs is warranted because these studies might identify or inform effective interventions to reduce excess fluoroquinolone prescribing.

Table 2. Logistic regression analysis of factors predicting fluoroquinolone use

N = 4,538	Odds ratio (95% CI)* for receipt of fluoroquinolone
Age (y)	
18–44	0.56 (0.48–0.66)
45–64	1
≥65	1.62 (1.38–1.91)
Year (later year)	1.40 (1.29–1.51)
Healthcare plan	
1	1
2	0.97 (0.77–1.21)
3	0.80 (0.66–0.97)
4	1.23 (0.93–1.62)

*CI, confidence interval.

Our study has a number of limitations. The cohort consists of patients seen in a particular geographic area. Antimicrobial drug use may vary significantly by region. The dataset consisted of patients enrolled in managed-care organization healthcare plans. Prescribing patterns for these patients may differ from those for other populations. The database captured only the first 3 ICD-9 codes associated with a patient visit, which we used to exclude patients with notable underlying conditions. Some coexisting conditions may have been coded as fourth or fifth diagnoses, or not coded at all, in which case our sample would not have excluded all patients with these coexisting conditions as we planned. We did not measure outcomes, such as return visits or deaths, to assess the effectiveness of the prescribed regimens. We were not able to exclude all patients with recent antimicrobial drug therapy. However, we did exclude patients with a recent visit for an acute respiratory infection or urinary tract infection, many of whom would have received an antimicrobial drug. We also did not have data on local antimicrobial resistance patterns to assess appropriateness of empiric therapy or changes in resistance over the course of the study.

In summary, our study demonstrates an increase in fluoroquinolone use to treat outpatient CAP among a cohort without complicating coexisting conditions. This increasing use of fluoroquinolones, especially in otherwise healthy patients whose infections are not likely to fail to respond to treatment or whose infections are not likely to be caused by resistant organisms, may threaten the future effectiveness of this drug class. Harmonization of expert guidelines regarding the role of fluoroquinolones in the outpatient management of CAP is recommended.

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Ebola Virus Antibody Prevalence in Dogs and Human Risk

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During the 2001–2002 outbreak in Gabon, we observed that several dogs were highly exposed to Ebola virus by eating infected dead animals. To examine whether these animals became infected with Ebola virus, we sampled 439 dogs and screened them by Ebola virus–specific immunoglobulin (Ig) G assay, antigen detection, and viral polymerase chain reaction amplification. Seven (8.9%) of 79 samples from the 2 main towns, 15 (15.2%) of 99 samples from Mekambo, and 40 (25.2%) of 159 samples from villages in the Ebola virus–epidemic area had detectable Ebola virus–IgG, compared to only 2 (2%) of 102 samples from France. Among dogs from villages with both infected animal carcasses and human cases, seroprevalence was 31.8%. A significant positive direct association existed between seroprevalence and the distances to the Ebola virus–epidemic area. This study suggests that dogs can be infected by Ebola virus and that the putative infection is asymptomatic.

Ebola virus causes fulminant hemorrhagic fever in both humans and nonhuman primates (1,2). The Zaire Ebola virus species (Ebola virus–Z), 1 of the 4 known species of Ebola virus, occurs in central Africa and kills 80% of infected persons within a few days (3,4). Ebola hemorrhagic fever occurs in rare epidemics, in which the index patient is often infected by an animal source, which indicates that Ebola hemorrhagic fever is a zoonotic disease (5). During the past 3 years, 5 Ebola outbreaks due to Ebola virus-Z have struck the region of central Africa, including Gabon and Republic of Congo, and caused 334 deaths among the 428 reported human cases (5). In previous studies, we showed that each extended outbreak could be subdivided into several independent epidemic clusters or chains of transmission, which resulted from close contact with an Ebola virus-Z–infected animal carcass. Epidemiologic observations and genetic analyses identified gorilla, chimpanzee, and duiker carcasses as the main sources of human cases (5). Once the species barrier has been crossed between animals and humans, the disease spreads among humans by direct physical contact.

Some human cases in the recent outbreak in the Gabon/Republic of Congo region did not have a documented source of exposure to Ebola hemorrhagic fever. Similarly, 14 (4.9%) of the 284 cases in the 1976 Sudan outbreak (6) and 55 (17.4%) of the 316 cases during the 1995 outbreak in Kikwit (7), Democratic Republic of Congo (DRC, former Zaire), had no direct physical contact with an infected person or known infected carcass. These observations point to other routes of transmission (e.g., human-human respiratory tract infection through droplets and aerosols) or may suggest that other, unidentified animal sources may be involved in Ebola virus transmission to humans.

Ebola hemorrhagic fever outbreaks occurred in villages where people keep domestic animals, including dogs. The dogs are not fed and have to scavenge for their food. They eat small dead animals found near the villages and also internal organs of wild animals hunted and slaughtered by villagers. Some dogs are also used for hunting in the dense forested area. Although canine infection by Ebola virus has never been documented, domestic dogs' behavior and diet place them at risk.

We examined whether pet dogs could have been infected by Ebola virus and their potential role as primary or secondary sources of human infection. We conducted a large-scale serologic survey to determine the prevalence of Ebola virus infection in pet dogs in an Ebola virus–epidemic area of Gabon.

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Methods

Study Populations

We sampled 439 dogs divided into 4 groups (Table 1). The first group comprised 102 dogs living in France (negative controls). The second group comprised 258 dogs sampled in the area of Gabon hit by the 2001–2002 Ebola outbreak. This group was subdivided into 2 clusters, 1 of 159 dogs from villages located between Mekambo and Ekata and between Mekambo and Mazingo (Figure 1, Table 1) and another of 99 dogs from Mekambo city, where human cases were also reported. The third group comprised 50 dogs from Libreville, the capital of Gabon, and 29 dogs from Port Gentil, Gabon's second largest town, located on the Atlantic Coast (Figure 1, Table 1). Although these 2 Gabonese towns are both located >600 km from the Ebola virus–epidemic area, several human cases of Ebola infection, imported from the disease-epidemic area, were observed in Libreville during the 1996–1997 outbreak.

Sampling

Sampling was conducted in 3 ways. 1) Dogs in Libreville and Port Gentil were sampled in a veterinary clinic. Blood was collected in 5-mL dry Vacutainers (VWR International, Fontenay-sous-bois, France), and serum was prepared by centrifugation. Serum specimens were stored at –20°C until they were sent to the Centre International de Recherches Médicales de Franceville (CIRMF), Gabon, where they were stored at –80°C until testing. 2) Dogs from the Ebola virus–endemic area were sampled in the villages. An experienced veterinary team was located at Mekambo, where field laboratory facilities were set up; blood samples were collected on a daily basis in the vicinity of the village by using 5-mL dry Vacutainers and medetomidine anesthesia. The tubes were then transported to Mekambo each evening, and serum was decanted from whole blood. Serum samples were kept in liquid nitrogen in 1-mL aliquots at Mekambo until they were transported to CIRMF. Serum samples were then stored at –80°C until serologic testing, antigen detection, and RNA amplification were carried out. 3) Dogs in France were sampled in the Laboratoire des Dosages Hormonaux of the Ecole Nationale Vétérinaire de Nantes, France.

Dog owners were interviewed on their pets' activities (e.g., participation in hunting) and health history. The focus of the interviews was on potential Ebola virus–

Table 1. Results of testing pet dogs for Ebola-specific immunoglobulin G antibodies by location

Location	No. dogs tested	No. dogs positive
Mekambo/Ekata		
Ekata	38	10
Ilahounene	15	1
Mendemba	3	1
Ntolo	11	3
Mekouma	12	1
Malassa	5	0
Mbeza	13	6
Total	97	22
Mekambo/Mazingo		
Mazingo	5	1
Massombo	1	0
Ego poma	4	1
Grand Etoumbi	7	3
Zoula	15	3
Ibea	12	3
Imbong	10	4
Etakangaye	8	3
Total	62	18
Mekambo	99	15
Libreville	50	5
Port gentil	29	2
France	102	2
Total	439	64

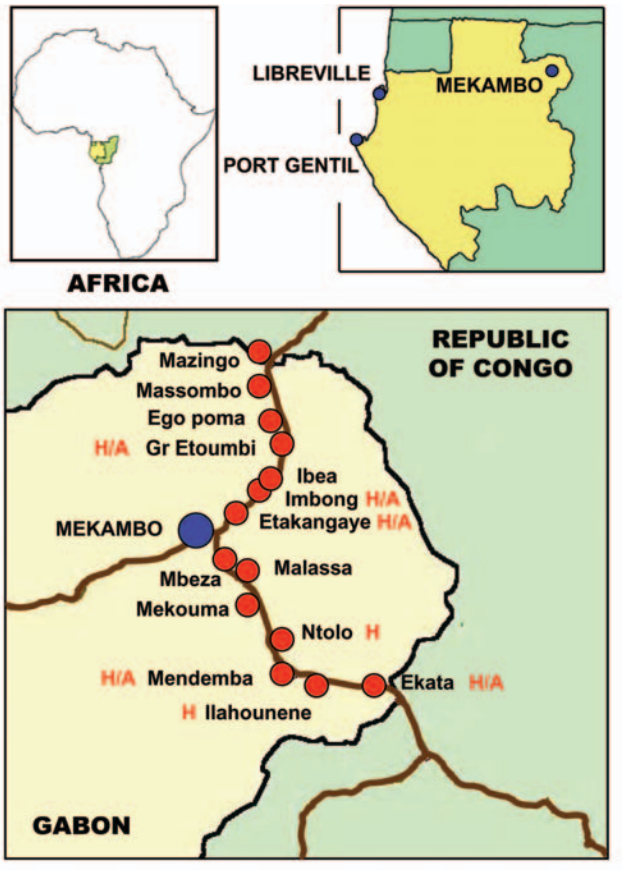

Figure 1. Locations of the main towns of Gabon (Libreville and Port Gentil) and the villages in the Ebola virus-epidemic area during the 2001-2002 outbreak in Gabon. The villages where human cases of Ebola infection were observed are indicated by "H." The villages where both human patients and infected animal carcass were observed are indicated by "H/A."

exposure events, including human cases that occurred in the village and among dog owners.

Laboratory Investigations

Ebola virus–specific immunoglobulin (Ig) G was detected by using a standard enzyme-linked immunosorbent assay (ELISA) method as previously described (8). Briefly, Maxisorp plates (VWR International) were coated with Ebola virus–Z antigens diluted 1:1,000 in phosphate-buffered saline (PBS), overnight at 4°C. Control plates were coated with uninfected Vero cell culture antigens in the same conditions. Sera diluted 1:400 in 5% nonfat milk in PBS-Tween 20 (0.1%) were added to the wells and incubated overnight at 4°C. IgG binding was visualized by using a peroxidase-labeled anti-dog IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) and the TMB detector system (Dynex Technologies, Issy-les-Moulineaux, France). Optical density (OD) was measured at 450 nm with an ELISA plate reader. For each sample we calculated the corrected OD as the OD of the antigen-coated well minus the OD of the corresponding control well. The cut-off value (CO) was calculated as follows: $CO = M + 3\sigma$, where M is the average of the corrected OD of the 102 negative controls from France, and σ is the standard deviation. Samples were considered positive when the corrected OD was above the cut-off.

Samples were used for antigen detection (9) and for viral polymerase chain reaction (PCR) amplification (10). Three positive and 3 negative serum specimens were also used for virus isolation (9). Briefly, Maxisorp plates were coated with a cocktail of 7 monoclonal antibodies against Ebola virus–Z antigens; control plates were coated with normal mouse ascitic fluid produced from the parent myeloma cell line. Serum was then added to the wells, followed by hyperimmune rabbit Ebola polyvalent antiserum and then peroxidase-conjugated goat antibodies against rabbit IgG. The TMB Microwell peroxidase substrate system was used to measure OD. For the detection of viral mRNA, total RNA was isolated from serum with the QIAmp viral RNA kit (Qiagen, Courtaboeuf, France), and cDNA was synthesized from mRNA as previously described. Two pairs of degenerate primers corresponding to the L-gene of Ebola virus were used for 2 rounds of amplification, which yielded a 298-bp fragment.

Statistical Methods

Confidence intervals for proportions were calculated by using the Clopper and Pearson method (11). Statistical comparisons between seroprevalence rates according to the sampling area were performed by using the Fisher exact test. The Cochran-Armitage test was used as a trend test for proportions, after checking for the goodness-of-fit of the underlying linear model (12). All tests used a 0.05 significance level. Statistical analyses were performed by using R software (R Development Core Team; 13).

Results

A total of 439 blood samples from dogs were screened for Ebola virus–specific IgG. Two (2%) of the 102 blood samples from dogs living in France had detectable Ebola virus–reactive IgG (Table 2). Seven of the 79 dogs sampled in Libreville and Port Gentil (8.9% prevalence rate), 15 of the 99 dogs sampled in Mekambo (15.2% prevalence rate), and 40 of the 159 dogs sampled in villages located within the Ebola virus–epidemic area (25.2% prevalence rate) had detectable IgG to Ebola virus antigens (Table 2).

During the 2001–2002 Ebola outbreak in Gabon, human cases of Ebola virus infection appeared only in certain villages within the Ebola virus–epidemic area (Figure 1). The prevalence of Ebola virus–reactive IgG among dogs from the villages where humans cases occurred was 27.2%, compared to 22.4% among dogs from villages where no human cases were noted (Table 2). In some cases, hunters had brought back to the village an Ebola virus–infected animal carcass found in the forest. This carcass was the source of human infection in the village, and the disease then spread from human to human, both within the village and to other villages by population movement (Figure 1). Thus, only secondary human cases were observed in some villages, with no identified animal source. The prevalence rate among dogs from villages with both an animal source and human cases was as high as 31.8%, compared to 15.4% among dogs from villages with human cases but no identified animal source (Table 2).

Table 2. Prevalence rates of Ebola-specific immunoglobulin G antibodies in pet dogs from different areas and villages

Area/village characteristic	No.	No. positive	Seroprevalence* (%)	95% confidence interval (%)
France	102	2	2	0.2–6.9
Major towns (Libreville and Port Gentil)	79	7	8.9	3.6–17.4
Mekambo	99	15	15.2	8.7–23.8
Ebola virus–epidemic area (villages)	159	40	25.2	18.6–32.6
Villages with human cases	92	25	27.2	18.4–37.4
Villages without human cases	67	15	22.4	13.1–34.2
Villages with human cases and animal source	66	21	31.8	20.9–44.4
Villages with human cases, without animal source	26	4	15.4	4.4–34.9

*Seroprevalence rates were compared by using the Fisher exact test with a 0.05 confidence level.

The seroprevalence rate was significantly lower in France (2.0%) than in Gabon (Table 2). In particular, it was lower than in the 2 major towns ($p = 0.043$), in Mekambo ($p = 0.001$), and in the Ebola virus–epidemic area ($p < 0.001$). The seroprevalence rate in the major towns (8.9%) was significantly lower than that in the Ebola virus–epidemic area ($p = 0.003$). Using scores from 1 to 4 for the canine prevalence rates in France, major towns, Mekambo and Ebola virus–epidemic areas, we observed a significant positive trend of linear increase (Cochran-Armitage test: $p < 0.0001$) (Figure 2A).

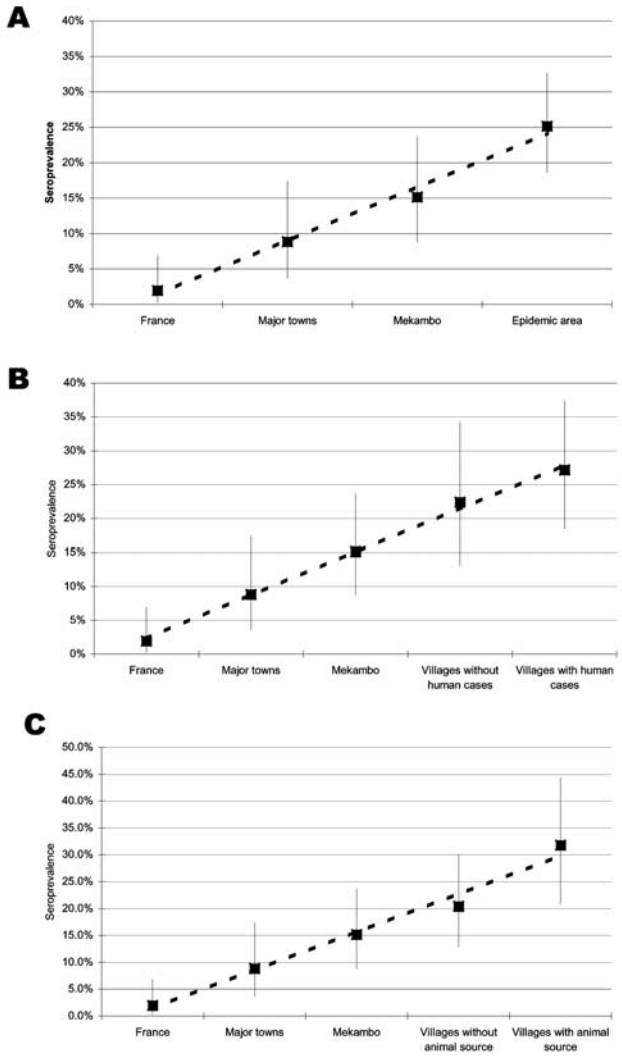

Figure 2. Seroprevalence of Ebola virus in dogs sampled in different areas: A) France, major towns of Gabon, Mekambo (a town close to the disease-epidemic area) and villages in the epidemic area; B) France, major towns of Gabon, Mekambo, villages without human cases and villages with human cases; C) France, major towns of Gabon, Mekambo, villages with and without an animal source. Estimates are represented by squares, bounded by their 95% Clopper and Pearson confidence intervals. The dashed line is the linear trend in proportion.

The seroprevalence rates in dogs increased linearly as the sampling area approached the sites of human cases, as confirmed by the highly significant Cochran-Armitage test for trends in proportions ($p < 0.0001$), which used a score of 1 for France, 2 for major towns, 3 for Mekambo, 4 for villages from the disease-epidemic area without human cases, and 5 for villages from the Ebola virus–epidemic area with human cases (Figure 2B). The result was confirmed when restricted to the 3 latter areas ($p = 0.04$).

In parallel, the seroprevalence rates in dogs increased linearly as the sampling area approached animal sources, as confirmed by a significant Cochran-Armitage test ($p < 0.0001$), using a score of 1 for France, 2 for major towns, 3 for Mekambo, 4 for villages where no animal source was observed (with or without human cases), and 5 for villages where an animal source was observed (with human cases) (Figure 2C). Again, the result was confirmed when restricted to the 3 latter areas ($p = 0.01$).

Neither Ebola virus antigens nor nucleotide sequences were detected in any of the positive or negative dog blood samples. We also failed to isolate the virus from 3 positive and 3 negative samples on VeroE6 cells.

Discussion

We investigated the potential involvement of domestic dogs in the occurrence or dissemination of Ebola virus hemorrhagic fever in humans. Based on a large serologic survey of dogs in the 2001–2002 Ebola outbreak area in Gabon, we found evidence that dogs can be infected by Ebola virus, a finding that raises important human health issues. The ELISA method was based on the use of Ebola virus–Z antigens. Although cross-reactions can occur with antibodies to other subtypes, the presence of these subtypes in our samples is unlikely because only the Zaire subtype circulates in the study area: all patients and nonhuman primates tested in this part of central Africa were infected by the Zaire subtype alone. The 2 positive dogs in France, an apparently Ebola virus–exempt part of the world, could be attributed to false-positive reactions due to the calculation of the positivity cut-off and the 1:400 serum dilution step used in the tests.

We found that 40 of 159 dogs living in the 2001–2002 Ebola virus–epidemic area had detectable Ebola virus–specific IgG, indicating either true infection or simple antigenic stimulation. All the tests were standardized at the 1:400 serum dilution, and most serum specimens had high OD values even at higher dilutions, confirming the specificity of the reactions. These data are consistent with observations we made during the different Ebola outbreaks that occurred in Gabon and the Republic of Congo in recent years. We observed that some dogs ate fresh remains of Ebola virus–infected dead animals brought back to the villages, and that others licked vomit from

Ebola virus–infected patients. Together, these findings strongly suggest that dogs can be infected by Ebola virus, and that some pet dogs living in affected villages were infected during the 2001–2002 human Ebola virus outbreak. No circulating Ebola antigens or viral DNA sequences (tested by PCR) were detected in either positive or negative serum specimens, and attempts to isolate virus from these samples failed. These findings indicate either old, transient Ebola infection of the tested dogs, or antigenic stimulation.

Symptoms did not develop in any of these highly exposed animals during the outbreak, a finding that tends to support antigenic stimulation, asymptomatic, or very mild Ebola virus infection. Wild animals, especially gorillas and chimpanzees, can also be infected by Ebola virus, but the infection is highly lethal and causes huge outbreaks and massive population declines (5,14). Other animals such as guinea pigs (15), goats (16), and horses (17) remain asymptomatic or develop mild symptoms after experimental infection, but Ebola virus infection has never been observed in these species in the wild. Thus, dogs appear to be the first animal species shown to be naturally and asymptomatically infected by Ebola virus. Asymptomatic Ebola infection in humans has also been observed during outbreaks (18) but is very rare. Although dogs can be asymptomatically infected, they may excrete infectious viral particles in urine, feces, and saliva for a short period before virus clearance, as observed experimentally in other animals. Given the frequency of contact between humans and domestic dogs, canine Ebola infection must be considered as a potential risk factor for human infection and virus spread. Human infection could occur through licking, biting, or grooming. Asymptomatically infected dogs could be a potential source of human Ebola outbreaks and of virus spread during human outbreaks, which could explain some epidemiologically unrelated human cases. Dogs might also be a source of human Ebola outbreaks, such as the 1976 Yambuku outbreaks in Democratic Republic of Congo (19), the 1995 Kikwit outbreak, some outbreaks that occurred in 1996 and 2004 in Gabon and Republic of Congo (5), and the 1976 (6), 1979 (20), and 2004 (21) outbreaks in Sudan, the sources of which are still unknown. Together, these findings strongly suggest that dogs should be taken into consideration during the management of human Ebola outbreaks. To confirm the potential human risk of Ebola virus–infected dogs, the mechanisms of viral excretion (i.e. body fluids and virus kinetics of excretion) should be investigated during experimental canine infection. This research would also offer insights into the natural resistance of dogs.

The canine seroprevalence rates in Libreville and Port Gentil, the 2 main towns of Gabon, were significantly higher than that observed in France, which suggests antigenic stimulation in these towns where no endemic cases of Ebola infection have been observed. Epidemiologic investigations showed that most seropositive dogs in Libreville and Port Gentil had probably never had contact with an infected source (dead animal or human case-patient), and that they had never visited the Ebola virus–epidemic area, in theory ruling out true infection. They may therefore have come into contact with free viral antigens, transmitted by aerosol or, to a lesser extent, experienced conjunctival exposure to virus-laden droplets of urine, feces, or blood of the unknown natural host. Ebola virus has been shown to be experimentally transmissible to rhesus monkeys by inhalation (22) and conjunctival exposure (23). Moreover, accidental transmission of Ebola virus to 2 rhesus monkeys that had no direct contact with experimentally infected monkeys was observed in a biocontainment laboratory, which also suggests aerosol, conjunctival, or oral transmission (24).

The Ebola virus reservoir species appears to extend throughout central Africa, both in rural and urban areas and might therefore be a small terrestrial mammal or a flying animal (bat or bird). No good candidate species has yet been identified, despite extensive studies (25,26). Epidemiologic observations during the 1976 outbreaks in Democratic Republic of Congo and Sudan identified bats as a potential reservoir (6,20), and Ebola virus nucleotide sequences and Ebola virus–like virus capsids were detected in rodents in the Central African Republic (27). The discovery of Ebola virus–positive pet dogs in undeclared affected areas suggests that these animals live in close contact with the Ebola virus reservoir, and this finding should help to narrow the search.

One striking result of this study is the significant increasing gradient of canine seroprevalence from France to the Ebola virus–epidemic area, including from villages with and without human cases in the area. The Cochran-Armitage test for trends in proportions showed that seroprevalence increased linearly from France (2%), to major towns (8.9%), then to Mekambo (15.2%), and then to villages in the Ebola virus–epidemic area (25.2%). This trend is supported by the increasing seroprevalence as the sampling area approached human cases and animal sources (Cochran-Armitage test, $p < 0.0001$). These findings suggest that canine seroprevalence could reflect contact with the virus and, thus, virus activity in a given area and also the risk for human infection.

The virus appears to jump from its natural host to humans only in specific, but unknown, conditions. Seroprevalence rates in dogs might serves as an indicator of Ebola virus in regions in which no animal deaths or human cases have been observed.

In conclusion, this study offers the first evidence that dogs might be asymptomatically infected by Ebola virus in

the wild. This finding has potential implications for preventing and controlling human outbreaks. The increasing canine seroprevalence gradient from low-risk to at-risk Ebola virus–endemic areas indicates that this seroprevalence might be used as an epidemiologic indicator of virus circulation in regions where no other means of virus detection are available.

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Probable Psittacosis Outbreak Linked to Wild Birds

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In autumn 2002, an outbreak of probable psittacosis occurred among residents of the Blue Mountains district, Australia. We conducted a case-control study to determine independent risk factors for psittacosis by comparing exposures between hospitalized patients and other residents selected randomly from the telephone directory. Of the 59 case-patients with laboratory results supportive of psittacosis, 48 participated in a case-control study with 310 controls. Independent risk factors were residence in the upper Blue Mountains (odds ratio [OR] 15.2, 95% confidence interval [CI] 5.6–41.7), age of 50–64 years (OR 3.9, 95% CI 1.5–10.5), direct contact with wild birds (OR 7.4, 95% CI 2.5–22), and mowing lawns without a grass catcher (OR 3.2, 95% CI 1.3–8.0). Protective equipment is recommended for residents in areas frequented by free-ranging birds if contact with birds and their droppings is likely when performing outdoor activities such as lawn mowing.

Psittacosis is a human disease caused by infection with the bacterium *Chlamydophila psittaci*. The bacterium also causes avian chlamydiosis, a disease reported in psittacine birds such as parrots, cockatiels, and parakeets (1–3). *Chlamydophila psittaci* can be present in large numbers in the droppings of sick birds and in dust contaminated by infected droppings (4). The organism can remain infectious in the environment for months (1). Human infection usually occurs when a person inhales the bacterium shed in feces and secretions of infected birds (1–3,5). Sheep, goats, cattle, and reptiles can also be infected, but these animals have rarely been linked to human cases (1,3,6).

Psittacosis has an incubation period of 1 to 4 weeks, and manifestations of disease can range from asymptomatic infection to systemic illness with severe pneumonia (1,5,7). Untreated psittacosis has a reported case-fatality rate of 15% to 20% (1,3). Psittacosis is most commonly reported among people in close contact with domestic birds, such as bird owners, poultry farmers, veterinarians, and workers within pet shops and poultry-processing plants (1–3,5,8–13). Sporadic cases and an outbreak in Australia linked to contact with free-ranging (wild) birds have been reported; however, little information is available on the role of wild birds in the transmission of *Chlamydophila psittaci* to humans (14,15)

Psittacosis became a notifiable disease in New South Wales (NSW), Australia, in 2001, and 38 laboratory notifications were received by the state health department that year, an incidence of 5.7 cases per 1,000,000 population for NSW (16,17). In May 2002, clinicians at the Blue Mountains Hospital (BMH), in the Wentworth Area Health Service, NSW, a 1-hour drive west of Sydney's central business district, reported an increase in adult admissions for severe community-acquired pneumonia. From March to May 2002, a total of 160 persons with pneumonia were seen at the BMH emergency department, compared with 82 from March to May 2001. The population of the Blue Mountains is ≈80,000 persons, and the area includes a large national park. The lower Blue Mountains (altitude ≈160 m) is on the western outskirts of Sydney, and residences tend to have suburban-style yards. The upper Blue Mountains district (altitude ≈1,044 m) lies further west, receives more rain, and has more bush land; its residential areas have larger yards and are closer to bush land. Reports that patients had found increased numbers of dead free-ranging birds in their yards, handled dead birds, and occasionally mowed over dead bird carcasses prompted clinicians to suspect psittacosis, although no case had been confirmed by laboratory testing. We report on our investigation into the extent and most likely cause of this outbreak.

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Methods

We defined a suspected case of disease as community-acquired pneumonia (as primary clinical diagnosis), confirmed by chest radiograph, in a resident of the Blue Mountains 15 to 75 years of age who was admitted to a local hospital between March 1 and June 30, 2002. Patients with a history of congestive cardiac failure and chronic obstructive pulmonary disease were excluded. Active surveillance for suspected cases of psittacosis was initiated in the first week of June 2002. Surveillance was performed by review of patient medical records and daily contact with the emergency department and infection control staff at the Blue Mountains and other local hospitals. We attempted to contact all suspected case-patients by telephone to invite them to participate in the study and provide serum samples for laboratory testing.

We defined a probable case of disease as psittacosis in a suspected case-patient in which any of the following were demonstrated: seroconversion, a 4-fold rise in immunoglobulin (Ig) G titer by microimmunofluorescence (MIF), or a single or static high convalescent-phase MIF IgG titer to *Chlamydophila psittaci*.

We conducted a case-control study to identify independent risk factors for psittacosis in the outbreak. Only probable case-patients were included in the analysis. To identify controls, random digit dialing was used to select household telephone numbers from the randomly sorted Blue Mountains telephone directory. All randomly selected households were telephoned, and 1 person 15–75 years of age from every household was randomly selected and invited to participate in the study (18,19).

Interviews were conducted by trained interviewers in a computer-assisted telephone interview service, from June 18 to July 2, 2002, 7 days per week, during the day and the evening. Up to 10 attempts were made to contact each case-patient and control. Case-patients and controls completed a detailed telephone questionnaire, which included questions on demographics; contact with poultry, pet, and free-ranging birds; types of bird contact; other animal contact; and gardening and other outdoor activities undertaken in the 3 weeks before onset of illness (for case-patients) or the 3-week period April 1–21, 2002 (for controls).

Statistical analysis was conducted by using SAS Version 8.02 (SAS Institute Inc., Cary, NC, USA) (20). In univariate analysis, we compared characteristics and potential risk factors reported by probable case-patients and controls. Univariate analysis was performed by using chi-square tests and logistic regression analysis. When expected cell counts were <5, Fisher exact 2-sided p value was used. We performed multivariable logistic regression modeling using the backward stepwise elimination method to identify independent risk factors for psittacosis (21).

Serum samples were tested for *Chlamydia* and *Chlamydophila* species IgG (to endpoint titer) with a MIF assay (Chlamydia IgG SeroFIA, Savyon Diagnostics, Ashdod, Israel) using purified *Chlamydophila pneumoniae*, *Chlamydia trachomatis*, and *Chlamydophila psittaci* elementary bodies as antigen and a complement fixation (CF) assay using a *Chlamydia* genus-specific glycoprotein (Virion, Ruschlikon, Switzerland) (22,23). According to the manufacturer's instructions, MIF titers >1:64 for *Chlamydophila psittaci* and *Chlamydia trachomatis* and >1:512 for *Chlamydophila pneumoniae* were regarded as indicative of current or recent infection. Serum samples were also tested for *Chlamydia trachomatis* IgG and IgA antibodies by using an enzyme immunoassay (EIA) (Medac Diagnostika, Wedel, Germany). All paired samples were tested in parallel in a single laboratory. Complement-fixing antibodies to *Mycoplasma pneumoniae*, influenza A and B viruses, adenovirus, and *Coxiella burnetii* were determined. *Legionella pneumophila* serogroups 1–6 and *L. longbeachae* total antibodies were tested by using an in-house immunofluorescence assay. In the later stage of the outbreak, upper respiratory tract specimens were collected from patients with pneumonia for *Chlamydophila pneumoniae* polymerase chain reaction (PCR) and for respiratory virus isolation. PCR for *Chlamydophila pneumoniae* was performed according to the published method (24).

To identify birds with avian chlamydiosis, we invited all local veterinarians and wildlife workers by letter to submit sick or dead birds for testing at a regional veterinary laboratory. The avian diagnostic test was based on direct immunofluorescence of a conjugated monoclonal antibody against a common, shared, genus-specific lipopolysaccharide antigen (Chlamydia-CEL VET IF TEST, Cellabs Diagnostics Pty Ltd, Brookvale, NSW, Australia). Household contacts of all probable case-patients were asked to submit any birds that the patient was known to have had contact with before onset of illness.

Results

Of 95 suspected cases identified, 59 (62%) had serologic evidence of psittacosis (probable cases), 30 (32%) were seronegative, and 6 (6%) were lost to follow up. The first patient with a probable case of psittacosis was hospitalized on March 11, 2002, marking the start of the outbreak, which peaked in late April to early May, with the last probable case-patient admitted to the hospital on June 29 (Figure). Of the 59 patients with probable cases of psittacosis, 36 (61%) were men, 50 (85%) resided in the upper Blue Mountains (altitude 770–1044 m), and 32 (54%) were 50–64 years of age. The average length of hospital stay was 7 days (range 2–29). No deaths occurred, although 2 patients required intensive care with intubation and mechanical ventilation.

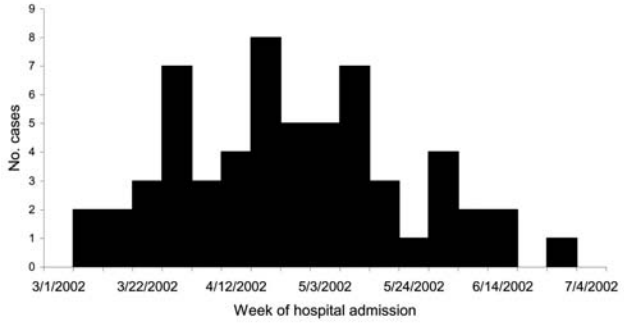

Figure. Hospitalized patients with cases of probable psittacosis, Blue Mountains, by week of hospital admission.

Case-Control Study

Of the 59 probable case-patients, 48 (81%) were interviewed for the case-control study, 2 refused to participate, 2 could not be contacted, and 7 were on vacation. Of 384 eligible controls contacted, 310 (80%) agreed to participate.

Symptoms reported by the 48 case-patients included fever (25 patients), weakness and tiredness (24 patients), sweats and chills (20 patients), aching muscles (16 patients), headache (16 patients), shortness of breath (9 patients), dry cough (8 patients), confusion (5 patients), vomiting (6 patients), diarrhea (5 patients), sore throat (3 patients), and loose cough (2 patients). In univariate analysis, when compared with controls, case-patients were significantly more likely to be male, to be 50–64 years of age, and to reside in the upper Blue Mountains (Table 1). Case-patients were also significantly more likely than controls to report direct contact with live or dead free-ranging birds, and a dose-response relationship existed between reported level of contact with free-ranging birds and disease (chi-square test for linear trend = 24.5, $p < 0.001$). In addition, case-patients were significantly more likely than controls to report lawn mowing without a grass catcher and to have spent more time performing this activity. Conversely, case-patients were significantly less likely than controls to report contact with caged or domestic birds, a history of asthma, and potting or weeding in the yard.

Case-patients were more likely than controls to report direct or indirect contact with the following bird species of the parrot family: crimson rosellas (odds ratio [OR] 4.4, 95% confidence interval [CI] 2.3–8.7, $p < 0.0001$), king parrots (OR 3.6, 95% CI 1.9–6.7, $p < 0.0001$), and gang gangs (OR 2.6, 95% CI 1.1–6.0, $p = 0.03$). Case-patients were also more likely than controls to report direct or indirect contact with doves (OR 4.2, 95% CI 1.8–10.3, $p = 0.003$), currawongs (OR 4.0, 95% CI 2.1–7.4, $p < 0.0001$), and magpies (OR 2.4, 95% CI 1.3–4.5, $p = 0.004$). Case-patients most commonly reported contact with crimson rosellas (73% of cases) and king parrots (60% of cases). Bird species were not included in multivariate analysis due to the likely unreliability of bird species identification.

In the multivariable logistic regression model, risk factors independently and positively associated with psittacosis were residence in the upper Blue Mountains, age of 50–64 years, direct contact with live or dead free-ranging birds, and lawn mowing without a grass catcher (Table 2). Risk factors independently and negatively associated with psittacosis were contact with caged or domestic birds, a history of asthma, and potting and weeding.

A number of persons with probable cases reported finding an unusually high number of dead free-ranging birds in their yards before onset of their illness; however, the bird carcasses had been disposed of before the investigation began. By the time the process for submission of birds for testing was arranged, winter had begun and few sick or dead birds were found. Of the 4 sick and 4 dead free-ranging birds submitted from the Blue Mountains area for testing, tissue of 1 king parrot tested positive for *Chlamydophila psittaci*.

Laboratory Results

Of the 59 seropositive case-patients, 35 (59%) had a seroconversion or a 4-fold rise in *Chlamydophila psittaci* MIF IgG titer, and 24 (41%) without acute-phase serum samples had elevated *Chlamydophila psittaci* MIF IgG titers in 1 or more convalescent-phase samples. Of these 24, 9 (15%) had a static high titer in 2 convalescent samples, 2 (3%) had high (but different) titers in 2 samples, and 13 (22%) had a high titer in a single convalescent sample. No evidence of infection by other respiratory pathogens was obtained in any of the *Chlamydophila psittaci*–seropositive patients. Alternative microbiologic diagnoses were made in 8 of the 30 (27%) *Chlamydophila psittaci*–seronegative patients, including 4-fold rises in serum antibodies specific to *M. pneumoniae* (2 cases), *L. longbeachae* (1 case), *L. pneumophila* serogroup 1 (1 case), and influenza B (2 cases). In 2 cases, *Streptococcus pneumoniae* was cultured from blood or sputum. *Chlamydophila pneumoniae* PCR and respiratory virus isolation were performed on upper respiratory tract samples taken from 8 case-patients when they arrived at the hospital, and all results were negative; later in the investigation, all 8 had serologic evidence of *Chlamydophila psittaci* infection.

Of the 48 probable case-patients included in the case-control study, 28 (58%) had a seroconversion or a 4-fold rise in *Chlamydophila psittaci* MIF IgG titer, 8 (17%) had a static high titer in 2 samples, 2 (4%) had high (but different) titers in 2 convalescent-phase samples, and 10 (21%) had a high titer in a single convalescent-phase sample.

Of the 35 patients with probable cases who seroconverted or showed a 4-fold rise in *Chlamydophila psittaci*

MIF IgG titer, 33 (94%) also had 4-fold rises in CF antibodies with a *Chlamydia* genus–specific glycoprotein, 1 had no rise, and 1 had insufficient serum for testing. In contrast, 3 of the 30 (10%) probable case-patients who were seronegative to *Chlamydophila psittaci* by MIF had 4-fold rises in CF antibodies.

Of the 59 probable case-patients with elevated *Chlamydophila psittaci* MIF antibody titers, 57 also had

Table 1. Potential risk factors for psittacosis among case-patients and controls in the Blue Mountains outbreak, Australia

Potential risk factor	Case-patients, N = 48 (%)	Controls, N = 310 (%)	Crude OR (95% CI)*	p value
Age group (y)				
15–49	10 (21)	155 (50)	Referent	
50–64	27 (56)	89 (29)	4.7 (2.2–10.2)	0.0007
65–75	11 (23)	66 (21)	2.6 (1.0–6.4)	0.04
Male sex	30 (63)	125 (40)	2.5 (1.3–4.6)	0.005
Resident of the upper Blue Mountains	42 (88)	104 (34)	13.9 (5.7–33.7)	<0.0001
History of asthma	1 (2)	64 (21)	0.08 (0.01–0.6)	0.0009
Employed	28 (58)	192 (62)	0.9 (0.5–1.6)	0.6
Outdoor employment	7 (15)	39 (13)	1.2 (0.5–2.8)	0.64
Bird contact				
Contact with caged or domestic birds	3 (6)	56 (18)	0.3 (0.1–1.0)	0.04
Contact with poultry birds	3 (6)	28 (9)	0.7 (0.2–2.3)	0.80
Visit to pet shop	9 (20)	39 (13)	1.6 (0.7–3.6)	0.25
Visit to aviary	0 (0)	3 (1)	Incalculable	1.0
Visit to zoo	0 (0)	3 (1)	Incalculable	1.0
Visit to poultry farm	0 (0)	4 (1)	Incalculable	1.0
Level of contact with free-ranging (wild) birds†				
No contact	9 (19)	137 (44)	Referent	
Only indirect contact‡	15 (31)	121 (39)	1.9 (0.8–4.5)	0.15
Any direct contact§	24 (50)	52 (17)	7.0 (3.1–16.1)	<0.0001
Yard exposures				
Lawn mowing				
Did not mow lawn	21 (44)	170 (55)	Referent	
Only mowed lawn with a grass catcher	4 (8)	75 (24)	0.4 (0.1–1.3)	0.14
Mowed lawn without a grass catcher	23 (48)	65 (21)	2.9 (1.5–5.5)	0.0017
Mowed lawn without a catcher for >1 hour	15 (31)	20 (6)	6.6 (3.1–14.1)	<0.0001
Pruning, cutting back branches	21 (45)	141 (47)	1.0 (0.5–1.7)	0.88
Potting or weeding	15 (31)	155 (50)	0.5 (0.2–0.9)	0.02
Watering yard	24 (50)	157 (52)	0.9 (0.5–1.7)	0.88
Raking yard	21 (45)	106 (35)	1.5 (0.8–2.8)	0.25
Soft landscaping	12 (25)	97 (31)	0.7 (0.4–1.5)	0.40
Hard landscaping	5 (10)	17 (5)	2.0 (0.7–5.7)	0.19
Mulching	9 (20)	56 (18)	1.1 (0.5–2.4)	0.84
Using compost	13 (27)	49 (16)	2.0 (1.0–4.0)	0.07
Using fertilizer	9 (20)	52 (17)	1.2 (0.5–2.6)	0.68
Home renovations or demolition	4 (9)	29 (9)	0.9 (0.3–2.7)	1.00
Bushwalking	13 (28)	91 (30)	1.0 (0.5–1.9)	1.00
Current or ex-smoker	23 (48)	141 (45)	1.1 (0.6–2.0)	0.76
General health self-rating				
Excellent to very good	23 (48)	182 (59)	Referent	
Good to fair	18 (37)	111 (36)	1.3 (0.7–2.5)	0.46
Poor to very poor	7 (15)	17 (5)	3.3 (1.2–8.7)	0.02
Yard attached to residence	48 (100)	302 (97)	Incalculable	0.61
Lived adjacent to bushland	33 (68)	184 (59)	1.5 (0.8–2.9)	0.27
Lived in a house	48 (100)	305 (98)	Incalculable	1.00
Lived in the Blue Mountains <5 years	11 (23)	38 (12)	2.1 (1.0–4.5)	0.07

*OR, odds ratio; CI, confidence interval.
†Chi-square test for linear trend = 24.5, p value < 0.001.
‡Indirect contact only with free-ranging (wild) birds: watching bird at bird feeder, watching birds in yard, seeing dead birds, watching birds in bird bath, bird flying in house.
§Any direct contact with free-ranging (wild) birds: catching birds, clipping birds' feathers, tending to sick birds, touching birds' feathers, cleaning up or touching birds' nest, cleaning up or touching bird droppings, handling dead birds.

Table 2. Multivariable logistic regression model of potential risk factors for psittacosis among 48 case-patients and 310 controls in the Blue Mountains outbreak, Australia

Potential risk factors for psittacosis	Adjusted* OR (95% CI)†	p value
Age group (y)		
15–49	Referent	
50–64	3.9 (1.5–10.5)	0.006
65–75	2.8 (0.9–8.8)	0.08
Resident of the upper Blue Mountains	15.2 (5.6–41.7)	<0.0001
History of asthma	0.1 (0.01-0.8)	0.03
Level of contact with free-ranging (wild) birds		
No contact	Referent	
Only indirect contact	2.6 (1.0–7.3)	0.06
Any direct contact	7.4 (2.5–22)	0.0003
Contact with caged or domestic birds	0.2 (0.04–0.8)	0.02
Lawn mowing		
Did not mow lawn	Referent	
Only mowed lawn with a grass catcher	0.4 (0.1–1.3)	0.12
Mowed lawn without a grass catcher	3.2 (1.3–8.0)	0.01
Potting or weeding	0.2 (0.1–0.5)	0.001

*After adjustment for the effects of age group, sex, region of residence, outdoor employment, history of asthma, level of free-ranging (wild) bird contact, contact with caged or domestic birds, lawn mowing, potting or weeding, smoking history, general health self-rating, duration lived in the Blue Mountains, soft landscaping, hard landscaping, using compost, and residence adjacent to bushland.
†OR, odds ratio; CI, confidence interval.

detectable *Chlamydia trachomatis* MIF IgG, generally at a lower titer. However, when an EIA was used, only 2 had *Chlamydia trachomatis* IgA, and 7 (12%) had *Chlamydia trachomatis* IgG detected. A similar proportion (10%) of the suspected case-patients who were seronegative for *Chlamydophila psittaci* had *Chlamydia trachomatis* IgG detected. Among the 59 probable case-patients, 21 had elevated *Chlamydophila pneumoniae*–specific IgG titers on MIF testing, although 7 showed a 4-fold rise or higher, and all were less than the rises in *Chlamydophila psittaci*.

Active surveillance identified 2 persons with laboratory evidence of psittacosis who were linked to the Blue Mountains outbreak but resided elsewhere. One person had stayed in a vacation home there for most weekends in the months preceding the outbreak. The other lived elsewhere in Australia but had been on vacation in the Blue Mountains in the weeks preceding the outbreak. Between March and June 2003, 11 other persons with psittacosis were identified in NSW; these patients all reported other exposures, such as contact with pet birds and aviaries.

Intervention

Media releases on June 11 and 12, 2002, identified psittacosis as a possible cause of the outbreak and advised residents of the Blue Mountains to avoid contact with free-ranging birds and their droppings, feathers, carcasses, and dust and to wear a particulate face mask and follow other prevention measures when performing outdoor activities likely to bring them in contact with free-ranging birds (1). Information on psittacosis and the outbreak were communicated by a telephone hotline, health department Web sites, and by fax to all medical doctors and hospitals in the Blue Mountains and adjacent regions (25)

Discussion

We identified a large outbreak of probable psittacosis among residents of a forested district of Australia. Contact with live or dead free-ranging birds and lawn mowing without a grass catcher explained 70% of the cases. Possible reasons for the cessation of the outbreak include the onset of colder winter weather, which may reduce the prevalence or transmissibility of the infection among free-ranging birds and yard exposure among humans.

This study had several limitations. Case-patients and controls were exposed to media speculation over the role of parrots in the outbreak, so that people who believed they had psittacosis may have been more likely to report parrot exposure. Among patients, 28 completed hypothesis-generating questionnaires early in the investigation (between May 30 and June 7), which may have provided them with additional prompts to recall parrot exposure. However, diagnosis was unknown at the time patients completed the questionnaire, and few had laboratory evidence of psittacosis at the time they completed the questionnaire. Potential recall bias may also be countered because similar proportions of case-patients and controls reported no difficulty recalling contact with birds (case patients = 83%, controls = 85%, OR 0.9, 95% CI 0.4–2.0, p = 0.7).

The serologic diagnosis of chlamydial infections is difficult because many assays lack specificity, and published data on the sensitivity and specificity of the MIF assay used in this study are limited (26). In this study, acute *Chlamydophila psittaci* infection, rather than *Chlamydophila pneumoniae* or *Chlamydia trachomatis* infection, was supported by 3 factors: 1) the seroconversion and 4-fold rises in *Chlamydophila psittaci* MIF IgG titers in samples from most case-patients (with most also

having 4-fold rises in CF antibodies to *Chlamydia*), compared with lower MIF IgG titers to *Chlamydophila pneumoniae* and *Chlamydia trachomatis*, 2) the similar background rates of *Chlamydia trachomatis*–specific IgG on EIA in samples from the *Chlamydophila psittaci* seropositive probable and seronegative suspected case-patients, and 3) history of bird exposure among case-patients.

Analysis of hospital admission and discharge data indicated that, compared with the NSW state average and with the lower Blue Mountains average, increased rates of pneumonia have been seen among residents of the upper Blue Mountains during the autumn of previous years (unpub. data). This excess in pneumonia may be due to seasonal outbreaks of psittacosis in the area. Free-ranging birds are plentiful in the Blue Mountains, and chlamydiosis has been clinically diagnosed among juvenile birds, in particular crimson rosellas, in the area during the autumn of previous years (M. Cannon, pers. comm.).

Reasons for the positive association between probable psittacosis and residence in the upper Blue Mountains are speculative. The residents of the upper Blue Mountains are slightly older than that of the lower Blue Mountains (unpub. data) and may be more likely to remain at home and be active in their yards than the slightly younger residents of the lower Blue Mountains, who tend to commute to work in Sydney every day. This age difference may result in greater proximity between residents and free-ranging birds in the upper Blue Mountains.

Although the reason probable psittacosis case-patients were more likely to be 50–64 years of age is unclear, younger people may have had milder infection or been less likely to seek medical attention and require hospitalization (27,28). That persons with congestive cardiac failure or chronic obstructive pulmonary disease were excluded from the study may explain why persons 65–75 years of age appeared less at risk of disease.

Why a history of asthma seemed protective against psittacosis is uncertain. Persons with a history of asthma may be less likely to perform outdoor yard activities that generate dust and pollen. That persons who reported contact with caged or domestic birds appeared protected against disease may be related to previous exposure to *Chlamydophila psittaci*. While the development of protective immunity following infection with *Chlamydophila psittaci* has not been proven, laboratory-confirmed cases of reinfection have seldom been reported (2,29). That disease was less likely to develop in persons who reported potting or weeding may be because these activities generate little contaminated dust compared with activities such as lawn mowing.

Reported symptoms of the probable psittacosis case-patients in this outbreak were similar to those described elsewhere (1–3,5,10,15). One other community-wide outbreak of psittacosis has been reported to be potentially associated with contact with free-ranging birds (15). In 1995, 16 cases of psittacosis among residents of a rural town in a forested area in southern Australia were linked to trimming and mowing lawns and time spent in a yard, which are thought to be proxies for exposure to infectious particles shed by free-ranging birds with chlamydiosis (15). While this 1995 study did not find a significant relationship between illness and handling of dead birds (OR 2.4, 95% CI 0.25–21.05), the OR supports the positive association between illness and handling live or dead free-ranging birds (adjusted OR 7.4, 95% CI 2.5–22, $p < 0.001$) found in our study. The 1995 study reported the risk of illness associated with lawn mowing was not linked to use of a grass catcher, though whether the authors differentiated between those who never used a catcher and those who sometimes used a catcher is not clear. Previous studies have linked lawn mowing with psittacosis and with primary pneumonic tularemia (15,30). In our case-control study questionnaire, we differentiated between persons who mowed only with, with and without, and only without a grass catcher. We found this probable psittacosis outbreak to be linked to lawn mowing only without the use of a grass catcher. We could find no evidence in the literature suggesting that the use of a grass catcher provides lawn mower operators some protection from inhaling infectious particles, though grass catcher marketing materials of lawn mower manufacturers in Australia suggest that grass catchers reduce the generation of dust and hence the amount of dust expelled into the operator's breathing zone (31).

This outbreak could have been missed altogether if not for alert clinicians. Laboratory evidence of psittacosis requires acute-phase and convalescent-phase (collected at least 6 weeks after onset) species-specific serologic testing for *Chlamydophila* or *Chlamydophila psittaci* PCR or culture. These tests require request by the treating doctor and referral to a specialized laboratory and enough concern to convince the patient to return for phlebotomy after recovery. In most clinical settings, underdiagnosis is therefore likely.

Clinicians should consider a diagnosis of psittacosis in persons with respiratory disease who reside in or have visited areas frequented by free-ranging birds. Health departments should also be alert for such outbreaks to facilitate diagnosis. Residents of areas frequented by free-ranging birds should avoid direct contact with dead or sick birds and bird droppings, use a particulate mask and gloves if contact is unavoidable, and avoid mowing lawns without a grass catcher. Further study is needed to identify effective measures, such as lawn mower design features, for reducing human exposure to *Chlamydophila psittaci* and other zoonoses.

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Effect of Regulation and Education on Reptile-associated Salmonellosis

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Reptiles have become increasingly common as domestic pets, and with them reptile-associated *Salmonella* infections in humans. From 1990 to 2000, a total of 339 reptile-associated *Salmonella* cases were reported in Sweden. In 1996, as part of its efforts to adapt its import regulations to those of the European Union, Sweden no longer required certificates stating that imported animals were free of *Salmonella*. A subsequent increase was noted in the incidence of reptile-associated cases from 0.15/100,000 in the period 1990–1994 to 0.79/100,000 in 1996 and 1997. After a public education campaign was begun through the news media, the incidence dropped to 0.46/100,000. Children were the most affected age group among patients (incidence 1.3/100,000). *Salmonella enterica* serotype Enteritidis was the most frequent serotype (24% of isolates), followed by *S*. Typhimurium (9% of isolates). Import restrictions and public information campaigns are effective public health measures against reptile-associated salmonellosis.

Salmonellosis is an important worldwide health problem, affecting both humans and animals. In the United States, *Salmonella* causes an estimated 1.4 million episodes of infection and 400 deaths annually in humans (1). *Salmonella* usually causes a moderate gastrointestinal disorder, but it may result in more severe disease, such as bacteremia or meningitis, sometimes with fatal outcome (2,3).

For decades, reptiles have been recognized as a source of human salmonellosis. *Salmonella* species were first isolated from snakes, turtles, and lizards in the 1940s (4,5), and more recent studies have shown that at least 50%–90% of these animals are carriers of *Salmonella* (6–8). The bacteria are excreted intermittently in the feces but can also be isolated from the cloacae, skin, and throat of water-living reptiles.

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Reptiles have become increasingly common as domestic pets. In Canada, pet turtle–associated salmonellosis was recognized as a serious health problem in the 1960s and 1970s, and the country banned imported turtles in 1975 (9).

Sweden has a long tradition of combating and controlling *Salmonella* in feed, animals, and humans, dating back to a large outbreak of salmonellosis in 1953 that affected >9,000 persons and caused 90 deaths (10,11). From 1970 to 1994, these control measures also included import restrictions on reptiles; anyone who wanted to import reptiles or turtles needed a certificate stating that the animals were free of *Salmonella*, and importing of turtles with shells <10 cm was not allowed.

In 1995, Sweden dropped its requirement for a *Salmonella* certificate and instead required an import permit issued by the Swedish Board of Agriculture. When Sweden became a member of the European Union (EU) in January 1995, a number of new rules were adopted. As a result of these changes, Sweden no longer required import permits for reptiles and turtles, and it also lifted the import ban on small turtles. The adaptation of import regulations for reptiles took effect on March 1, 1996.

Our study had 2 goals. First, we studied the impact of strict import regulations on the epidemiology of reptile-associated salmonellosis (RAS). Secondly, we assessed whether awareness campaigns can decrease the number of such cases.

Methods

Swedish Surveillance System for Salmonellosis

Salmonellosis is a reportable disease in Sweden. Case-patients, who need to have a *Salmonella*-positive stool or blood sample confirmed by a laboratory, are reported both by the physician who has seen the patient (clinical notification) and the laboratory that identified the bacterium

(laboratory notification). Notification is submitted concurrently to the Swedish Institute for Infectious Disease Control, the county medical officer, and the municipal Environmental Health Board. Clinical notification includes relevant epidemiologic information, including suspected source of infection. If the county medical officer's initial investigation indicates an environmental source of infection (such as food, water, or animals), the Environmental Health Board samples the suspected source.

Reptile-associated Cases

We reviewed all reported domestic cases (patient reported to be infected in Sweden) of salmonellosis from 1990 to 2000 for association with reptiles. A patient was considered to have RAS if reference was made to direct or indirect contact with a reptile or turtle, and the notification did not indicate other sources of infection. All *Salmonella* strains were sent to the national Salmonella Reference Laboratory at the Swedish Institute for Infectious Disease Control, which performed serotyping according to the Kauffman and White scheme (12). In the case of *Salmonella enterica* serotype Typhimurium or *S.* Enteritidis, the laboratory also carried out phage typing according to the Colindale scheme. The Department of Epidemiology at the Swedish Institute for Infectious Disease Control compiled and analyzed all of these sources of information, together with reports on outbreaks from county medical officers.

All data used in this study were compiled as part of routine national surveillance of communicable diseases, as regulated in the Swedish Communicable Disease Act. The research ethics committee at Karolinska Institutet, Stockholm, Sweden, approved the study.

Results

Age and Sex of Patients

Clinicians and laboratories reported a total of 339 RAS cases in Sweden from 1990 to 2000. Patients infected by turtles (n = 153) were younger (median age 8 years, mean age 16 years) than patients infected by lizards and snakes (n = 175) (median age 17 years, mean age 18 years) (Figures 1 and 2). Eleven case-patients had contact with both turtles and lizards or snakes. Before 1996, RAS was more common in male patients (65% of cases). This difference disappeared after 1996, and from 1996 to 2000, approximately as many female (47%) as male (53%) patients were affected (nonsignificant difference).

Serotypes

A total of 51 different serotypes were isolated from RAS-infected patients (Table 1). *S.* Enteritidis was the most frequent serotype, accounting for 24% of reported serotypes, followed by *S.* Typhimurium with 9%. Some serotypes were unique for patients with RAS.

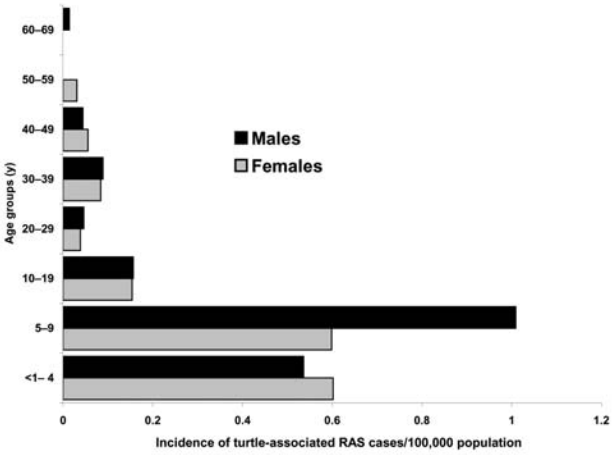

Figure 1. Age and sex distribution in turtle-associated salmonellosis cases. RAS, reptile-associated salmonellosis.

Cases before and after Adaptation of Importation Rules

Sweden has ≈9 million inhabitants; 4,500–5,200 report *Salmonella* infections each year. Less than 20% of reported cases (400–900 per year) are domestically acquired in Sweden.

Table 2 and Figure 3 present the number of RAS cases in proportion to all domestic cases. From a very low proportion of RAS (1.2%, 5–16 cases) from 1990 to 1994 when import restrictions were in place, the proportion increased to 4.5% (25 cases) in 1995, as "*Salmonella* certificates" were no longer needed. The proportion of RAS increased even more (to 11.6%, 68–71 cases) in the 2 subsequent years, when all reptile import regulations had ceased.

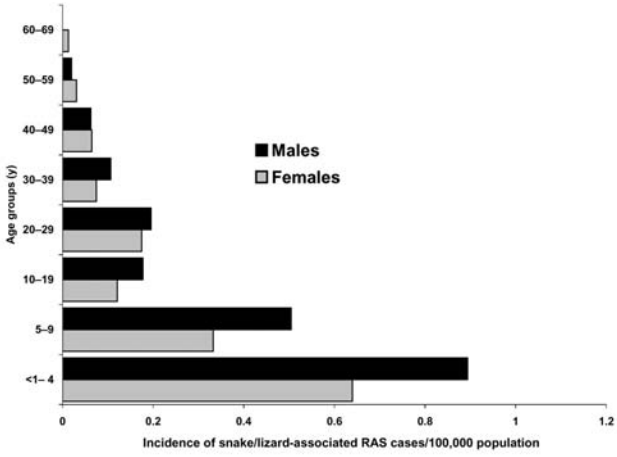

Figure 2. Age and sex distribution in snake/lizard-associated salmonellosis cases. RAS, reptile-associated salmonellosis.

Table 1. Reported serotypes of salmonellosis acquired in Sweden, 1990–2000

Serotype	No. of RAS* patients	No. of non-RAS patients	% of all RAS cases	% of all patients infected in Sweden	Type of reptile (could be >1 reptile/case)
Salmonella enterica Serotype Enteritidis	81	2,345	23.9	3.3	Turtle (46); tortoise (2); lizard (12); snake (28)
S. Typhimurium	30	2,056	8.9	1.4	Turtle (11); lizard (7); snake (16)
S. Subspecies III	30	8	8.9	78.9	Turtle (5); lizard (5); snake (25)
S. Subspecies I	19	41	5.6	31.7	Turtle (7); lizard (4); snake (8)
S. Newport	15	100	4.4	13.0	Turtle (3); lizard (4); snake (9)
S. Poona	15	19	4.4	44.1	Turtle (9); lizard (1); snake (5)
S. Saintpaul	12	53	3.5	18.5	Turtle (12)
S. Braenderup	12	30	3.5	28.6	Turtle (8); lizard(4)
S. Stanley	11	147	3.2	7.0	Turtle (11)
S. Muenchen	10	30	3.0	25.0	Turtle (4); lizard (1); snake (5)
S. Java	9	49	2.7	15.5	Turtle (5); snake (2); several reptiles (2)
S. Oranienburg	6	84	1.8	6.7	Turtle (2); lizard (3); snake (3)
S. Litchfield	6	12	1.8	33.3	Turtle (6)
S. Subspecies	5	177	1.5	2.7	Turtle (4); lizard (1)
S. Adelaide	5	33	1.5	13.2	Snake (5)
S. Chester	5	11	1.5	31.3	Turtle (2); snake (3)
S. Bovismorbificans	4	350	1.2	1.1	Turtle (3); snake (1)
S. Hadar	4	140	1.2	2.8	Turtle (3); snake (1)
S. Telelkebir	4	4	1.2	50.0	Lizard (2); snake (2)
S. Montevideo	3	55	0.9	5.2	Turtle (2); lizard (1)
S. Subspecies II	3	12	0.9	20.0	Turtle (1); lizard (1)
S. Subspecies IV	3	2	0.9	60.0	Lizard (3)
S. Infantis	2	324	0.6	0.6	Snake (2)
S. Bredeney	2	56	0.6	3.4	Turtle (2)
S. Heidelberg	2	44	0.6	4.3	Turtle (2)
S. Panama	2	35	0.6	5.4	Lizard (1); snake (1)
S. Abony	2	17	0.6	10.5	Turtle (2)
S. Ituri	2	5	0.6	28.6	Snake (2)
S. Napoli	2	1	0.6	66.7	Lizard (1)
S. Bonn	2	0	0.6	100.0	Turtle (1)
S. Nima	2	0	0.6	100.0	Snake (1)
S. Shubra	2	0	0.6	100.0	Turtle (1)
S. Agona	1	429	0.3	0.2	Snake (1)
S. Virchow	1	152	0.3	0.7	Turtle (1)
S. Thompson	1	54	0.3	1.8	Snake (1)
S. Blockley	1	51	0.3	1.9	Lizard (1)
S. Mikawasima	1	27	0.3	3.6	Turtle (1)
S. Muenster	1	7	0.3	12.5	Snake (1)
S. Oslo	1	3	0.3	25.0	Lizard and snake (1)
S. Reading	1	3	0.3	25.0	Snake (1)
S. Ibadan	1	2	0.3	33.3	Snake (1)
S. Monschaui	1	2	0.3	33.3	Snake (1)
S. Rubislaw	1	2	0.3	33.3	Snake (1)
S. Urbana	1	2	0.3	33.3	Turtle (1)
S. Farmsen	1	1	0.3	50.0	Snake (1)
S. Pomona	1	1	0.3	50.0	Turtle (1)
S. Abaetetuba	1	0	0.3	100.0	Turtle and lizard (1)
S. Lome	1	0	0.3	100.0	Snake (1)
S. Matadi	1	0	0.3	100.0	Turtle (1)
S. Spartel	1	0	0.3	100.0	Lizard (1)
S. Tamberma	1	0	0.3	100.0	Snake (1)
S. Veijle	1	0	0.3	100.0	Snake (1)
S. Windemere	1	0	0.3	100.0	Snake (1)
>1 serotype†	4	‡	1.2	–	Lizard (1); snake (3)
Not typed	2	‡	0.6	–	Snake (2)
Total no. of cases	339	6,974	100	100	–

*RAS, reptile-associated case of *Salmonella* infection, human.
†1 case respectively with *S.* Adelaide + *S.* Montevideo, *S.* Agoueve + *S.* Chester + *S.* Infantis, *S.* Ajiobo + *S.* Muenchen, and *S.* Mesbit + *S.* Montevideo.
‡Not available.

Table 2. Reptile-associated salmonellosis and its proportion of all salmonellosis cases by study period, Sweden, 1990–2000*†

Study period	Incidence per 100,000 (no.) of all salmonellosis	Incidence per 100,000 (no.) of RAS cases	Proportion (%) RAS (95% CI)	p value
Period 1 (1990–1994)	12.74 (4,405)	0.15 (52)	1.2 (0.9–1.5)	<0.001
Period 2 (1995)	6.32 (558)	0.28 (25)	4.5 (2.9–6.5)	Reference
Period 3 (1996–1997)	6.78 (1,199)	0.79 (139)	11.6 (9.9–13.5)	<0.001
Period 4 (1998–2000)	7.70 (2,046)	0.46 (123)	6.0 (5.1–7.1)	NS

*Figures only include patients infected in Sweden.
†RAS, reptile-associated salmonellosis; CI, confidence interval; NS, not significant.

In recognition of the growing problem with RAS, authorities alerted the public, mainly through the newspapers, starting in late 1997. In the 2 following years (1998–2000) the proportion of RAS cases decreased to 6.0% (43 and 34 cases), but it did not reach the low levels seen before 1995.

Of the 77 RAS cases diagnosed before the adaptation with EU importation rules in 1996, 26 (34%) persons had contracted the infection from turtles and 51 (66%) from snakes and lizards. After adaptation, the proportion of cases from the 2 sources was more even; of 262 diagnosed cases, 127 (48%) were due to contacts with turtles and 124 (47%) were due to contacts with snakes and lizards. Eleven (4%) of the case-patients after 1995 had a history of contact with several kinds of reptiles. Since no sampling of the animals was performed, we do not know which kind of reptile caused these infections.

Discussion

First, this report shows that an import restriction requiring certificates that animals are not carrying *Salmonella* is an effective public health measure for protecting the general population from RAS. Sweden noted a dramatic increase in the number of reported cases of RAS in 1996. No changes in the reporting system were made that could explain the increase in reported cases, making it likely that synchronization of import rules of animals and reptiles within the EU in March 1996 caused the sudden increase in the number of RAS cases in Sweden that year.

Secondly, our report indicates that actively informing the public about RAS can cause a decrease in cases. Immediately after the harmonization of rules, the availability of reptiles in pet stores increased, and many parents gave their children a pet turtle, lizard, or snake. The exact number of animals sold is unknown. No information about the risk of contracting salmonellosis was given to the families who bought the animals. In the United States, the Association of Reptilian and Amphibian Veterinarians (ARAV) produced a client education handout with basic facts on how to avoid transmission of *Salmonella* bacteria from reptiles to humans (13). The Centers for Disease Control and Prevention recommends that children under the age of 5 should avoid contact with reptiles and that households with children <1 year should not own reptiles.

Information about the risk of contracting salmonellosis from pet reptiles was communicated to the public from late 1997 and onwards, and the information given to the public in Sweden has closely followed ARAV guidelines. After the information activities began, the number of reported RAS cases decreased significantly in subsequent years. The decrease might have been even greater since it is likely that more patients with diarrhea would have informed their doctors of reptile contact once they knew of the association between reptiles and salmonellosis.

Third, our study demonstrates that RAS poses a threat to human health that cannot be ignored. The true number of such cases is hard to estimate. We have little hard data on the proportion of all *Salmonella* cases in the community being diagnosed, although according to conservative estimates, ≈90% of cases remain undiagnosed (11). The Swedish system, with its dual notifications from clinicians and laboratories, captures >99% of cases actually being diagnosed, and >95% of all diagnosed cases are clinically reported (A. Jansson, unpub. data). We assume that at least 50% of RAS patients have been reported as such, once a *Salmonella* infection has been diagnosed, for the following reasons: 1) only a few clinical notifications lack information on likely route of transmission, 2) domestically acquired salmonellosis is comparatively rare, and 3) most physicians are aware of this route of transmission. We estimated the true annual number of RAS in Sweden after

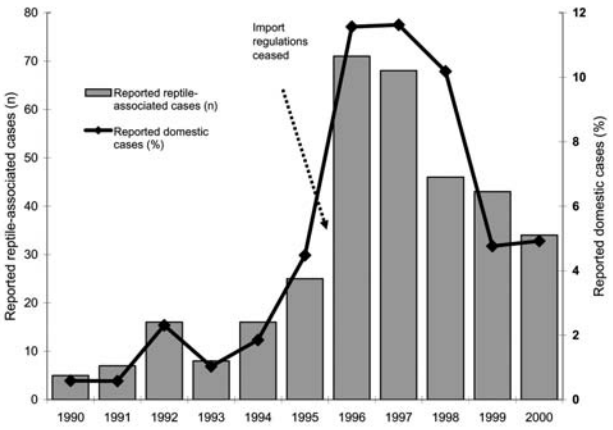

Figure 3. Reported cases of reptile-associated salmonellosis in Sweden, 1990–2000; total number of cases and proportion of domestic cases.

1996 to be ≈1,000 cases per year, or >10 cases per 100,000 inhabitants. The same rate applied in the United States would correspond to 30,000 to 40,000 cases per year. This number is substantially less than the 93,000 cases per year estimated by the Centers for Disease Control and Prevention (14), which hypothesized that 7% of all U.S. cases of salmonellosis were RAS.

The incidence of RAS in a country is naturally dependent on the magnitude of reptile imports. We therefore tried to obtain some estimates of the numbers of imported reptiles in the years under study. This attempt was unsuccessful because the responsible national authority (the Swedish Board of Agriculture) has no figures on the number of imported animals, only records on the importer. Furthermore, according to direct private import rules, anyone can legally bring ≤3 animals into the country without any registration. Although we have no indication that the numbers of imported reptiles decreased in the later years (rather, we think the opposite is true), we cannot rule out that some of the decrease in RAS cases was due to decreased imports.

Fourth, we obtained information on the epidemiology of RAS that is likely to hold true for other Western countries as well. Children are the most affected age group, with an incidence of 1.3/100,000 inhabitants. Those patients who had acquired salmonellosis from a turtle were younger than patients who acquired it from a snake or lizard, a reflection of the age groups for which turtles are bought as pets. Young boys 5 to 9 years of age who had contracted the disease from a turtle were shown to be at particular risk in this study.

In the United States during the 1970s, small pet turtles were a major source of RAS infections and accounted for 14% of all cases of salmonellosis in children <10 years of age. This fact was the reason for the ban on commercial distribution of pet turtles with a carapace length <4 inches. This ban has prevented an estimated 100,000 RAS cases among children in the United States yearly (14,15). Nevertheless, the incidence of RAS in the United States has been increasing because of an increase in pet reptile owners; experts estimate that the number of pet reptiles owners doubled from 1991 to 2001 (3).

In most cases included in this study, patients experienced gastrointestinal infection with symptoms severe enough to seek medical care; some even required hospitalization. Many of the serotypes isolated from the Swedish RAS patients were serotypes also commonly found in foodborne *Salmonella* infection. *S.* serotype Enteritidis and *S.* Typhimurium, the 2 most common serotypes found in humans in Europe and the United States (16,17), accounted for 33% of all RAS cases, while *S.* Subspecies III, known to be reptile-associated, caused 9% of cases. Some serotypes such as *S.* Abaetetuba, *S.* Bonn, *S.* Lome, *S.* Matadi, *S.* Nima, *S.* Shubra, *S.* Spartel, *S.* Tamberma, *S.* Veijle, and *S.* Windemere were only found in RAS cases and could therefore be assumed to be more reptile-associated than other serotypes. Previously known reptile-associated serotypes, including *S.* Poona, *S.* Stanley, *S.* Pomona, and *S.* Java, were also isolated from Swedish RAS patients (15). Some serotypes may be more species-specific; 24 of the 51 isolated serotypes were isolated from only 1 kind of reptile (turtle, snake, or lizard). In most of these cases, the numbers were too low to allow for any firm conclusions. However, *S.* Litchfield, *S.* Saintpaul, and *S.* Stanley seem to be associated with turtles and *S.* Adelaide with snakes. The wide variety of *Salmonella* serotypes in persons with RAS demonstrates that reptiles are well adapted to *Salmonella* and could harbor and transmit many different serotypes, which confirms that a risk is always involved in handling these kinds of animals.

Having the same close contact with pet reptiles and turtles as with cats and dogs, or even just keeping them in the private home, increases the risk for transmission of *Salmonella* bacteria from animals to humans. Pet stores, veterinarians, and other appropriate sources should provide better information on how to avoid these risks, such as the ARAV guidelines, to all prospective buyers of turtles and other reptiles.

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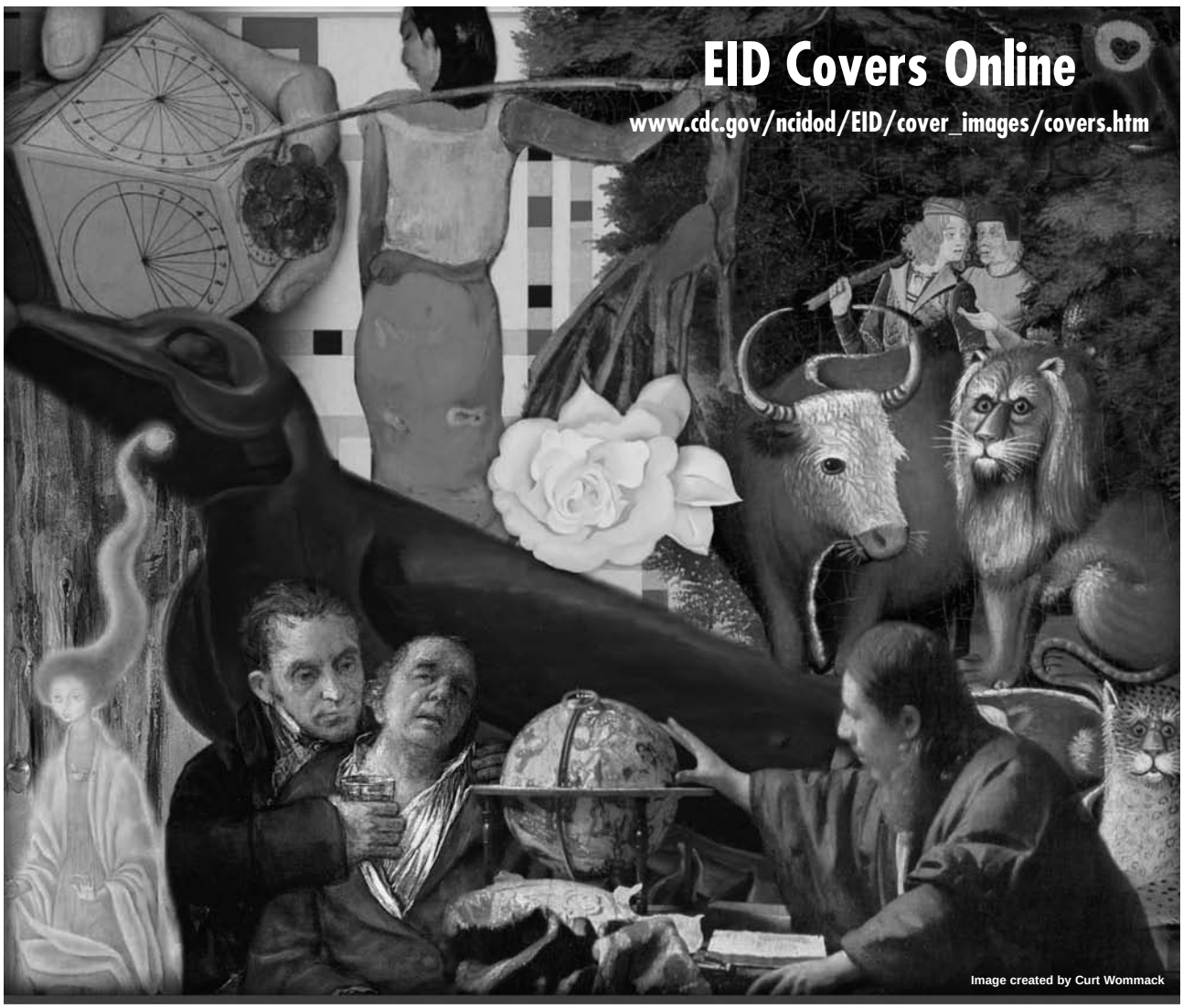

SARS Risk Perceptions in Healthcare Workers, Japan

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In coping with severe acute respiratory syndrome (SARS), infection control measures are a key aspect of protecting healthcare workers. We conducted a survey concerning perception of risk and countermeasures for SARS in 7 tertiary hospitals in Japan from July through September 2003, immediately after the SARS epidemic in neighboring countries. Based on 7,282 respondents out of 9,978 questionnaires administered, we found the perception of risk to be relatively high and the perception of countermeasures at the institutional level to be relatively low. Knowledge of preventive measures, concept of (opinions regarding) institutional measures, and perception of risk differed substantially among the 3 job categories, notably between physicians and nurses. The concept of institutional measures was the most important predictor of individual perception of risk. In view of the potential for future epidemics, planning and implementing institutional measures should be given a high priority.

Severe acute respiratory syndrome (SARS) has been reported in 30 countries with a total of 8,096 probable cases and 774 deaths as of July 31, 2003. A large proportion of these SARS outbreaks occurred in hospitals, and 21% of probable SARS cases involved healthcare workers (1). Protecting healthcare workers is essential from the standpoints of both public and occupational health. Experience in hospitals has suggested that appropriate infection control measures, including use of personal protective equipment, personal hygiene, and environmental measures, such as area isolation, protect healthcare workers from SARS (2,3). During the SARS epidemic, hospitals in affected areas emphasized training and issued guidelines on infection control and use of personal protective equipment (4–6). To prepare for future potential outbreaks of SARS and other emerging infectious diseases, implementing appropriate infection control measures in healthcare settings and assessing the efficacy of those measures in the postepidemic period are necessary.

Japan was one of the few Asian countries to be spared from the SARS epidemic in 2003. Although Japan did not experience cases of SARS, healthcare workers in Japan likely felt insecure in their work environment because of the situation in neighboring countries. Quah et al. (7) reported that the anxiety level of the general population in Singapore was low at the height of the SARS epidemic (55% of the respondents reported a low anxiety level). In contrast, Nickell et al. (8) reported that, in a teaching hospital in Toronto, the SARS outbreak had substantial psychological effects on healthcare workers, whose General Health Questionnaire scores suggested that "a probable case of emotional distress" was more than double the level of the general population. However, the level of anxiety (i.e., perception of risk) among healthcare workers has yet to be evaluated in Japan. Infection control measures and other administrative support also must be examined at the institutional level, which may influence the perception of risk among healthcare workers.

Another point of interest is the comparison between the overall preparedness of Japan for SARS and the preparedness of other countries. Thus, we joined an international collaborative effort to study the perception of risk and countermeasures for SARS among healthcare workers and conducted a survey concerning those issues among healthcare workers in Japan. The objective of the present analysis was 2-fold: 1) to assess healthcare workers' perception of risk, knowledge of preventive measures, and perception of infection control measures at the institutional level and 2) to evaluate the interrelationships among these factors, with a focus on institutional measures.

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Materials and Methods

Study Population

The study population comprised 9,978 healthcare workers working at 7 tertiary-level hospitals distributed throughout Japan; 4 of the hospitals are university-affiliated, 2 are municipal, and 1 is private. The study participants held a wide range of jobs in each institution. The questionnaire was administered from July through September 2003. Overall, 7,463 healthcare workers responded to the questionnaire (crude response rate 74.8%). After missing or invalid responses for sex, age, or job category were excluded, 7,282 were finally analyzed (valid response rate 73.0%) (Table 1).

Table 1. Demographic characteristics of respondents*

Variable	n (%)
Age, y (mean 35.6 ± SD 11.2)	
<35	3,963 (54.4)
≥35	3,319 (45.6)
Sex	
Women	5,077 (69.7)
Men	2,205 (30.3)
Job category	
Physicians	1,370 (18.8)
Nurses	3,274 (45.0)
Others†	2,638 (36.2)
Tenure at this job, y (mean 11.1 ± SD 9.6)	
<10	3,884 (54.1)
≥10	3,292 (45.9)
Type of facility	
University hospitals (4 facilities)	5,163 (70.9)
Municipal hospitals (2 facilities)	1,344 (18.5)
Private hospital (1 facility)	775 (10.6)
Total	7,282 (100.0)

*SD, standard deviation.
†Others include nursing assistant, social worker, pharmacist, clinical and radiologic technologist, physical therapist, occupational therapist, speech therapist, managerial staff, clerk, educational and research staff, building maintenance staff, cleaner, nutritionist, and licensed cook.

Questionnaire

This study formed part of an international collaborative study involving healthcare workers in Singapore, China, Taiwan, Canada (Toronto), and Japan. The questionnaire was developed in English at the National University of Singapore, translated into Japanese, and adapted to accommodate background conditions (i.e., no outbreak). The questionnaire was anonymous, and procedures involving human participants were approved by the institutional review board of the University of Occupational and Environmental Health, Japan.

The questionnaire included 24 items regarding knowledge of preventive measures (15 items), concept of (opinion regarding) institutional measures (4 items), and perception of risk (5 items) (online Appendix 1 available from http://www.cdc.gov/ncidod/EID/vol11no03/04-0631_app1.htm). These 24 items were measured on a 7-point scale for responses (strongly agree, agree, probably agree, probably disagree, disagree, strongly disagree, and not applicable). In the statistical analyses, we dichotomized this scale into positive response (strongly agree, agree, and probably agree) and negative response (strongly disagree, disagree, and probably disagree) after excluding "not applicable."

To assess knowledge of preventive measures, we analyzed responses to questions regarding the effectiveness of measures to avoid contracting SARS (personal protective equipment, personal hygiene, environmental measures). The 15 items are shown in Appendix 1. The correct response to each item was designated on the basis of World Health Organization (WHO) guidelines (2) and other findings. The correct responses for the 15 items were a positive response for all items except "paper mask" and "gauze mask," which required a negative response. To assess concept (opinion) of institutional measures, we used 4 items regarding "clear policies and protocols," "specialist available," "adequate training," and "effectiveness." To assess perception of risk, we used 5 items regarding "avoidance of patient," "acceptance of risk," "little personal control," "fear," and "job change," as indicators (Appendix 1).

We quantified the degree of concept of institutional measures and that of knowledge of preventive measures by calculating the institutional (I) and knowledge (K) scores. The I-score was defined as the total number of positive answers to the 3 specific questions regarding "clear policies and protocols," "specialist available," and "adequate training"; the maximum possible I-score was 3 points. "Effectiveness" was excluded from the calculation of the I-score because it could be looked upon as a combined, general concept of institutional measures. The K-score was defined as the total number of correct (either positive or negative) answers to the 15 questions regarding the knowledge of preventive measures; thus the maximum possible K-score was 15 points. The K-score was categorized as high (11–15 points), middle (6–10 points), or low (0–5 points). Cronbach's α was 0.87 for the K-score and 0.76 for the I-score, which indicated a high degree of internal consistency for each score.

Statistical Analysis

The chi-square test was used to evaluate differences in the proportion of respondents according to job category (physician, nurse, and other), sex, age, and type of facilities. The Student *t* test was used to evaluate differences in the mean value between 2 groups, and analysis of variance was used to evaluate differences in the mean value among 3 groups. Logistic regression analyses were used to identify factors associated with the overall concept of the effectiveness of institutional measures ("effectiveness") and

perceptions of risk ("avoidance of patient" and "acceptance of risk") as the dependent variables. The independent variables were the I-score (0, 1, 2, and 3 points), the K-score (low, middle, and high), age (<35 years old, ≥35 years old), sex (men, women), fear (–, +), and type of facility (nonuniversity hospital, university hospital). The logistic regression model was applied to each of the 3 job categories and to all participants. Spearman's correlation coefficients among 6 independent variables were <0.26, and no strong correlations were seen among them. Data were analyzed by using SPSS, version 11.5J (SPSS Inc., Chicago, IL, USA) for Windows and SAS V8 (SAS Institute Inc., Cary, NC, USA). All reported p values are 2-tailed, and $p < 0.05$ was considered statistically significant.

Results

Levels of knowledge of preventive measures, concept of institutional measures, and perception of risk are shown in Table 2 (complete data are available in online Appendix 2 from http://www.cdc.gov/ncidod/EID/vol11no03/04-0631_app2.htm). The proportion, mean score, or both were calculated for each item or category according to job category (physician, nurse, and other), sex, age, and type of facility, as well as the total. As shown in Table 3, the distribution of job categories was significantly different between the 2 types of facility (university hospitals and nonuniversity hospitals), with a higher proportion of physicians and lower proportion of nurses in university hospitals. The corresponding proportion did not differ substantially between municipal and private hospitals, so we categorized the 2 types as nonuniversity hospital for further analyses.

For knowledge of preventive measures, the overall correct response rates were, in descending order, area isolation (98.1%), hand washing (98.0%), alcohol rubs (93.3%), prominent notices (89.9%), N95 mask (86.9%), gloves (79.3%), gowns (67.0%), surgical masks (64.2%), temperature checks (60.9%), hair cover (59.7%), paper mask (59.0%), goggles (56.2%), gauze mask (54.5%), shoe cover (53.3%), and limiting visitors (35.3%). The

Table 2. Knowledge of preventive measures, concept of institutional measures, and perception of risk by job category, sex, age, and type of facility*

	Job category			Sex		Age		Type of facility		
Questionnaire item	Physicians	Nurses	Other	Men	Women	<35 y	≥35 y	University	Nonuniversity†	Total
Knowledge of preventive measures										
Area isolation	96.0	99.3	97.8	96.3	98.9	98.1	98.2	97.8	98.9	98.1
Hand washing	95.6	99.2	97.8	96.0	98.9	98.1	97.9	97.8	98.7	98.0
Alcohol rubs	87.0	94.6	95.1	89.8	94.9	93.2	93.5	93.2	93.6	93.3
Prominent notices	86.8	91.2	89.9	86.1	91.6	89.7	90.2	89.5	91.0	89.9
N95 mask	86.2	89.5	83.8	85.7	87.5	86.4	87.6	85.6	90.1	86.9
Gloves	73.7	82.6	78.2	74.8	81.3	78.0	81.0	77.3	84.3	79.3
Gowns	63.5	74.5	58.8	62.0	69.1	65.8	68.5	63.9	74.4	67.0
Surgical mask	64.5	62.5	66.4	63.6	64.5	64.6	63.8	63.8	65.1	64.2
Temperature checks	51.2	61.5	65.4	58.1	62.2	61.6	60.1	60.5	61.9	60.9
Hair cover	55.1	63.9	56.6	56.3	61.1	56.9	63.1	56.1	68.3	59.7
Paper mask	64.3	62.3	51.6	61.5	58.0	56.9	61.8	59.5	57.9	59.0
Goggles	57.7	56.3	55.3	56.7	56.0	51.9	61.7	52.9	64.2	56.2
Gauze mask	58.5	58.6	46.7	54.4	54.5	51.5	58.3	54.3	54.8	54.5
Shoe cover	50.6	55.5	51.9	50.7	54.4	50.7	56.5	49.4	62.6	53.3
Limiting visitors	29.9	41.3	30.5	31.6	36.9	33.4	37.6	32.6	41.9	35.3
Concept of institutional measures										
Clear policies and protocols	62.8	70.6	59.4	62.6	66.4	61.6	69.7	62.8	71.2	65.2
Specialist available	42.6	59.6	50.4	45.4	56.5	48.8	58.3	49.0	62.8	53.0
Adequate training	29.4	48.9	31.9	31.7	42.4	35.2	43.8	35.7	47.1	39.1
Effectiveness	27.2	34.0	29.5	30.6	31.7	28.1	34.6	28.4	37.6	31.1
Perception of risk										
Avoidance of patient	86.9	93.4	92.1	87.3	93.7	93.1	90.0	91.1	93.3	91.7
Acceptance of risk	69.5	64.7	60.9	67.0	63.1	62.3	66.6	64.0	64.8	64.3
Little personal control	59.8	61.7	59.8	59.2	61.3	60.6	60.7	60.5	61.1	60.6
Fear	48.9	60.6	52.1	48.7	58.2	55.8	54.7	52.2	62.9	55.3
Job change	14.3	34.1	24.7	15.7	31.9	30.7	22.5	24.1	33.8	27.0

*Data are presented as percentages, number of positive responses divided by number of respondents answering each question (except for paper mask and gauze mask, where negative responses were counted). Positive responses include "probably agree," "agree," and "strongly agree"; negative responses are "probably disagree," "disagree," and strongly disagree." Detailed information, including n's, distribution of scores, and p values (based on chi-square test for difference in proportion, *t* test for difference in 2 means, and analysis of variance [ANOVA] for differences in 3 means), is available in the full table in online Appendix 2.
†Nonuniversity includes municipal hospitals (2 facilities) and private hospitals (1 facility).

Table 3. Job categories by type of facility

Type of facility	Physicians, n (%)	Nurses, n (%)	Others, n (%)	p value*	Total
University hospital (4 facilities)	1,116 (21.6)	2,225 (43.1)	1,822 (35.3)	<0.001	5,163 (100.0)
Nonuniversity hospital (3 facilities)	254 (12.0)	1,049 (49.5)	816 (38.5)		2,119 (100.0)
Municipal hospital (2 facilities)	153 (11.4)	688 (51.2)	503 (37.4)		1,344 (100.0)
Private hospital (1 facility)	101 (13.0)	361 (46.6)	313 (40.4)		775 (100.0)

*p value based on chi-square test between university and nonuniversity hospitals.

correct response rate differed significantly among job categories for all items except for goggles. As a general trend, physicians ranked third for 9 items, nurses ranked first for 10 items, and others ranked second for 7 items. The K-score distribution and mean indicated the highest score for nurses, intermediate for physicians, and lowest for others. The correct response rate differed significantly between men and women for all items except goggles, gauze mask, and surgical mask. As a general trend, women ranked higher than men for 13 out of 15 items. Accordingly, the K-score distribution and mean indicated a significantly higher score for women. This trend was observed in physicians but not in nurses when the analysis was conducted separately for each group (data not shown). The correct response rate differed significantly between the 2 age categories for 8 items. As a general trend, older workers (≥35 years old) ranked higher for 12 of the 15 items. However, neither the K-score distribution nor the mean K-score indicated a higher score for older workers. The correct response rate differed significantly between the 2 types of facilities for 9 items. As a general trend, nonuniversity hospital ranked higher for 14 of the 15 items. Accordingly, the K-score distribution and mean indicated a significantly higher score for nonuniversity hospital.

For concept of institutional measures, the overall proportion of positive responses were, in descending order, clear policies and protocols (65.2%), specialist available (53.0%), adequate training (39.1%) (concept of respective institutional measures), and effectiveness (31.1%) (overall concept of effectiveness of institutional measures). For all items, the positive response rate differed significantly among the 3 job categories, with nurses consistently ranked the highest. The I-score distribution and mean indicated the highest score for nurses, intermediate for physicians, and lowest for others. For all items except for the overall concept of effectiveness, the rate of positive responses was significantly higher for women than men. The I-score distribution and mean indicated a higher score for women than men. For all items, the positive response rate was significantly higher for older workers than younger workers. Accordingly, the I-score distribution and mean indicated a significantly higher score for older workers than younger workers. For all items, the positive response rate was significantly higher for nonuniversity hospital than university hospital. Accordingly, the I-score distribution and mean indicated a significantly higher score for nonuniversity hospital than university hospital.

For perception of risk, the overall positive response rates were, in descending order, avoidance of patient (91.7%), acceptance of risk (64.3%), little personal control (60.6%), fear (55.3%), and job change (27.0%). The positive response rate differed significantly among the job categories for all items except little personal control. Nurses ranked highest for avoidance of patient (93.4%), whereas physicians ranked highest for acceptance of risk (69.5%). Nurses showed the highest level of fear (60.6%) and physicians the lowest (48.9%). Nurses had the highest tendency to consider job change (34.1%) and physicians the lowest (14.3%). The positive response rate differed significantly between men and women for all items except little personal control. Compared to men, women had a significantly higher proportion of positive responses to avoidance of patient (93.7% vs. 87.3%, $p < 0.001$), fear (58.2% vs. 48.7%, $p < 0.001$), and job change (31.9% vs. 15.7%, $p < 0.001$) and lower proportion of positive responses to acceptance of risk (63.1% vs. 67.0%, $p = 0.002$). The positive response rate differed significantly between the 2 age categories for all items except little personal control and fear. Compared to younger workers, older workers had a lower proportion of positive responses to avoidance of patient (90.0% vs. 93.1%, $p < 0.001$) and job change (22.5% vs. 30.7%, $p < 0.001$), and a higher proportion of positive responses to acceptance of risk (66.6% vs. 62.3%, $p < 0.001$). The positive response rate differed significantly between university hospital and nonuniversity hospital for all items except acceptance of risk and little personal control. Compared to university hospitals, nonuniversity hospitals had a significantly higher proportion of positive responses to avoidance of patient (93.3% vs. 91.1%, $p = 0.002$), fear (62.9% vs. 52.2%, $p < 0.001$), and job change (33.8% vs. 24.1%, $p < 0.001$).

As shown in Table 4, logistic regression analyses indicated that effectiveness (as overall conception of effectiveness of institutional measures) was positively associated with the I-score in all 3 job categories and with age in 1 job category (others). Effectiveness was negatively associated with fear in 2 job categories (physicians and others) and with type of facility in 2 job categories (nurses and others). Avoidance of patient was positively associated with fear in all 3 job categories, with gender in 1 job category (others), and with K-score in 1 job category (physicians).

Table 4. Factors associated with concept of institutional measures (effectiveness) and perception of risk (selected factors) by job category

Variable*	OR (95% CI)†			
	Physician (N = 1,370)	Nurse (N = 3,274)	Other (N = 2,638)	Total (N = 7,282)
Effectiveness				
I-score (0,1,2,3)	1.87 (1.65–2.13)	1.83 (1.69–1.98)	2.02 (1.84–2.22)	1.90 (1.80–2.01)
Age (≥35 year)	1.23 (0.93–1.62)	1.19 (0.99–1.41)	1.42 (1.15–1.74)	1.25 (1.11–1.41)
K-score (low, middle, high)	0.89 (0.72–1.10)	1.01 (0.88–1.17)	0.96 (0.82–1.11)	0.97 (0.88–1.06)
Type of facility (university)	0.82 (0.59–1.15)	0.71 (0.60–0.84)	0.75 (0.60–0.92)	0.74 (0.65–0.83)
Sex (women)	0.79 (0.53–1.18)	0.63 (0.38–1.06)	0.97 (0.79–1.18)	1.00 (0.88–1.14)
Fear (+)	0.61 (0.46–0.80)	0.90 (0.76–1.06)	0.58 (0.47–0.70)	0.72 (0.64–0.81)
Avoidance of patient				
Fear (+)	1.91 (1.33–2.76)	2.56 (1.89–3.48)	2.32 (1.66–3.24)	2.31 (1.91–2.80)
K-score (low, middle, high)	1.42 (1.10–1.84)	1.01 (0.78–1.30)	1.23 (0.98–1.56)	1.19 (1.03–1.37)
Sex (women)	1.27 (0.76–2.13)	1.95 (0.91–4.21)	2.05 (1.50–2.82)	1.93 (1.59–2.33)
Age (≥35 year)	1.00 (0.70–1.42)	0.58 (0.42–0.79)	0.84 (0.60–1.17)	0.81 (0.67–0.97)
Type of facility (university)	0.91 (0.57–1.43)	0.83 (0.59–1.16)	0.75 (0.52–1.08)	0.82 (0.66–1.01)
I-score (0,1,2,3)	0.80 (0.68–0.94)	0.87 (0.75–0.99)	0.80 (0.69–0.93)	0.81 (0.75–0.88)
Acceptance of risk				
I-score (0,1,2,3)	1.26 (1.11–1.42)	1.08 (1.01–1.16)	1.24 (1.14–1.35)	1.18 (1.12–1.24)
Age (≥35 year)	1.05 (0.81–1.36)	1.27 (1.06–1.51)	1.11 (0.93–1.33)	1.10 (0.99–1.22)
Fear (+)	1.01 (0.78–1.30)	1.14 (0.97–1.33)	1.44 (1.21–1.71)	1.21 (1.09–1.34)
Sex (women)	1.01 (0.71–1.43)	0.65 (0.38–1.12)	0.85 (0.71–1.02)	0.82 (0.73–0.92)
K-score (low, middle, high)	0.90 (0.74–1.10)	1.05 (0.92–1.20)	1.04 (0.91–1.18)	1.03 (0.95–1.12)
Type of facility (university)	0.81 (0.58–1.14)	0.98 (0.83–1.15)	1.20 (0.99–1.45)	1.04 (0.93–1.17)

*Goodness-of-fit was satisfactory: ranged from [goodness-of-fit statistics = 0.49 with 8 df (p = 0.99)] for (effectiveness) x (physician) to [goodness-of-fit statistics = 12.71 with 8 df (p = 0.12)] for (nurse) x (avoidance), except for [goodness-of-fit statistics = 18.98 with 8 df (p = 0.02)] for (nurse) x (effectiveness) and [goodness-of-fit statistics = 18.38 with 8 df (p = 0.02)] for (others) x (effectiveness).
†OR, odds ratio calculated by logistic regression; CI, confidence interval.

Avoidance was negatively associated with I-score in all 3 job categories and with age in 1 job category (nurses). Acceptance of risk was positively associated with I-score in all 3 job categories, with age in 1 job category (nurse), and fear in 1 job category (others). Hence, the I-score was a significant positive predictor of effectiveness (as overall conception of effectiveness of institutional measures) in all 3 job categories, a significant negative predictor of avoidance of patient in 2 job categories (physician and others), and a significant positive predictor of acceptance of risk in all 3 job categories.

Discussion

A substantial number of probable SARS cases were concentrated in Asian countries during the previous SARS epidemic (5,327 cases in China, 1,755 cases in Hong Kong, 346 cases in Taiwan, and 238 cases in Singapore as of July 31, 2003) (1). Accordingly, strict policies and administrative measures for infection control (e.g., mandatory quarantine and training of healthcare workers in infection control measures) were implemented in these countries (9–11). In contrast, no probable SARS cases were recorded in Japan, and thus administrative measures for infection control tended to be hypothetical (i.e., most countermeasures at the institutional level were voluntary) (12,13). As such, the Japanese situation is distinct from that in other Asian countries, and various aspects of knowledge, perception, and attitudes of healthcare workers regarding SARS are likely to differ between Japan and other Asian countries. To clarify this issue, we assessed the level of knowledge of preventive measures, concept of institutional measures, and perception of risk and their interrelationships in healthcare workers in Japan.

SARS Knowledge, Concept of Institutional Measures, and Perception of Risk

Regarding knowledge of preventive measures, most respondents assigned relatively high importance to hand hygiene and area isolation but saw personal protective equipment as being of relatively low importance. This finding may be partly due to healthcare workers' not having previously used some of the protective equipment recommended for use with SARS patients (3). The use of personal protective equipment as countermeasures for SARS has been rightly advocated by various authors (10,14,15). Thus, adequately training healthcare workers in the use of personal protective equipment is an important aspect of reducing the incidence of SARS infection.

Regarding the concept of institutional measures, 40% of respondents believed that they had received adequate training; for example, less than half felt that they had adequate training in the use of masks. During the SARS epidemic, medical institutions were required by authorities to provide adequate training to healthcare workers in affect-

ed countries (5,6,10,11). Because no outbreaks were in Japan, however, Japanese institutions have not been forced to implement sufficient measures to adequately cope with future outbreaks of SARS and other emerging diseases.

Regarding perception of risk, although we did not compare healthcare workers with an external group, more than half (55%) of the healthcare workers surveyed indicated that they were afraid. Furthermore, a high proportion of healthcare workers preferred to avoid the patient (92%), although almost two thirds accepted the risk (64%). When these 2 items were cross-classified, 55% of respondents showed a mixed attitude (i.e., avoidance of patient [+] and acceptance of risk [+]), 32% showed a disloyal attitude (i.e., avoidance of patient [+] and acceptance of risk [–]), and 6% showed a loyal attitude (i.e., avoidance of patient [–] and acceptance of risk [+]). These results indicate a high level of fear and anxiety with complex psychology in Japanese healthcare workers, even in the absence of an epidemic.

Significant differences were seen in the level of knowledge and attitudes among the 3 job categories. Nurses showed the best knowledge of preventive measures and concept of institutional measures, while physicians showed the highest acceptance of risk. Both sex and job characteristics may have influences in this regard. Ninety-eight percent of nurses were women, whereas 84% of physicians were men. Quah et al. reported that, in Singapore, women showed better practice of SARS preventive measures than men among the general population (7). Similarly, our results indicated a higher level of knowledge regarding preventive measures for female physicians compared to male physicians. However, this trend was not observed within the nurse job category, although the number of male nurses was sufficiently small that separating the effect of sex was difficult. In terms of job characteristics, nurses may receive more official training in infection control than physicians, under the assumption that physicians are already knowledgeable. In fact, compared to physicians, nurses have higher levels of compliance with universal precautions (16) and hand-washing (17,18) in their respective countries. However, nurses tend to have higher job turnover rates than physicians, which reflect less stability or security in their profession. These factors directly and indirectly influence the response pattern among the 3 job categories.

Interrelatedness of Knowledge, Concept of Institutional Measures, and Perception of Risk

In the logistic regression model, K-score, an indicator of knowledge of preventive measures, was not a significant predictor of perception of either risk or concept of institutional measures. This finding implies that professional knowledge has little, if anything, to do with positive perception of risk (in terms of accepting risk and not avoiding patients) and concept of institutional measures. However, the importance of providing accurate knowledge cannot be discounted solely on this ground. In contrast, I-score, an indicator of concept of institutional measures, was a significant positive predictor of concept of effectiveness and acceptance of risk and a significant negative predictor of avoidance of patient. In other words, a collective assertion of 3 specific institutional measures (clear policies and protocols, specialists available, and adequate training) had the greatest effect on a person's 2 different aspects of perception of risk and concept of the effectiveness of institutional measures. These findings corroborate earlier studies reporting that administrative support enhances compliance with universal precautions (19–21) and hand washing (17,18). Therefore, we infer that perception of institutional measures affects perception of risk and related behaviors.

Fear was a significant negative predictor of concept of effectiveness in 2 job categories (physicians and others) and a significant positive predictor of avoidance of patient in all 3 job categories. These findings were in line with our expectations and signal the need to reduce fear as a practical goal. Older age was a significant positive predictor for the concept of effectiveness in 2 job categories (nurses and others). Among nurses, older age was also a significant negative predictor for avoidance of patient and a significant positive predictor for acceptance of risk. Age has previously been shown to be a positive predictor for practicing SARS preventive measures among the general population (7). Hence, older age seems to correlate with an increased ability to cope with emergency situations related to infectious diseases. Type of facility (university hospital) was a significant negative predictor for the concept of effectiveness in 2 job categories (nurses and others). Although confined to the 7 facilities studied, university hospitals may have been less stringent in the formulating or implementing infection control measures, which in turn affected the overall concept of effectiveness of measures among healthcare workers.

Limitations

Our study has several limitations. First, the cross-sectional nature of the study prevents assertion of cause and effect. Our conclusion, particularly on interrelationship among individual factors, is based on inferences. Second, responder bias may have been in play, i.e., only workers with a strong interest in SARS may have been motivated to respond, although the fairly high response rate counteracts this argument to an extent. Third, K-score may not accurately reflect knowledge of preventive measures. For example, workers who, in practice, had accurate knowl-

edge about shoe covers as personal protective equipment may have answered incorrectly because they had been taught conflicting information. In fact, the Infectious Disease Surveillance Center (IDSC), Japan, categorizes shoe cover as optional personal protective equipment (22). However, among the personal protective equipment considered in this study, only alcohol rubs (WHO) (2) and shoe cover (IDSC, Japan) (22) are considered optional, and the effect of conflicting information should not be strong. Fourth, we considered the difference in type of facility (university or nonuniversity) but did not consider differences by facility (hospital A or B) or type of unit (internal medicine, surgery, and others), which may be related to differences in job descriptions (even within the same job category) as well as the study variables. Such effects caused by affiliation constitute a separate theme worth further investigation, which will be pursued.

We found that the level of anxiety among healthcare workers in Japan was relatively high and that the implementation of preventive measures at the institutional level was not perceived to be sufficient. However, a collective assertion of 3 specific institutional measures stood out as the most important predictor for individual perception of risk, including avoidance of patient and acceptance of risk, as well as concept of general effectiveness of institutional measures. In view of the potential for future epidemics of SARS or other emerging infectious diseases, the planning and implementation of institutional measures should be given a high priority.

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Longitudinally Profiling Neutralizing Antibody Response to SARS Coronavirus with Pseudotypes

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The severe acute respiratory syndrome–associated coronavirus (SARS-CoV) spike protein (S) is a major target for neutralizing antibodies. Retroviral SARS-CoV S pseudotypes have been constructed and used to develop an in vitro microneutralization assay that is both sensitive and specific for SARS-CoV neutralizing antibodies. Neutralization titers measured by this assay are highly correlated to those measured by an assay using replication-competent SARS-CoV. No cross-neutralization occurred with human sera known to contain antibodies to coronavirus strains OC43 and 229E. The pseudotype assay was used to profile neutralizing antibody responses against SARS-CoV S in sequential serum samples taken from 41 confirmed SARS patients during the 2003 outbreak in Hong Kong and shows long-lasting immunity in most recovered patients. The pseudotype assay does not require handling live SARS virus; it is a useful tool to determine neutralizing titers during natural infection and the preclinical evaluation of candidate vaccines.

The coronavirus that causes severe acute respiratory syndrome (SARS-CoV) is a new human pathogen for which a vaccine may be urgently required should a new outbreak occur. Studying the magnitude and longevity of the neutralizing antibody response during natural infection will help establish correlates of protection to be generated by immunization. Humoral immunoglobulin (Ig) G, IgM, and IgA responses to SARS-CoV have been studied extensively (1–7). However, studies of neutralizing antibody responses during natural infection have been limited (8,9), partially because neutralization assays must be performed at biosafety level 3 or higher.

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The SARS-CoV genome encodes 4 structural proteins, the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins (10). The S protein is the major surface antigen of the virus, and the neutralizing antibody response is primarily directed against this protein. Monoclonal antibodies to the S protein neutralize the virus and have been mapped (11–14). By vaccinating hamsters with a recombinant parainfluenza virus vector, Buchholz et al. found that the expression of M, E, or N, in the absence of S, did not induce a neutralizing antibody response (15). Preclinical studies of SARS-CoV vaccines provide evidence that generating a strong neutralizing antibody response to SARS-CoV S may protect against SARS infection (16–19).

Retroviral and lentiviral pseudotypes have been employed in lieu of replication-competent virus to study neutralizing antibody responses to viral infection (20,21). Pseudotype viruses encode marker genes and bear foreign viral envelopes (22). The transfer of marker genes to target cells depends on the function of the envelope protein; therefore, the titer of neutralizing antibodies against the envelope can be measured by a reduction in marker gene transfer. Lentiviral pseudotypes bearing the SARS-CoV spike protein were first described by Simmons et al. to study viral entry (23). Other studies have used SARS-CoV S pseudotyped viruses for identifying receptors (24), examining viral tropism (25–27), and measuring neutralizing antibody responses (18,28–30). Yang et al. constructed lentiviral pseudotypes harboring S, M, or E proteins and found that only S supported viral entry into target cells (26).

The aim of this study was to establish a neutralizing antibody assay using murine leukemia virus (MLV) pseudotypes bearing the SARS-CoV S envelope, MLV(SARS), and to profile neutralizing antibody responses to SARS-CoV natural infection during a relatively long period in a cohort of Hong Kong patients who had recovered from the disease.

Materials and Methods

Patient Samples

A total of 166 blood samples were obtained from 41 patients (68% female) 11–80 years of age who were admitted to the Prince of Wales Hospital, Hong Kong, from March to May 2003. All study patients fulfilled the World Health Organization criteria for having a probable case of SARS. Samples from 7 of the 41 patients were tested for SARS-CoV by reverse transcription–polymerase chain reaction (RT-PCR) in a study previously described (31), and 4 patients had positive results. Pneumonia developed in all 41 patients, and 6 required intensive care. None of these patients died of the infection. For most patients, multiple samples were obtained at sequential times covering the acute, convalescent, and recovered phase of the disease. This study was approved by the Prince of Wales Hospital local institutional ethics committee.

Plasmids and Cell Lines

Construction of the plasmid pCAGGS-S harboring full-length SARS-CoV S from the Urbani strain has been described previously (23). The MLV gag/pol construct, pCMVi, and the green fluorescent protein (GFP) reporter construct, pCNCG, have been described (32). Vesicular stomatitis virus envelope protein (VSV-G) expression vector pMDG has been described previously (33). HIV constructs were used as described (34).

All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax and high glucose (Gibco, Paisley, Scotland, UK), supplemented with 10% fetal calf serum and penicillin/streptomycin. To make the quail QT6/ACE2 cell line, the gene encoding the receptor for SARS-CoV, human angiotensin-converting enzyme 2 (ACE2) (35), was cloned from a human primary kidney cDNA library (Invitrogen, Paisley, Scotland, UK) using 21-mer primers designed to the start and stop of ACE2, and subcloned into pcdna3.1+. QT6 cells were transfected by using lipofectamine 2000 and selected with G418, and a bulk ACE2-positive, G418-resistant population was grown.

Viral Vector Production and Infection of Target Cells

Confluent plates of 293T cells were split 1:4 the day before transfection. Each plate of 293T cells was transfected with 1 μg gag/pol construct, 1.5 μg of enhanced GFP reporter construct, and 1.5 μg envelope-expressing construct by using the Fugene-6 transfection reagent (36). Supernatant was harvested 48 h and 72 h posttransfection, filtered through 0.45-μm filters, and stored at –80°C. MLV and HIV vector titer were measured on 293T, TE671, and QT6/ACE2 cells and are presented as infectious units (IU) per milliliter. Briefly, cells were infected with vector, and eGFP titers were determined 72 h later by fluorescence-activated cell sorter (FACS).

Neutralization Assays

Live Virus

Patient serum samples were heat inactivated at 56°C for 30 min and serially diluted from 1:10 in culture medium. Fifty PFU of SARS Frankfurt strain were added to the serum dilution and incubated for 1 h at 37°C. We added 5×10^4 Vero E6 cells per well to the virus and serum mix, and the mixture was incubated in 96-well plates for 4 days, after which neutralization was assessed by cytopathic effect (CPE). The neutralization endpoint was taken as the last well in which complete neutralization was observed. Serum samples were assayed in duplicate, and positive results were confirmed in separate assays.

Pseudotype

Patient serum samples were heat inactivated at 56°C for 30 min, 2-fold serially diluted from 1:10 in culture medium, and mixed with MLV(SARS) virions (≈100 IU) at a 1:1 vol/vol ratio. After incubation at 37°C for 1 h, 100 μL of each dilution was added to QT6/ACE2 cells seeded at 1×10^4 cells per well in 96-well flat-bottomed tissue culture plates seeded 24 h previously. GFP-positive cells were counted 48 h later by fluorescence microscopy. Neutralizing antibody titers are presented as geometric mean titers of assays performed in triplicate.

Results

Production of MLV S Pseudotypes

Retroviral particles pseudotyped with SARS-CoV S were made by cotransfection of an S-expressing plasmid, pCAGGS-S, with plasmids encoding MLV or HIV gag-pol and GFP vector genome in 293T cells. Culture supernatants were used to infect human TE671, 293T, and quail QT6/ACE2 cell lines. VSV-G pseudotyped MLV particles, MLV(VSV), and HIV particles, HIV(VSV), were used as controls. MLV(VSV) and HIV(VSV) pseudotypes infected all 3 cell lines tested. MLV(SARS) and HIV(SARS) pseudotypes infected 293T (which have a low level of endogenous ACE2 expression) and QT6/ACE2 but not TE671 cells (Figure 1). The highest titer (3.5×10^5 IU/mL) was obtained with the combination of QT6/ACE2 cells and MLV(SARS), so this system was employed for all subsequent assays.

Validation of Pseudotype Microneutralization Assay

A blinded panel of 50 samples comprising sera from healthy persons, patients infected with other human coronaviruses (OC43 and 229E), patients infected with influenza

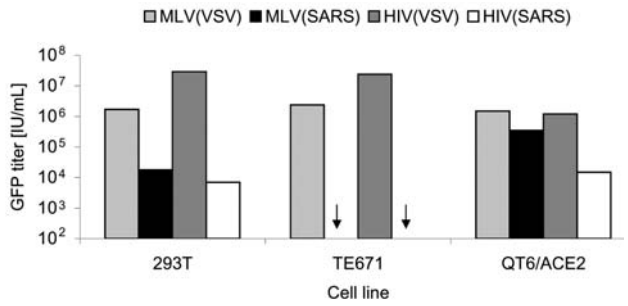

Figure 1. Infectivity of retroviral severe acute respiratory syndrome–associated coronavirus (SARS-CoV) spike protein (S) pseudotypes on target cells. SARS-CoV S-mediated infection of human 293T, TE671, and quail QT6/ACE2 was assessed. Murine leukemia virus (MLV) or HIV pseudotypes bearing either the pantropic vesicular stomatitis virus envelope protein (VSV-G) as a positive control, or the SARS-CoV S, were added to target cells. After 72 h, green fluorescent protein (GFP)–positive cells were counted by fluorescence-activated cell sorter analysis. Infection titers are given as infectious units per milliliter (IU/mL). Arrow indicates that infection titer was less than the detection limit, 10^2 IU/mL.

virus, and persons who were convalescent from SARS was provided by the Health Protection Agency (HPA), United Kingdom, for the validation of our pseudotype neutralization assay. For 12 samples positive for both assays, 90% and 50% inhibitory concentration (IC_{90} and IC_{50}) pseudotype neutralizing titers were compared with titers obtained at HPA by neutralization assay using replication-competent SARS-CoV. Logarithmic plots of pseudotype versus live virus neutralization titers are shown in Figure 2. Correlation coefficients for pseudotype IC_{90} and IC_{50} titers versus live SARS-CoV neutralization titers were 0.69 and 0.78, respectively. MLV(SARS) entry into QT6/ACE2 cells was not substantially inhibited by sera from healthy persons or from persons with human coronavirus OC43 and 229E antibodies. MLV(VSV) infection was not inhibited by any sera (data not shown). The pseudotype assay was thus shown to be both sensitive and specific for SARS-CoV neutralizing antibodies, with no evidence for cross-reaction with the other human coronaviruses. Although the live virus assay was based on the Frankfurt SARS-CoV isolate, and the pseudotype assay was based on the Urbani isolate, they gave equivalent titers, including analysis of serum from the person from whom the Frankfurt isolate was made.

Neutralizing Antibody Response to SARS-CoV S

Blood samples from the Hong Kong cohort of patients were tested for neutralizing antibodies to the SARS-CoV S protein by using the pseudotype neutralization assay. Figure 3 shows the number of patients positive for neutralizing antibodies and the mean neutralizing antibody titer displayed by week after onset of fever. Samples taken during the convalescent and recovered phase (after day 28 following onset of fever) are grouped into longer time blocks (29–100 days, 101–200 days, and >201 days). In the first week after onset of fever, all patient samples tested were negative for neutralizing antibody. Appearance of neutralizing antibody was first seen in week 2 with 9 (64%) of 14 patients becoming positive. Geometric mean IC_{90} neutralizing antibody titers ranged from negative (≤10) to 40. In week 3, all patients were positive for neutralizing antibodies with titers from 10 to 200. IC_{90} titers peaked during week 4 (mean titers 28–640) but persisted in some patients for >200 days after onset of fever. Figure 4 shows the longitudinal profiles of neutralizing antibody responses to SARS-CoV S in 4 representative patients for whom serially collected blood samples were available for testing.

Discussion

We have developed a retroviral pseudotype-based assay that facilitates the accurate determination of neutralizing antibody responses to SARS-CoV without the use of replication-competent virus. Since the neutralization titers measured on replication-competent SARS-CoV and pseudotypes are highly correlated, this assay can be widely applied in routine diagnostics and used for the preclinical evaluation of candidate vaccines and immune therapies for SARS, without the pathogen itself being handled. This advantage is important because nosocomial infections have arisen from laboratory handling of SARS-CoV in Taiwan, Singapore, and Beijing (37). A lack of good, quantitative assays for SARS-CoV replication in vitro also

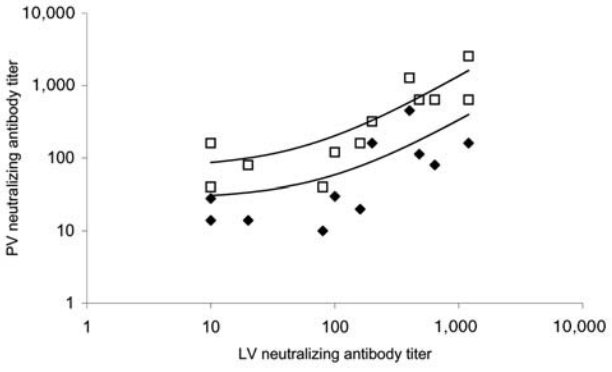

Figure 2. Correlation of neutralizing antibody titers measured by plaque reduction assay with titers measured with pseudotype assay. LV, neutralizing antibody titer by using replication-competent severe acute respiratory syndrome-associated coronavirus (SARS-CoV) (live virus); PV, neutralizing antibody titer by using pseudotype virus; PV_{90} (filled black diamonds), 90% neutralizing antibody titer by using murine leukemia virus (MLV) (SARS) pseudotype virus; PV_{50} (open squares), 50% neutralizing antibody titer. Logarithmic trendlines were fitted to the data by using Microsoft Excel 2003 (Microsoft Corp., Redmond, WA, USA). Correlation coefficients for LV versus PV_{90} and LV versus PV_{50} are 0.69 and 0.78, respectively.

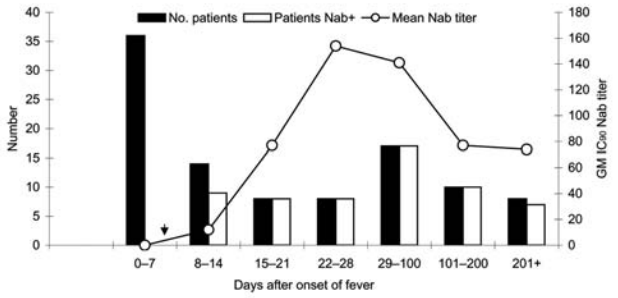

Figure 3. Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) neutralizing antibody-positive rate by time of blood sample collection (days after onset of fever). Black bars represent the number of patients tested for neutralizing antibodies (Nab). White bars represent the number of patients whose assayed samples were positive for neutralizing antibodies (Nab+). Samples are considered positive for Nab if the 90% neutralizing antibody titer determined by using murine leukemia virus (MLV) (SARS) pseudotypes is ≥10. Line plot with open white circles shows the geometric mean (GM) Nab titer within each time frame. IC_{90}, 90% inhibitory concentration.

makes the pseudotype assay, with its easily interchangeable reporter genes, a more flexible platform with which to study neutralization and cell tropism.

Our assay detected neutralizing antibodies generated during both the acute and convalescent phases of SARS infection. When looking for neutralizing antibody responses, previous researchers have predominantly tested samples taken during the convalescent phase of the disease, whereas we found that during the period 8–14 days after onset of fever, 9 patients in our cohort had neutralizing responses to SARS S protein. Viral load, as measured by real-time RT-PCR, for 19 of the patients in our cohort, was previously shown to peak at approximately day 4 or 5 after onset of fever and then decreased to barely detectable around the time of seroconversion (38), which suggests that the neutralizing antibody response may play a role in viral clearance. This finding has implications for diagnostics and surveillance, since positive diagnoses for neutralizing antibodies can be made earlier in infection and as a complement to testing for IgG responses by enzyme-linked immunosorbent assay. SARS has yet to manifest itself as a seasonal epidemic threat like influenza, which makes mass vaccination an unlikely scenario. The rapid detection of neutralizing antibodies seen in this study suggests that localized vaccination with an effective vaccine is likely to help control the spread of SARS-CoV during an outbreak, if vaccine elicits as rapid a response as live virus.

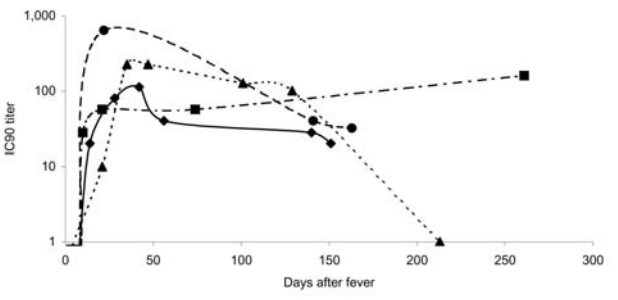

Figure 4. Neutralizing antibodies to severe acute respiratory syndrome-associated coronavirus spike protein in sequential blood samples from 4 representative patients. Lines represent profiles of individual patients. Filled black symbols represent geometric mean titers at individual time points. IC_{90}, 90% inhibitory concentration.

This article also reports longitudinal neutralizing antibody profiles in patients with SARS by using blood samples collected at serial time points (up to day 287). A broad spectrum of longitudinal profiles is seen in patients, and neutralizing antibody levels persist in many recovered persons for several months (Figure 4). In only 1 patient did we find a complete loss of neutralizing antibody titer after a sharp rise, which began at the end of the acute phase (day 10). In a second patient, IC_{90} neutralizing antibody titers attained 640 by day 22 after onset of fever, followed by a decline; however, in another patient, neutralizing antibody was detectable at day 261 after onset of fever. Maintenance of neutralizing antibody titers will have important implications for vaccine design.

Gao et al. (39) tested in rhesus macaques an adenoviral vaccine that was made up of the S1 spike fragment, M, and N; the test showed that strong neutralizing antibody responses were generated, some of which appeared early after vaccination. We have shown that some patients convalescing from SARS have similar responses before full recovery, which suggests that this level of vaccine-induced neutralizing antibodies may be protective. Initial preclinical studies in mice and hamsters are encouraging and show that neutralizing antibodies are sufficient to protect against live virus challenge (16–19,40). Candidate vaccines for SARS must be moved from the preclinical evaluation phase to clinical trials in human volunteers as rapidly as possible, since the possibility of further SARS outbreaks is uncertain. The method used here to analyze natural infection can be applied to clinical trials of candidate vaccines, and we expect this test to be equally applicable to animal sera.

Acknowledgments

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Dr. Temperton is a postdoctoral research fellow at the Wohl Virion Centre, Division of Infection and Immunity, University College London, United Kingdom. His research interests include viral pseudotypes, viral neutralization studies, and vaccines.

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SARS-related Perceptions in Hong Kong

Joseph T.F. Lau,* Xilin Yang,* Ellie Pang,* H.Y. Tsui,* Eric Wong,* and Yun Kwok Wing*

To understand different aspects of community responses related to severe acute respiratory syndrome (SARS), 2 population-based, random telephone surveys were conducted in June 2003 and January 2004 in Hong Kong. More than 70% of respondents would avoid visiting hospitals or mainland China to avoid contracting SARS. Most respondents believed that SARS could be transmitted through droplets, fomites, sewage, and animals. More than 90% believed that public health measures were efficacious means of prevention; 40.4% believed that SARS would resurge in Hong Kong; and ≈70% would then wear masks in public places. High percentages of respondents felt helpless, horrified, and apprehensive because of SARS. Approximately 16% showed signs of posttraumatic symptoms, and ≈40% perceived increased stress in family or work settings. The general public in Hong Kong has been very vigilant about SARS but needs to be more psychologically prepared to face a resurgence of the epidemic.

The severe acute respiratory syndrome (SARS) epidemic affected ≈30 countries, resulting in 8,422 cases and 916 deaths globally (1). Approximately 20.8% (1,755) of the cases and 32.8% (300) of the deaths occurred in Hong Kong. The World Health Organization issued a travel advisory warning against visiting Hong Kong from April 2 to May 23, 2003 (2). School classes were suspended from March to May 2003 (3). More than 90% of Hong Kong residents frequently wore face masks in public places from March through May 2003, and 33.6% worried that they or their family members would contract the disease (4). A number of hypotheses have been generated about different modes of transmission of SARS (5–7). However, responses to many of these issues have not yet been formulated. From December 16, 2003, through April 30, 2004, another 14 new SARS cases were reported in 4 areas in China (8–10). Public health measures played an important role in the control of the spread of SARS in the community (11,12). Whether SARS will reappear in some parts of the world is not known.

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Studies of the psychological effect of disastrous events at a general population level have been reported. Some studies investigated the effect of the September 11, 2001, terrorist attack in the United States (13–16). Longitudinal studies found that some of the mental health problems could become chronic (15,17). Similar studies have been conducted for other disasters, such as the 1995 Sarin attack in the Tokyo subway system (18) and the terrorist attacks in Israel (19). SARS-related psychological problems have been reported to be prevalent in the general population (20,21).

The first objective of our study was to investigate how members of the general population in Hong Kong perceived different aspects of SARS and how people would react to a possible resurgence of SARS ≈6 months after the end of the epidemic (survey 1). The second objective was to assess the mental health effects on the general population at the end phase of the epidemic and to investigate relationships among various reactions, perceptions, and mental health effects (survey 2).

Participants and Methods

The study population was composed of Chinese-speaking residents of Hong Kong (22) who were 18–60 years old. We conducted 2 independent cross-sectional telephone surveys to achieve the 2 aforementioned objectives. Survey 1 was conducted from December 30, 2003, to January 17, 2004, and survey 2 from May 27, 2003, to June 1, 2003. Telephone numbers were randomly selected from up-to-date residential phone directories. Nearly 100% of the Hong Kong residents have telephones at home (Hong Kong Office of the Telecommunications Authority, pers. commun.), and other local studies have used telephone surveys for data collection (21).

Interviewers called between 6:00 p.m. and 10:30 p.m. to avoid undersampling workers and students. If a telephone call was not answered, at least 2 follow-up calls were made at different hours on weekdays. An eligible household member, whose birthday was closest to the date of the interview, was invited to participate in the study. Verbal informed consent was obtained from participants.

Ethical approval was obtained from the Chinese University of Hong Kong. The response rate, defined as the number completing the survey divided by the number of valid households contacted, was 65% for survey 1 and 57.7% for survey 2. Relevant sociodemographic characteristics of respondents are shown in Table 1.

Respondents in survey 1 were asked about SARS-related perceptions, different public health measures currently practiced, and one's anticipated public health and emotional responses if SARS were to return to Hong Kong. Respondents in survey 2 were asked about psychological effects of SARS. These included whether respondents felt horrified, helpless, or apprehensive; had sleeping and psychosomatic problems; had increased smoking and alcohol consumption; or had perceived stress. The Chinese version of the Impact of Event Scale (IES) (23,24) and the mental health (5 items) and the vitality (4 items) subscales of the Medical Outcomes Study 36-Item Short Form Health Survey (SF-36) (25–27) were also used in survey 2.

In survey 1, multivariate logistic regression analysis, using univariately significant responses as input variables, was performed. In survey 2, 2-staged stepwise linear and logistic regression models were used. SPSS for Windows Release 11.0.1 (SPSS Inc., Chicago, IL, USA) was used and p values < 0.05 were considered significant.

Results

Current SARS-related Preventive Behavior

At the time of the first survey, 66.7% and 68.6% of the respondents, respectively, would avoid visiting hospitals or mainland China (Table 2). More than 80% would make a health declaration to customs, use a mask on a flight, or see a doctor when traveling overseas if they had influenza, while 38.7% would see a local doctor in mainland China under such circumstances (Table 2). Women were more likely than men to avoid visiting China or avoid seeing a local doctor if they had influenza when traveling overseas ($p < 0.05$) (Table 2).

SARS-related Perceptions

From 65.0% to 89.3% of respondents believed that SARS could be transmitted through droplets, fomites, and sewage systems; by eating wild animal meat; or by rats, cockroaches, or pets, while 49.2% of respondents believed that SARS is transmittable through aerosols (Table 3). Of all respondents, >90% believed that using a mask in public places, disinfecting living quarters, and frequent hand washing are efficacious means of SARS prevention (Table 3).

A total of 40.4%, 68.9%, and 29.1% of the respondents, respectively, believed that resurgence of SARS would occur in Hong Kong, in mainland China, or overseas in the coming 6 months. In addition, 69.8% of respondents believed that even if this resurgence occurred, it would not be a major outbreak, and 80.3% believed that the government would be able to control the epidemic under such circumstances (Table 2).

In the event that a few new cases of SARS were reported in Hong Kong, >70% of all the respondents would wear

Table 1. Sociodemographic characteristics of respondents*

	Survey 1			Survey 2		
	Men (n = 428), %	Women (n = 435), %	Total (N = 863), %	Men (n = 407), %	Women (n = 411), %	Total (N = 818), %
Age group (y)						
18–29	25.6	22.4	24.0	35.5	24.1	29.8
30–44	37.2	44.8	41.0	33.0	50.5	41.8
45–60	37.2	32.8	35.0	31.5	25.4	28.4
Education level						
≤9 y	23.7	32.5	28.1	24.0	32.0	28.0
10–12 y	48.4	44.9	46.6	44.4	47.3	45.9
Post secondary	27.9	22.6	25.2	31.6	20.7	26.1
Marital status						
Single	39.2	25.8	32.4	44.0	28.3	36.1
Married/divorced/widowed	60.8	74.2	67.6	56.0	71.7	63.9
Employment status						
Full time	71.1	42.3	56.6	65.4	41.4	53.3
Housewife/student	10.8	50.8	31.0	14.3	42.1	28.2
Other	18.1	6.9	12.5	20.4	16.5	18.5
Monthly income (HKD)						
≤4,000	24.6	50.2	37.9	–	–	–
4,001–12,000	42.1	27.8	34.7	–	–	–
12,001–20,000	18.5	11.2	14.7	–	–	–
≥20,001	14.9	10.8	12.7	–	–	–

*HKD, Hong Kong dollar (1 US$ = 7.8 HKD). –, data not collected in survey 2.

Table 2. Perceptions related to resurgence of severe acute respiratory syndrome (SARS) and associated behaviors (survey 1 data)

Perceptions	Men (n = 428), %	Women (n = 435), %	Total (N = 863), %	p value*
Resurgence of SARS				
There will be a resurgence of SARS in Hong Kong in the coming 6 months	37.4	43.4	40.4	0.069
There will be a resurgence of SARS in China in the coming 6 months	66.6	71.3	68.9	0.138
There will be a resurgence of SARS overseas in the coming 6 months	29.9	28.3	29.1	0.613
No major outbreaks even if SARS returns to Hong Kong	71.3	68.3	69.8	0.340
The government could control SARS if there were a few sporadic new SARS cases in Hong Kong	80.3	80.2	80.3	0.958
Preventive behavior if a few new SARS cases were reported in Hong Kong				
Would frequently wear a mask in public places	70.7	71.7	71.2	0.730
Would avoid going to crowded places	71.5	77.9	74.8	0.031
Would avoid going to mainland China	69.6	79.7	74.7	0.001
Would not allow children to go to school	13.5	12.1	12.8	0.536
Would avoid going to hospitals	67.3	76.3	71.8	0.003
Would avoid contacts with medical personnel	35.8	38.4	37.1	0.437
Would avoid contacts with tourists coming from mainland China	31.5	37.8	34.7	0.051
Perceived emotional responses if a few new cases were reported in Hong Kong				
Would be in a state of panic	14.0	23.0	18.6	0.001
Would be very depressed	12.1	17.7	14.9	0.020
Am still emotionally disturbed because of SARS	33.0	39.5	36.3	0.047
Current preventive behavior				
Would avoid visiting hospitals to prevent contracting SARS	65.0	68.3	66.7	0.311
Would avoid visiting China to prevent contracting SARS	64.4	72.8	68.6	0.008
Would make a health declaration if crossing the border and had influenza	79.3	84.3	81.9	0.058
Would see a local physician if had influenza in mainland China	37.6	39.8	38.7	0.498
Would see a local physician if had influenza overseas	79.3	84.8	82.1	0.039
Would wear a mask if had influenza when traveling by air	87.3	91.0	89.2	0.079

*Chi-square test.

a mask in public places and avoid visiting crowded places, mainland China, or hospitals (Table 2); 12.8% of respondents would not allow their children to attend school. A total of 37.1% of respondents would avoid contacting medical personnel, and 34.7% would avoid contacting visitors from mainland China. Furthermore, 18.6% of the respondents indicated that they would be in a state of panic, and 14.9% would be very depressed. Approximately 36.3% of the respondents felt emotionally disturbed because of SARS.

Female respondents were more likely than male respondents to perceive SARS to be transmittable through different modes (rats and cockroaches, animal meat, and sewage) or to perceive efficacy in disinfecting living quarters, washing hands frequently, and using traditional Chinese medicine for SARS prevention ($p < 0.05$) (Table 3). Women were also more likely than men to be in a state of panic and be depressed or emotionally disturbed because of SARS ($p < 0.05$) (Table 3).

Factors Predicting Public Health Measures for Preventing SARS

Multivariate results show that sex, marital status, believing that SARS would be transmitted through fomites or aerosols, perceiving that older people were more susceptible to SARS, perceiving that a resurgence would occur in Hong Kong or in China, and current emotional disturbance because of SARS were associated with visiting hospitals or visiting mainland China (online Appendix Table 1, available from http://www.cdc.gov/ncidod/EID/vol11 no03/04-0675_app1.htm). Sex; education level; marital status; believing that SARS was transmitted through droplets, fomites, pets, or sewage; anticipation of a resurgence in SARS in Hong Kong or overseas; and the perceived ability of the government to control the resurgence of SARS were associated with being emotionally disturbed by SARS or in a state of panic if SARS returned to Hong Kong (online Appendix Table 1).

Table 3. Perceptions related to mode of transmission, medical development, and epidemiology of severe acute respiratory syndrome (SARS) (survey 1 data)

% agreeing with statements	Men (n = 428), %	Women (n = 435), %	Total (N = 863), %	p value*
Mode of transmission				
SARS is transmittable through respiratory droplets	86.7	88.5	87.6	0.416
SARS is transmittable through fomites	87.1	87.6	87.4	0.847
SARS is transmittable through aerosols	47.2	51.3	49.2	0.232
SARS is transmittable through rats and cockroaches	70.6	79.3	75.0	0.003
SARS is transmittable through pets	66.6	63.4	65.0	0.333
SARS is transmittable through sewage	86.7	92.0	89.3	0.012
There is a high likelihood of contracting SARS through wild animal meat	77.4	87.6	82.6	<0.001
Perceived efficacy of preventive measures				
Wearing masks in public places could effectively prevent SARS	92.0	93.3	92.7	0.471
Disinfecting living quarters could effectively prevent SARS	96.0	98.6	97.3	0.018
Frequent hand washing could effectively prevent SARS	96.3	99.3	97.8	0.002
Vaccination against influenza could effectively prevent SARS	44.6	47.7	46.2	0.363
Intake of traditional Chinese medicine could effectively prevent SARS	36.6	44.2	40.5	0.023
Medical development				
SARS vaccines would be developed in a year	47.2	44.7	45.9	0.462
No effective drugs available to treat SARS	82.9	80.5	81.7	0.345
Epidemiology of SARS				
Old people are more likely than others to contract SARS	68.9	70.5	69.7	0.613
SARS mortality rate >50% for patients >60 years old	44.7	47.6	46.2	0.400

*Chi-square test.

Mental Health Effects of SARS

A total of 92.5% of the respondents regarded the SARS epidemic in Hong Kong as severe or very severe. High percentages (65.4%, 55.5%, and 65.0%, respectively) of respondents felt helpless, horrified, and apprehensive because of SARS or worried that they or family members would contract the virus, and 48.4% of respondents perceived that their mental health had severely or moderately deteriorated because of the SARS epidemic (Table 4).

Using the cutoff values of the IES of the combined intrusion and avoidance subscale (28), we observed that 13.3% of male respondents and 18.0% of female respondents ($p = 0.060$), respectively, had moderate or severe posttraumatic stress symptoms (1.3% and 1.5%, respectively, of the male and female respondents had severe symptoms) (Table 4). Female respondents had higher mental health quality of life (QOL) and vitality QOL subscale scores ($p < 0.05$).

A total of 36.8% and 37.8%, respectively, of the respondents perceived that the level of stress related to work and family had increased as a result of the SARS epidemic, and 26.5% of the respondents were facing increased financial stress. Among current smokers, 12.9% had increased their frequency of smoking during the SARS epidemic compared with the pre-SARS period. Among those who consumed alcohol, 4.7% of male respondents and 14.8% of female respondents had increased their frequency of drinking (Table 4).

Of the respondents, 11.5% had trouble falling or staying asleep because they had been preoccupied by thoughts related to SARS. In the month preceding the survey, 18.6% of the respondents reported that they slept restlessly (Online appendix Table 1). A total of 6.9% of respondents had psychosomatic symptoms such as sweating, nausea, trouble breathing, or pounding heartbeats when thinking about the SARS epidemic (Table 4). When the situations before or during the SARS epidemic were compared, we observed that 4.2% of respondents had family members in need of psychological or psychiatric services, 6.1% reported poorer sexual functioning, 37.2% reported a poorer social life, 20.1% of those employed reported difficulty in concentrating on their work, and 26.5% of respondents reported poorer emotional states of their family members (Table 4).

Table 4. Psychological and related effects of severe acute respiratory syndrome (SARS) (survey 2 data)*

	Men, %	Women, %	Total, %	p value†
General mental health effect of SARS				
SARS perceived to be severe or very severe	91.4	93.7	92.5	0.216
Felt horrified because of SARS (agree or strongly agree)	65.4	80.3	72.9	<0.001
Felt apprehensive because of SARS (agree or strongly agree)	55.5	69.1	37.7	<0.001
Felt helpless about SARS (agree or strongly agree)	65.0	63.7	64.4	0.703
Worried or worried very much about oneself or family members contracting SARS	41.3	57.2	49.3	<0.001
IES cutoff (posttraumatic stress symptoms)	13.3	18.0	15.7	0.060
Worsened self-assessed mental health effect of SARS (very much or somehow)	42.6	54.1	48.4	0.001
Sleeping/psychosomatic problems				
Experienced trouble falling or staying asleep because of SARS (sometimes or often)	9.3	13.6	11.5	0.054
Sleep was restless in the last month (sometimes or often)	15.3	21.9	18.6	0.015
Experienced sweating, trouble breathing, nausea, or heart pounding because of SARS	5.2	8.5	6.9	0.059
Substance use				
Increased frequency of smoking‡	13.2	11.5	12.9	0.820
Increased frequency of drinking alcohol§	4.7	14.8	6.8	0.062
Perceived increased stress because of SARS				
Increased or much increased work stress	35.4	38.2	36.8	0.403
Increased or much increased family stress	38.6	37.0	37.8	0.639
Increased or much increased financial stress	25.1	28.0	26.5	0.344
Other problems				
Family members in need of psychology or psychiatry services	4.7	3.7	4.2	0.539
Difficult or very difficult to concentrate at work¶	18.8	21.8	20.1	0.409
Worsened or much worsened sexual life	6.2	5.9	6.1	0.855
Worsened or much worsened social life	31.0	43.4	37.2	<0.001
Family member with worsened or much worsened emotional states	26.0	26.9	26.5	0.783

*IES, Impact of event scale.
†Chi-square test.
‡Among those who were smokers.
§Among those who drank alcohol.
¶Among those who were currently working full time and part time.

Factors Predicting Mental Health Effects

Stage 1 Analysis (Stepwise Regression of Sociodemographic Variables)

The relevant sociodemographic variables (Table 1) were entered as input variables in stepwise linear and logistic regression models to predict IES scores, mental health, and vitality QOL scores and various psychological effects (e.g., whether one had trouble falling asleep) (online Appendix Tables 2 and 3, available from http://www.cdc.gov/ncidod/eid/vol11no03/04-0675_app2.htm and http://www.cdc.gov/ncidod/eid/vol11no03/04-0675_app3.htm).

Stage 2 Analysis (Adjusted for Variables Significant in Stage 1)

Those who felt horrified, apprehensive, and helpless because of SARS were more likely to report posttraumatic stress symptoms (as measured by IES) or have a lower mental health QOL and vitality QOL scores (online Appendix Table 2). Those who felt apprehensive because of SARS were more likely to report sleeping problems and experience overall negative mental health effects (online Appendix Table 3). Feeling helpless because of SARS was associated with sleeping problems, while worrying about contracting SARS was associated with overall negative mental health and psychosomatic symptoms.

Increased work-related and family-related stress, but not increased financial stress, were associated with IES and mental health QOL and vitality QOL outcomes (online Appendix Table 2). Increased work-related stress was also associated with sleeping problems, psychosomatic symptoms, and a poorer social life. Increased family-related stress was associated with a poorer social life, worsened mental health, and the need for psychological/ psychiatric services (online Appendix Table 3). Financial stress was associated with worsened sexual functioning and worsened mental health.

A poorer social life was associated with IES (intrusion and hyperarousal) (online Appendix Table 2), sleeping problems, worsened sexual functioning, and a negative overall effect on mental health (online Appendix Table 3).

Worsened emotional states of family members was significantly associated with subscales of the IES (intrusion and avoidance) and QOL subscales, sleeping problems, worsened overall mental health effects, and worsened sexual and social life.

Discussion

The general public in Hong Kong did not perceive the possibility of a resurgence of SARS. The degree of vigilance was high when respondents were asked about current preventive behaviors and hypothetical situations of having a few new SARS cases reported in Hong Kong. The entire city was expected to react strongly to a resurgence of SARS. However, some precautions may be unwarranted and could have a negative economic effect (29). Approximately 20% of respondents believed that they would be in a state of panic, 37% were still emotionally disturbed by SARS, and 4% had family members in need of psychological or psychiatric services. Thus, the general public needs to be better prepared psychologically to be able to avoid possible panic and emotional disturbances in a resurgence of SARS.

More than 90% of respondents perceived that mask use, frequent hand washing, and disinfection of living quarters are efficacious means of SARS prevention. Although the droplet theory of transmission has been widely accepted by the scientific community, other theories involving fomites (30), aerosols (5), sewage (31), rats (7), and wild animals (32) remain controversial. No conclusions have been reached regarding these topics. Information provided by health workers has also shown marked variations (33). In the absence of confirmed "top-down" official information, the general public has apparently been forming their own attitudes in a "bottom-up" manner. Similarly, another study claimed that laypersons in Hong Kong, Taiwan, and Toronto used "naive knowledge models" that were either incomplete or faulty in conceptualizing the symptoms, threat, spread, and prevention of SARS (34). Another study also reported substantial misinformation and false beliefs related to the existence of SARS in the general public (20). Therefore, it is important to understand how perceptions were formed during a newly emerging epidemic.

If one compares the results of this study with those obtained in March 2003, SARS-related perceptions and behaviors changed sharply over time (21,35). The results of several studies show that most of the general public had always believed that SARS could be transmitted through droplets, and increasingly more people believed that SARS is transmittable through fomites, but opinions about aerosol transmission of SARS remained split (20,21,35). Different studies had similar conclusions that perceptions such as perceived efficacy and perceived susceptibility were predictive of the use of preventive measures and emotional responses (20,21,35).

In survey 2, the prevalences of avoiding hospitals and China were 66.7% and 68.6%, respectively, which are comparable with the results obtained in another study conducted in May 2003 (21). More than 80% of respondents in this study would use a mask if they had influenza while traveling, while another study conducted from April 22 to April 29, 2003, documented that ≈70% would do so (36). A third study reported that ≈50% of the general public practiced at least 5 of 7 studied types of preventive measures (20). Preventive behaviors were thus prevalent throughout different phases of the epidemic.

A study conducted on approximately April 1, 2003 (20), reported that 12.6% of the respondents were quite or very anxious. Our survey 2, which was conducted at the ending phase of the epidemic, showed that ≈16% of the respondents had moderate or severe posttraumatic stress symptoms. Another study conducted from April 11 to May 19, 2003 (37), documented that ≈68% of healthy control participants experienced negative SARS-related effects. Our study showed that ≈48% assessed their mental health as being worse because of SARS. Also, 20% of the respondents worried about finances, whereas ≈27% of the respondents had financial stress. Emotional disturbance (our survey 2) and anxiety level (20) were associated with use of preventive measures. Psychological stress was prevalent throughout different phases of the epidemic.

Sex differences in perceptions and responses were observed. Men and women may have reacted differently to the incomplete evidence available when forming their views about the spread and control of SARS. Women were more likely than men to believe that SARS could be transmitted through different modes or that different methods could effectively prevent SARS.

A sizable proportion of the population felt horrified, apprehensive, or helpless because of the SARS epidemic in Hong Kong. Approximately 40%–50% of the respondents reported that their mental health status had been worsened, and 40% felt that their levels of work- and family-related stress had increased during the epidemic. The SARS epidemic exerted adverse effects on multiple aspects of social, family, sexual, and occupational domains. Those who smoked and drank in Hong Kong also increased their frequency of smoking and drinking. Thus, the mental health effect was prevalent and pervasive. Longitudinal studies are therefore required to understand the long-term mental health effects of SARS. Similar effects had been documented in studies conducted after the September 11, 2001, terrorist attack in the United States (15,38,39). Some similarities may exist in the community responses of different large-scale disasters.

Married persons tended to have a worsened mental health status because of SARS. Married people usually have a lower prevalence of psychological problems and a better support system compared with single people. However, ≈25% of respondents reported that their family members were emotionally affected by the epidemic, and ≈40% reported increased family stress. When an infectious epidemic is being faced, the worries of cross-infection and the well-being of family members are critical in determining the mental health effects of the epidemic on a person. Mental health services should take into account mutual influences among family members. Increased work-related stress was another predictor of mental health effects. Business activity decreased sharply, and the job security of many people was threatened. Similarly, social life was reported as worse among 40% of respondents. The effect of SARS was not confined to physical and psychological aspects, but it also affected socioeconomic and social aspects, which in turn determined the psychological well-being of persons.

This study had several limitations. First, data were self-reported and are subject to reporting biases. However, the interviews were anonymous. Second, some questions were asked about behavior in response to a potential resurgence of SARS, rather than measuring actual behavior because we were investigating how the general public would respond to a possible resurgence of SARS. Third, the response rates of the studies were moderate (≈58% in survey 1 and 65% in survey 2), and no data were available from nonresponders. The response rates were comparable with those of other survey studies in Hong Kong (40,41), and the age composition of the 2 samples was comparable with those of the Hong Kong census figures. Furthermore, we were not able to ascertain the previous psychological conditions of the respondents. However, results of the study should reflect the direct effect of SARS, rather than the general psychological status of the respondents. Some important factors, such as intensity of media exposure, were not measured in the study. However, many variables in this study (e.g., perceived reaction to resurgence and some psychological responses variables) have not been reported elsewhere.

SARS may return to some parts of the world, and preparative work is warranted. Up-to-date SARS-related knowledge should be collated and disseminated to the general public to promote effective public health measures and avoid unnecessary panic in case of a resurgence. Sex differences and concerns for family members and work need to be considered by relevant information campaigns. The perception of the general public changes rapidly over time and needs to be monitored closely. Bioterrorism may be similar to SARS in many ways. The results of this study predict that, in cases of bioterrorism, the general public would form their perceptions based on weak evidence, and the effect on mental health would also be evident. Modifying perceptions of the public would facilitate control of the disaster and alleviate panic among the general population. Further studies on the process of perception formation and its consequences on psychological responses in newly emerged epidemics are warranted.

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West Nile Virus Risk Assessment and the Bridge Vector Paradigm

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In the northeast United States, control of West Nile virus (WNV) vectors has been unfocused because of a lack of accurate knowledge about the roles different mosquitoes play in WNV transmission. We analyzed the risk posed by 10 species of mosquitoes for transmitting WNV to humans by using a novel risk-assessment measure that combines information on the abundance, infection prevalence, vector competence, and biting behavior of vectors. This analysis suggests that 2 species (*Culex pipiens* L. and *Cx. restuans* Theobald [Diptera: Cilicidae]) not previously considered important in transmitting WNV to humans may be responsible for up to 80% of human WNV infections in this region. This finding suggests that control efforts should be focused on these species which may reduce effects on nontarget wetland organisms. Our risk measure has broad applicability to other regions and diseases and can be adapted for use as a predictive tool of future human WNV infections.

Since its first appearance in North America in 1999, West Nile virus (WNV) has spread across the continent and into Central America. It has infected >17,000 persons and caused >670 deaths (1,2). Reducing the number of human cases of WNV through vector control depends on efficiently using limited resources (3), which requires understanding which vectors are most important in transmitting WNV to humans.

Previous research has suggested that different mosquito species play different roles in spreading WNV. *Culex pipiens* L. and *Cx. restuans* Theobald mosquitoes are thought to be the primary amplification vectors of WNV in birds in the northeastern and north-central United States (4) because they are primarily ornithophilic, or bird-biting, are abundant, and have the highest prevalences for WNV in this region (5). These species have not been considered important in transmitting WNV to humans because of their feeding habits (3,4). Instead, other mosquitoes, which take a larger fraction of their blood meals from mammals, are thought to be bridge vectors in transmitting WNV to humans (3,4). Species that have been proposed as bridge vectors include members of the genera *Aedes* and *Ochleratus* and other *Culex* species (3,4). However, classification of mosquito species as enzootic or bridge vectors was previously based primarily on qualitative categories and did not incorporate other data that are critical to determining the risk for human infection from each species. In this study, we integrate quantitative information on the abundance, WNV infection prevalence, vector competence, and biting behavior of the most important vectors in the northeast and north-central United States to predict the risk for human infection from each species.

Materials and Methods

The probability or risk that a species of mosquito will infect a human with WNV can be estimated as

$$\text{Risk} = A \times F_m \times P \times C_v$$

where A is the abundance, F_m is the fraction of blood meals taken from mammals, P is the WNV infection prevalence, and C_v is an index of vector competence (the fraction of WNV-infected mosquitoes that will transmit virus in a subsequent bite). Our equation for Risk (capitalized to denote our calculated expression) is an estimate of the relative number of WNV-infectious bites on mammals by each mosquito species. We discuss the data we used for each variable in turn.

We used abundance data from 2 counties near the original 1999 outbreak (Suffolk and Rockland) in New York

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State during the period 2000–2003, totaling ≈7,195 trap-nights and 378,000 mosquitoes. Mosquitoes were collected by using Centers for Disease Control and Prevention (CDC) light traps baited with CO_2 (dry ice) from evening until the next morning, which includes the peak activity periods for the mosquitoes considered here. While some mosquitoes are underrepresented in CDC light trap collections (e.g., *Ochleratus trivittatus* [6,7]), baiting traps with CO_2 and trapping during both dusk and dawn minimizes this bias (3).

Mosquitoes from these traps were identified to species with 1 exception; *Cx. pipiens* and *Cx. restuans* adults are difficult to distinguish in the field and are usually counted and submitted for testing after being pooled and labeled as *Cx. pipiens/Cx. restuans*. As a result, we present the 2 species as a pair. We averaged their vector competencies and fraction of blood meals from mammalian hosts, which were examined for each species separately by using a molecular identification protocol (8) and identification of mosquitoes as larvae to separate the 2 species. Identification of a subset of trapped adult mosquitoes by experienced entomologists (9) suggests that although year-to-year variability occurs, these species had approximately equal abundance averaged over the past 4 years (L. Kramer et al., unpub. data). Both species have similar feeding behavior (15%–22% of blood meals come from mammalian hosts, averaging the data weighted by sample size from [9,10] and L. Kramer et al., unpub. data) and breed in similar habitats (containers such as tires, gutters, catch basins, polluted surface pools). As a result, combining these species in our analysis should not detract from our ability to determine which vectors transmit WNV to humans; nor should it alter strategies that should be taken to control vector populations.

To increase the sensitivity of our analyses, we used WNV testing data from all of New York State from 2000 to 2003 to estimate the infection prevalence for each species. However, we only included data from mosquitoes trapped with CDC light traps because prevalences were higher from mosquitoes caught in gravid traps, and these traps primarily capture *Culex* mosquitoes that have already fed at least once. Although the large-scale averaging we performed ignores important spatial and temporal variation, the larger dataset is required to accurately estimate prevalence for species that are rarely infected (i.e., all non-*Culex* species). A correlation analysis of mosquito species' prevalences at the county and state level suggested that the 2 datasets were comparable (Rockland vs. New York State, r = 0.92 and Suffolk vs. New York State, r = 0.97). Mosquitoes were tested for WNV RNA by using reverse transcription–polymerase chain reaction (RT-PCR) (11) in groups (pools) of 20 to 50, and infection prevalence of each species is expressed as the minimum infection rate (MIR), where MIR = 1,000 × (pools testing positive for WNV/total number of mosquitoes tested). This calculation assumes that each pool contains only 1 infected mosquito, which is >99% likely for MIRs <3 and pools of 50. Occasionally, MIRs >3 have been recorded for *Cx. pipiens/Cx. restuans* (5), which could lead to an underestimate of the true prevalence and Risk for this species pair.

Blood meals from mosquitoes trapped in New York and New Jersey were identified to vertebrate order by using PCR or the heteroduplex method (9,10,12). We calculated the fraction of each species' blood meals that came from mammals as a relative estimate of the probability that the species would feed on humans (13). We believe this approximation is valid because identification of mammalian blood meals to the species level showed that all of the species considered here feed on humans (10,14). In addition, all of the identified mammalian blood meals from *Cx. pipiens* and *Cx. restuans* were from ground-dwelling mammals (9,10, Kramer et al., unpub. data). As a result, we believe that mammalian blood meals identified from *Cx. pipiens* and *Cx. restuans* were not a result of these normally ornithophilic mosquitoes' occasionally biting arboreal mammals. Lastly, recent research showed that North American *Cx. pipiens* are actually hybrids between more ornithophilic *Cx. pipiens* and more opportunistically feeding *Cx. molestus* and *Cx. quinquefasciatus* (15), which might help explain their feeding on humans and other mammals.

Finally, previous research has shown that mosquitoes may be infected with WNV (i.e., test positive) but not transmit virus when feeding, at times because the virus is not present in the salivary glands (4). The probability that the virus will be transmitted with a bite, given that a mosquito tests positive for WNV, differs among species and has been incorporated into the analysis through the vector competence C_v (see Table for sources). Three species, *Culiseta melanura*, *Ochlerotatus canadensis*, and *Oc. trivittatus*, have not been tested for vector competence. For these species, we used values for their congeners and also present the Risk for these species if their C_v was 1, i.e., if every infected mosquito transmitted the virus when feeding (see Table).

Results

The species-pair *Cx. pipiens* + *Cx. restuans* accounts for >80% of the total Risk, a surrogate for human WNV infections in this region, over this time period (Table). The threat of this species-pair is ≈16 times higher than that for the 4 other important species, *Oc. japonicus*, *Ae. vexans*, *Oc. trivittatus*, and *Cx. salinarius*. This finding is a result of the high WNV prevalence and abundance of this species-pair, which more than compensates for the relatively small fraction of mammalian blood meals of these primarily bird-

Table. Risk of mosquito species transmitting West Nile virus (WNV) to humans

Species	Relative abundance	WNV MIR*	Vector competence† (reference)	Fraction mammal‡	Risk	% Risk
Aedes vexans	20.7	0.05	0.17 (16)	0.86 (126)	0.14	4.5
Coquillettidia perturbans	11.3	0.01	0.11 (17)	0.83 (191)	0.01	0.5
Culex pipiens + *Cx. restuans*	37.2	0.95	0.38 (16–18)	0.19 (373)	2.52	80.2
Cx. salinarius	0.6	0.85	0.36 (17)	0.67 (91)	0.12	3.9
Culiseta melanura	5.2	0.17	0.28 (19) §	0.11 (141)	0.03	0.8
Ochlerotatus canadensis	14.9	0.00	0.55 (16,18)¶	1.00 (107)	0.00	0.0
Oc. japonicus	0.5	0.33	0.93 (16)	0.95 (57)	0.16	5.0
Oc. sollicitans	2.0	0.07	0.16 (16)	1.00 (28)	0.02	0.7
Oc. trivittatus	7.6	0.05	0.55 (16,18)¶	0.64 (115)	0.14	4.4

*MIR, minimum infection rate.
†The fraction of WNV-infected mosquitoes that will transmit virus in a subsequent bite.
‡Number of mosquito blood meals identified in parentheses (9,10, Kramer et al., unpub. data).
§Vector competence value taken from study on *Cs. inornata*. Risk increases to 0.09 and 3.0%, assuming a maximum vector competence of 1.0.
¶Genus average used. Risk with a vector competence of 1.0 would be 0 and 0% for *Oc. canadensis* and 0.25 and 8.0% for *Oc. trivittatus*.

biting mosquitoes (Table). In contrast, while some of the species previously suggested as important bridge vectors have high infection rates (*Cx. salinarius*), are abundant (*Aedes vexans*, *Oc. canadensis*), or are extremely efficient vectors in the laboratory (*Oc. japonicus*), none make up >5% of the total Risk for human WNV infections.

Discussion

Integrating 4 important aspects of disease transmission into a single measure of Risk suggests that 2 mosquito species that were previously overlooked as vectors for transmission to humans may in fact be the most important. Current WNV management guidelines (3) call for broadly controlling mosquitoes by using both insecticides and water flow management. Our results argue for focusing mosquito control efforts on *Cx. pipiens* and *Cx. restuans*, which primarily breed in a small subset of habitats (tires, gutters, catch basins, polluted surface pools) that are different from those of many other vectors (20). This focus could substantially reduce the detrimental effects of mosquito control on nontarget species, especially in wetlands. In addition, focusing control on these habitats and species should improve the effectiveness of control measures and reduce the number of human WNV infections. Finally, reducing the densities of these mosquito species should also decrease transmission of WNV between birds. This management strategy has the dual benefit of decreasing the severity of WNV epidemics in birds and the subsequent spillover to mammals.

These results should be placed within their proper spatial and geographic context. The most important vectors for transmitting WNV to humans in other regions of the United States are likely to be different. *Cx. quinquefasciatus* and *Cx. nigripalpus* are the predominant vectors of WNV between birds in the southeastern United States (17,21), and *Cx. tarsalis* and *Cx. quinquefasciatus* play this role in much of the western United States (19). Broad feeding habits, host switching from birds to mammals in the fall, or both (22–24) make these 3 species likely to also be the dominant vectors in transmitting WNV to humans in these regions. Similarly, while our analysis of vectors in the northeastern United States determined the most important vectors for human WNV infections by averaging over several years and a multicounty scale, other vectors may be more important on a local scale (e.g., *Cx. salinarius* near a salt marsh [25]) or during portions of the transmission season. Our results should be verified at smaller temporal and spatial scales because averaging over data in which abundance and infection rates negatively covary can produce biased results (26).

The validity of our conclusions rests on the assumptions we have made and the data on which they are based. Of primary importance is the relative number of feedings of *Cx. pipiens* and *Cx. restuans* on humans, which is based on abundance estimates generated by using CO_2-baited CDC light traps and mosquito blood meals identified from mammalian hosts (as well as a small number from humans). Recent work by Gingrich and Casillas (25) strengthens our results and suggests that feedings by *Cx. pipiens* on humans may be more common than was previously thought. These researchers compared the landing rates of mosquitoes on humans (which were then captured with an aspirator and identified) with the abundance of mosquitoes trapped with CO_2-baited CDC light traps at 4 sites in Delaware. When data from their Table 1 were used, the ratio of mosquitoes caught after landing on a human to those caught by using CO_2-baited CDC light traps is 0.36 for *Cx. pipiens*, 0.40 for *Cx. salinarius*, and 0.07 for *Ae. vexans* (25, Table). This finding suggests that in terms of feeding on humans, *Cx. pipiens* are relatively underrepresented by CO_2-baited CDC light traps compared to *Ae. vexans*, which implies that *Ae. vexans* may be less important and *Cx. pipiens* more important than our analyses have shown. Of the species considered in both

our study and that of Gingritch and Casillas, only *Oc. canadensis* is relatively underrepresented by CDC light traps compared to *Cx. pipiens*, with a human landings to CDC light trap ratio of 1.31. However, this species is rarely infected with WNV and does not represent an important vector for transmitting WNV to humans (Table).

One strength of our Risk measure is that it can be applied to other locations and at other scales simply by applying the risk equation to data from the desired scale and region if analyzed appropriately (26). In addition, the Risk equation can be used as a predictive index to forecast the relative number of future human WNV infections, which could be useful for short-term planning and resource allocation. The sum of the Risk equation over all (i = 1 to n) mosquito species multiplied by human population density in the area considered should estimate the number of short-term future human WNV infections:

Predicted human infections =

$$(\text{human density}) \times \sum_{i=1}^{n} A \times F_m \times P \times C_v$$

We are currently testing the usefulness of this index in predicting the relative number of human WNV infections between locations and over the mosquito season.

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Methicillin-resistant *Staphylococcus aureus* in Horses and Horse Personnel, 2000–2002

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Methicillin-resistant *Staphylococcus aureus* (MRSA) infection was identified in 2 horses treated at a veterinary hospital in 2000, prompting a study of colonization rates of horses and associated persons. Seventy-nine horses and 27 persons colonized or infected with MRSA were identified from October 2000 to November 2002; most isolations occurred in a 3-month period in 2002. Twenty-seven (34%) of the equine isolates were from the veterinary hospital, while 41 (51%) were from 1 thoroughbred farm in Ontario. Seventeen (63%) of 27 human isolates were from the veterinary hospital, and 8 (30%) were from the thoroughbred farm. Thirteen (16%) horses and 1 (4%) person were clinically infected. Ninety-six percent of equine and 93% of human isolates were subtypes of Canadian epidemic MRSA-5, *spa* type 7 and possessed SCC*mec*IV. All tested isolates from clinical infections were negative for the Panton-Valentine leukocidin genes. Equine MRSA infection may be an important emerging zoonotic and veterinary disease.

Methicillin was first introduced in human medicine in the 1950s for the treatment of penicillin-resistant staphylococci, and within a few years, methicillin-resistant isolates of *Staphylococcus aureus* (MRSA) were identified (1). Since then, MRSA has emerged as an important problem in human medicine internationally, especially in the hospital setting (2–6). Methicillin resistance is mediated by production of an altered penicillin-binding protein (PBP2a), which confers resistance to all β-lactam antimicrobial agents. The gene that encodes this altered PBP, *mecA*, resides on a large, mobile genetic element called the staphylococcal chromosomal cassette *mec* (SCC*mec*). Three types of SCC (types I, II, and III) were originally described in hospital-acquired MRSA strains, most of them isolated before 1990. A fourth type (type IV) was recently described, initially in community-acquired MRSA isolates (2–4). Although MRSA have been typically hospital acquired (5,6), reports of community-acquired MRSA in persons have increased (7–9). In Canada, 6 epidemic clones, designated CMRSA (Canadian epidemic MRSA) 1 through 6 based on pulsed-field gel electrophoresis (PFGE), are recognized (10,11). In addition to PFGE, SCC*mec* typing and DNA sequencing of the X region of the protein A gene (*spa* typing) can be used to further differentiate strains for epidemiologic analysis (12).

The role of MRSA in veterinary medicine has not been well characterized. Isolation of MRSA has been reported in horses, cattle, and dogs (13–20). In addition, MRSA infection in 2 horses treated at the Ontario Veterinary College Veterinary Teaching Hospital (OVC-VTH) was first documented in 2000. To evaluate the apparent emergence of MRSA infection and colonization in horses, nasal swabs from horses and persons at OVC-VTH and a select group of farms from southern Ontario were cultured.

Materials and Methods

Veterinary Hospital Facility

The Large Animal Clinic at OVC-VTH is a tertiary-care referral center with a caseload of ≈2,000 horses per year. Hospital personnel that are in contact with horses include senior clinicians (internists, surgeons, other specialists), residents, interns, veterinary technicians, agricultural assistants, and veterinary students.

Sampling Procedures

A limited organized MRSA screening of horses at OVC-VTH was performed from October 1 to October 5,

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2000, after the first 2 clinical cases were recognized. A more thorough screening program was performed from May 17 to November 16, 2002, after further cases were recognized. With this program, nasal swabs were collected from all horses at the time of admission, weekly during hospitalization, and at the time of discharge. Intermittent screening of horses on 1 Ontario breeding farm (farm A) was performed from May 11 to September 15, 2002, after infected horses were identified. Nasal swabs were collected from horses at 9 other Ontario farms after MRSA infection in a resident horse was identified at OVC-VTH. Nasal swabs were collected from all horses that were present on these farms on the day of sampling.

Voluntary screening of OVC-VTH Large Animal Clinic personnel was performed on 2 occasions in 2002 in response to identification of clusters of nosocomial MRSA colonization in horses. Periodic nasal cultures of horses and personnel were initiated at farm A from May 11 to September 15, 2002.

For persons, cotton-tipped swabs were used to sample both anterior nares. For horses, the swab was inserted ≈10 cm into 1 anterior nare and rubbed against the mucosa as the swab was removed. Swabs were placed in liquid Stuart's or Amies medium and stored at 4°C until processing. Colonization or infection identified within 72 h of admission was classified as community acquired.

MRSA Identification, Characterization, and Typing

Nasal swab samples were injected onto mannitol-salt agar with 2 μg/mL oxacillin and incubated aerobically at 35°C for 48 h. Colonies were identified as *S. aureus* based on colony morphology, Gram stain appearance, ability to ferment maltose, and positive tube coagulase test or latex agglutination test (Pastorex Staph Plus, Bio-Rad Laboratories Ltd, Mississauga, Canada).

Screening for methicillin resistance in all *S. aureus* was by growth on Mueller-Hinton agar with 4% NaCl and 6 μg/mL oxacillin. Confirmation of methicillin resistance was by detection of PBP 2a by using the MRSA SCREEN antibody kit (Denka Seiken Co. Ltd, Tokyo, Japan) (21).

Antimicrobial susceptibility testing was performed by broth microdilution as per NCCLS guidelines (22). Detection of inducible *erm* gene-mediated clindamycin-resistance (MLS_B phenotype) was performed by using the double disk diffusion method as described in M100-S14 NCCLS 2004 Informational Supplement (23).

Isolates were typed by using PFGE after DNA extraction and *Sma*I digestion (24). PFGE images were read manually by 1 investigator (B.W.), and related isolates were divided into subtypes by using an arbitrary naming system based on previously described principles (25). Further typing by DNA sequence analysis of the X region of the protein A gene (*spa* typing) and SCC*mec* typing were performed, as has been described, on isolates from those with clinical infections, atypical isolates from colonized horses, and a random sample of isolates from colonized horses (12,26).

Isolates from those with clinical infection were tested for the presence of the Panton-Valentine leukocidin (PVL) genes by polymerase chain reaction (PCR) and by molecular beacon with the *lukF* component of *pvl*. Amplification of the *pvl* gene was performed by using the following primers: LukS-PV: GGCCTTTCCAATACAATATTGG; and LukF-PV: CCCAATCAACTTCATAAATTG.

Thermal cycling consisted of initial heating at 95°C for 5 min followed by 35 cycles of denaturation (1 min at 94°C), annealing (30 s at 57°C), and extension (1 min at 72°C). The beacon experiment was carried out using the following beacon and primers: *lukF* beacon: 5′ 6-FAM d (CGCGAAGAATTTATTGGTGTCCTATCTCGATCGCG) DABCYL 3′, LukF F: 5′-GCCAGTGTTATCCAGAGG-3′, LukF R: CTATCCAGTTGAAGTTGATCC-3′.

Quantitative real-time PCR mixture contained 1x I.Q. supermix (Bio-Rad Laboratories Ltd., Hercules, CA, USA), 0.1 μmol/L each molecular beacon, 0.5 μmol/L of each primer, and DNA template. The thermal cycling program consisted of 10 min on a spectrofluorometric thermal cycler at 95°C, followed by 45 cycles of 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C.

Statistical Analysis

Duration of carriage by adult horses versus foals was compared by using the Mann-Whitney test. The incidence of intermittent MRSA shedding by adult horses and foals was compared by using Fisher exact test. A p value of <0.05 was considered significant for all comparisons.

Results

In total, MRSA was isolated from 79 horses and 27 persons. Two equine isolations occurred in 2000, 5 in 2001, and 72 in 2002. Of the 79 equine cases, 27 (34%) were in horses that had been hospitalized at OVC-VTH, 41 (52%) were from 1 thoroughbred farm (farm A), and 11 (14%) were from other Ontario farms. Twenty-four of the 79 (30%) horses were adults; the remaining 55 (70%) were <1 year of age.

Clinical infections developed in 13 (16%) horses at 1 or more body sites. Incision or wound infections (n = 6), infection from intravenous catheter (n = 2), bacteremia (n = 2), pneumonia (n = 1), infection from surgical implant (n = 1), septic arthritis (n = 1), omphalophlebitis (n = 1), gluteal abscess (n = 1), and osteomyelitis (n = 1) were identified. In 2 neonatal foals, MRSA was isolated from the nares, blood, and intravenous (jugular) catheter; septic arthritis developed in 1 of the foals. The intravenous catheter was thought to be the original site of infection in

both cases. Ten (85%) of 13 clinically affected horses had a history of contact with colonized persons; 2 of these also had contact with infected or colonized horses. No history of known contact with colonized humans or horses was present in 3 (15%) cases. Although many of the clinically infected horses were seriously ill and required prolonged hospitalization, MRSA was implicated directly in the death of 1 horse as a result of severe osteomyelitis.

The clinical significance of 3 isolates was unclear. One was from a routine prebreeding uterine swab from a mare without any apparent clinical abnormalities or history of reproductive disease. Two were from uterine swabs from 2 mares following abortions. Whether MRSA was the cause of abortion in either case was unclear. The remaining 63 (80%) horses had subclinical infection and were nasal carriers.

Typing was performed on all 27 OVC-VTH isolates, 37 (90%) of 41 farm A isolates, and all 11 isolates from other Ontario farms. The remaining 4 isolates, all from colonized horses, were lost before being typed. Of the 75 tested isolates, including all 13 isolates from clinical infections, 72 (96%) were identified as CMRSA 5. This strain is closely related to an international epidemic strain designated the "archaic clone" (ATCC BAA-38) (11). Nine different subtypes of CMRSA-5 were identified from horses. All 12 tested isolates from horses with clinical infections contained SCC*mec*IV, were *spa* type 7, and did not contain PVL genes. The 2 isolates from postabortion uterine swab specimens also contained SCC*mec*IV and were negative for PVL genes; however, 1 was *spa* type 7 and the other *spa* type 235. Five isolates from colonized horses were tested, and all also contained SCC*mec*IV and were *spa* type 7. All 3 non-CMRSA-5 isolates were obtained from farm A and were similar to, but distinguishable from, CMRSA-2, *spa* type 2 and contained *SCCmec*IV.

MRSA infection or colonization was suspected of being nosocomial in 17 (63%) of 27 OVC-VTH cases and community-acquired in 3 (11%) cases. Origin of infection was unclear in the remaining 7 (26%) cases. In these cases, the nasal swab specimen was not collected on admission; MRSA was isolated from the first sample collected >72 hours after admission, and horses were admitted from farm A during a time when numerous colonized horses were on the farm.

Sixty-eight of the equine isolates were obtained during periods of organized screening at OVC-VTH or on horse farms. The remaining 11 isolates were from clinical specimens submitted directly by primary care veterinarians to a diagnostic laboratory and are excluded from prevalence calculations. In 2000, MRSA was isolated from the nasal passages of 2 (4%) of 57 horses, including the 2 initial clinical cases. In 2002, MRSA was isolated from 25 (8%) of 320 horses at OVC-VTH and 41 (13%) of 321 horses on farm A. Of the 9 other farms evaluated after identification of an infected or colonized horse at OVC-VTH, MRSA was only identified on 1 farm, where 3 (5%) of 64 of horses were colonized. MRSA was not isolated from any of 277 horses from 8 other Ontario farms.

MRSA was isolated from 27 persons; 17 (14%) of 125 of tested OVC-VTH personnel, 8 (12%) of 67 of farm A personnel, 1 owner of a horse with an MRSA wound infection and the spouse of a colonized OVC-VTH clinician. Three human isolates were obtained in 2000, and 24 were obtained in 2002. Only 1 (4%) was from a source with clinical infection, an OVC-VTH veterinarian with a tattoo site infection. That person was infected with CMRSA-5 subtype H12, a strain that contained SCC*mec*IV, was *spa* type 7, and was PVL negative, and that strain was isolated from 2 horses that had been under that person's care for a week before the wound infection developed. All but 1 colonized person (96%) had previous contact with 1 or more MRSA-positive horses; in 24 (89%) of 27 persons, recent contact with a horse infected with an indistinguishable subtype was documented. The colonized spouse of the colonized OVC-VTH clinician reported no contact with horses; however, isolates from both of these persons were indistinguishable from an isolate recovered from a horse under that clinician's care. CMRSA-5 was isolated from 26 (96%) of 27 persons. One person harbored both CMRSA-5 and the CMRSA-2–like isolate in his nose at the same time. This person was a veterinarian from farm A, which was the origin of the 3 horses colonized with this strain. Nine different subtypes of CMRSA-5 were identified among human isolates.

All but 2 of the human isolates were obtained during organized screening of personnel from OVC-VTH or selected Ontario horse farms. In 2000, MRSA was isolated from 2 (10%) of 21 humans at the OVC-VTH. In 2002, MRSA was isolated from 15 (12%) of 127 persons at OVC-VTH and 8 (12%) of 68 from farm A.

Antimicrobial susceptibility testing was performed on 67 of the 72 equine and all 26 human CMRSA-5 isolates, and the 5 CMRSA-2–related strains. Five of the equine CMRSA-5 isolates were unavailable for testing. All 101 MRSA isolates tested were susceptible to ciprofloxacin, clindamycin, fusidic acid, linezolid, mupirocin, quinupristin-dalfopristin, and vancomycin. The range of oxacillin MIC was 1–>32 μg/mL, and although 21.8% of strains had oxacillin MIC of ≤2 μg/mL at 24 h, all such strains grew on the NCCLS oxacillin screen agar and produced the PBP2a protein. Isolates of both CMRSA-5 and CMRSA-2–related strains that were erythromycin-resistant were found to be inducibly resistant to clindamycin when challenged by using the double disk approximation test. The remaining susceptibility test results are presented in the Table.

Table. In vitro antimicrobial susceptibilities of 101 methicillin-resistant *Staphylococcus aureus* isolates from humans and horses*

	MIC in μg/mL			% of isolates	
Antimicrobial agent	50%	90%	Range	Intermediate	Resistant
Tetracycline	>16	>16	≤4–≥16	0	96
Doxycycline	8	8	≤4–8	78.2	0
Minocycline	≤4	≤4	≤4–8	14.8	0
Erythromycin	>8	>8	≤0.5–>8	0	86.1
Gentamicin	>16†	>16†	≤2–>16†	9.9	88.1
Rifampicin	>4	>4	≤1–>4	10.9	71.2
TMP/SMX	8/152	>8/152	≤2/38–8/152	NA	79.2

*MIC, minimum inhibitory concentration; TMP/SMX, trimethoprim-sulfamethoxazole; NA, no intermediate MIC category in NCCLS guidelines.
†All strains were susceptible to gentamicin at a concentration of 500 μg/mL.

Discussion

This study has identified the largest number of reported cases of clinical MRSA infection in horses and horse personnel. It also identified extensive nasal colonization in horses and horse personnel from a veterinary hospital and horse farm, nosocomial infection in a veterinary hospital setting with clinical illness in horses, and for the first time, clinical infection in 1 person working with infected horses. The subtyping information and timing of isolation provide solid evidence supporting both human-to-horse and horse-to-human transmission.

The prevalence of MRSA colonization in horses at the OVC-VTH was 4% in 2000 and 8% in 2002; however, care must be taken when interpreting these data because screening was performed during 2 periods that followed identification of clinical MRSA infection in horses at the facility. Similar limitations are present with the prevalence data regarding MRSA colonization on breeding farms, which ranged from 0% to 13% and were based on screening after identification of MRSA infection or colonization at OVC-VTH in horses from these farms. The prevalence of human colonization at OVC-VTH and 1 Ontario horse farm is of concern, particularly because of the likelihood of transmission between horses and humans on these farms. As with horses, the prevalence data in humans must be interpreted with care because of the nature of sampling. Further studies are required to determine the prevalence of MRSA infection and colonization in horses and humans at veterinary hospitals and equine farms. One well-recognized human *S. aureus* strain, CMRSA-5, a relatively uncommon isolate in Canada (11), has the ability to colonize the nose of horses and to spread between horses and between horses and persons on farms and within a veterinary hospital setting. The PFGE pattern of the CMRSA-5 isolates was similar to the PFGE patterns published in a previous report of MRSA infection in horses (13). This finding, along with the isolation of CMRSA-5 from horses in Prince Edward Island, Canada (J.S. Weese et al., unpub. data) and Colorado, USA (P. Morley, pers. comm.) that did not have any contact with colonized Ontario horses or horse personnel, further suggests that CMRSA-5 may be more disseminated in the horse population beyond Ontario.

Why MRSA has emerged in the equine population is unclear. It may reflect increased exposure of horses to MRSA-infected persons, a unique ability of CMRSA-5 to colonize horses, the increasing use of certain antimicrobial drugs in veterinary medicine, or a combination of these factors. Several recent investigations in humans suggest that the fluoroquinolones themselves may actually predispose patients to infection with or carriage of MRSA (27,28). Two case-control studies examining risk factors for MRSA found a significant association between fluoroquinolone exposure and MRSA isolation or infection (4,29).

Whether fluoroquinolone use in horses has facilitated emergence of MRSA is not known, since no current data exist on fluoroquinolone use in veterinary medicine. Enrofloxacin is widely used in some sectors of the Ontario horse population, particularly racing horses; however, it is rarely prescribed at OVC-VTH and uncommonly used on breeding farms (J.S. Weese, unpub. data).

While MRSA has been considered primarily a hospital-associated pathogen in humans (10), the increasing incidence of community-acquired infection is concerning (8,9,30,31). Similar to the equine and equine-associated cases reported here, community-acquired infection with SCC*mec*IV strains has been reported in humans (32–34). Most reports of community-acquired-MRSA in humans involve skin and soft tissue infections, and production of PVL has been implicated as the possible virulence factor in community-associated MRSA infection in humans (31,35). None of the isolates from clinical equine or equine-associated infections in this study contained PVL genes; however, definitive conclusions regarding the role of PVL in equine-associated infection cannot be made with the reasonably small number of clinical isolates evaluated here. Isolates in this study were also multidrug resistant, in contrast to results of many reports of community-associated MRSA in humans (36,37). Therefore, determining the origin of these isolates (community versus hospital) is not straightforward and requires further study.

The lack of proven, safe, and acceptable options of eradication of nasal colonization in horses creates potential management problems. To date, isolation of infected hors-

es and use of barrier precautions have been employed (J.S. Weese, unpub data). However, such methods may be difficult on farms, particularly if colonization is prolonged.

Our study has shown that MRSA infection may be an emerging disease in horses, which agrees with earlier reports (13,14). MRSA infection also may become an important nosocomial problem in the veterinary hospital setting and become endemic on horse farms, particularly in foals. Because of the extensive movement of horses, especially thoroughbreds and standardbreds, between and within Canada and the United States, MRSA colonization and infection may be more widespread than recognized. Emergence of MRSA as an equine pathogen is of additional concern because horses may be a community reservoir of MRSA and source of infection or reinfection for persons. In view of the size of the North American horse population and the frequent close contact between many persons and horses, this concern must not be dismissed. Further study is required to clarify the role of this pathogen in equine disease and transmission between horses and humans.

Dr. Weese is an associate professor in the Department of Clinical Studies, Ontario Veterinary College, University of Guelph. His current research interests include multidrug-resistant bacteria (particularly interspecies transmission of such bacteria), zoonotic diseases, and veterinary infection control.

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Malaria Risk in Travelers

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Imported malaria has been an increasing problem in several Western countries in the last 2 decades. To calculate the risk factors of age, sex, and travel destination in Swedish travelers, we used data from the routine reporting system for malaria (mixture of patients with and without adequate prophylaxis), a database on travel patterns, and in-flight or visa data on Swedish travelers of 1997 to 2003. The crude risk for travelers varied from 1 per 100,000 travelers to Central America and the Caribbean to 357 per 100,000 in central Africa. Travelers to East Africa had the highest adjusted odds ratio (OR = 341, 95% confidence intervals [CI] 134–886) for being reported with malaria, closely followed by travelers to central Africa and West Africa. Male travelers as well as children <1–6 years of age had a higher risk of being reported with malaria (OR = 1.7, 95% CI 1.3–2.3 and OR = 4.8, 95% CI 1.5–14.8) than women and other age groups.

Imported malaria has been an increasing problem in Sweden and other Western countries in the last 2 decades. Two possible reasons for this increase are the increase in the number of travelers to tropical countries, as well as a growing number of immigrants from malaria-endemic countries (1–3). Even though the number of malaria cases has been declining during the past years in Sweden (4), the risk for travelers is still evident and should be a concern for physicians who give pretravel advice or evaluate a returning traveler with fever.

Several studies have assessed malaria risk in travelers to specific countries (5–8). The risk of a traveler's acquiring malaria has been considered highest in sub-Saharan Africa and Papua New Guinea, intermediate on the Indian subcontinent, and low in Southeast Asia and Latin America. The numbers assigned to the relative risk in these regions, however, are quite variable (9–12). The total number of travelers is often unknown, and most reports based on national reporting data therefore lack a denominator (2,9). Hence, making a risk assessment on the basis of such figures is difficult. Other approaches to achieve risk estimates are case-control studies. In 1 such Danish study, the country-specific risk for acquiring malaria varied from 714 per 100,000 travelers to Ghana to 2.5 per 100,000 to Thailand (13).

To our knowledge, no previous study based on national data over an extended period has related the number of cases of malaria diagnosed in returning travelers from malaria-endemic areas to continuously collected data on the total number of travelers to that same area. Through access to one of Europe's largest ongoing surveys on travel patterns (14) and to data on reported malaria, we analyzed the risk factors for malaria in returning Swedish travelers from 1997 to 2003.

Materials and Methods

Cases and Controls

Cases were derived from the routine Swedish reporting system. Malaria is a reportable disease in Sweden, and all patients in whom the disease is diagnosed are reported from both the clinician treating the patient and the microbiology laboratory confirming the diagnosis. By using a unique personal identification number, issued to all Swedish residents, the 2 reporting sources can be linked. Thus, all cases reported from 1997 to 2003 were included. Since routine surveillance data were not sufficiently detailed regarding information on prophylaxis, the patients included a mixture of those with and without adequate prophylaxis. To assess travel risk specifically, newly arrived immigrants and refugees, who lack a personal identification number, were excluded from the study.

Controls were obtained from the Swedish Travel and Tourist Database, a commercial ongoing survey, based on a randomized selection of 2,000 members of the Swedish population each month (14). These persons are interviewed by telephone in regard to all overnight travel (business as well as pleasure) in the preceding month, and the data are weighted and extrapolated to estimate the total numbers of Swedish travelers. When the number of respondents has been too low to give a reliable estimate of

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the total number of travelers to a specific country, regions (rather than single countries) are given in this database. This practice was used for all malaria-endemic countries in this report, except for Thailand, where country-specific data were available for the period 2001–2003.

Out of the total database, which contained detailed information from almost 170,000 interviews, information about all respondents who had traveled to malaria-endemic areas from 1997 to 2003 was extracted. To evaluate data quality in the travel database, the weighted and extrapolated estimates of total numbers of travelers to Thailand, India, The Gambia, and South Africa were compared with in-flight or visa data on Swedish travelers. These figures were obtained by courtesy of the embassies of the respective country in Sweden, except for The Gambia, where the figures were supplied by the Central Statistics Department of the Gambia, (through the courtesy of the Swedish embassy in Dakar, Senegal). For each study participant (patients and controls), we used the following information: age, sex, year and month of infection (patients); year and month of travel (controls); country of infection (patients); and country/region of travel (controls). No data on any illness were available for controls. The latest available information on annual malaria incidence among the local population in the studied countries and regions was accessed from the World Health Organization (WHO) (15).

Statistics

The risk for disease per 100,000 travelers, with 95% confidence intervals (95% CI), was calculated by using reporting data as numerator and the estimated total numbers of travelers from the travel database as denominator. For the 4 specific countries mentioned above, the risk per 100,000 travelers was also calculated by using in-flight and visa data as denominator. Since malaria is a rare disease in Sweden and controls were chosen randomly from the entire Swedish population, we could use odds ratios (OR) with corresponding 95% CI as relative risk estimates to assess the association between risk factors (age, sex, and travel destination) and outcome (being reported with malaria).

Each risk factor was first analyzed in a univariate model. To adjust for confounding, we then used a multivariate logistic regression model with these variables, and we also included month of travel or infection. The parameter with the lowest OR in each category was used as reference in the models. Likelihood ratio statistics were used to assess whether each variable in the model contributed significantly to the model and to test for interaction. All analyses were performed with Stata 6.0 software (Stata Corporation, College Station, TX, USA).

The travel database contains aggregated data only. Reportable data are regulated by the Communicable Disease Act and contain full personal identification. The subset of the reporting database abstracted for this study did not contain any information that could be linked to a specific person. The study was approved by the Ethics Committee of the Karolinska Institute, Stockholm, Sweden.

Results

From 1997 to 2003, a total of 975 persons were reported with malaria in Sweden; 118 of them were newly arrived immigrants or refugees and thus excluded from further analysis (Table 1). Of the remaining 857 persons, 348 were infected with *Plasmodium falciparum*, 178 with *P. vivax*, 47 with *P. ovale*, and 15 with *P. malariae* (Tables 2 and 3). In 269 patients, the report did not contain data on *Plasmodium* species. Most of these patients were seen in 1997 before the full implementation of a new reporting system that year. Little variation occurred, either in the number of reported cases or the species distribution over the period, except in the last year studied (2003), which had <65% of the mean of reported cases for the preceding years. This low figure in 2003 was mainly due to a decreased number of reported *P. falciparum* cases.

A total of 16,255 persons with overnight travel abroad were recorded in the travel database for the period 1997–2003. Of these, 881 (projected to a total of 3.5 million travelers) had traveled to malaria-endemic countries or regions, as defined by WHO (16) and were included as controls (Tables 3 and 4). Of the travel destinations, east Asia (mainly Thailand) was dominant.

Three quarters of all cases and 93% of the *P. falciparum* cases were seen in travelers from sub-Saharan Africa (Table 4). The crude risk for travelers to different regions varied from 1 per 100,000 travelers to Central America and the Caribbean to 357 per 100,000 in central Africa. In the multivariable analysis, OR for being diagnosed with any malaria species after return to Sweden was calculated for various risk factors (Table 1). Compared to the reference region (Central America and the Caribbean), travelers to East Africa had the highest OR for being reported with malaria, closely followed by travelers to central Africa and

Table 1. Reported cases of malaria in Sweden, by group of patients, 1997–2003

Category of infection	1997	1998	1999	2000	2001	2002	2003	Total
Immigrants/refugees	10	16	23	14	13	24	18	118
Travel associated	157	144	124	112	127	111	82	857
Total	167	160	147	126	140	135	100	975

Table 2. Reported travel-associated cases of malaria in Sweden, 1997–2003

Source of infection	1997	1998	1999	2000	2001	2002	2003	Total
Plasmodium falciparum	10	56	55	60	58	65	44	348
P. malariae	0	0	1	5	4	4	1	15
P. ovale	2	11	4	8	7	10	5	47
P. vivax	12	34	33	19	40	21	19	178
Unspecified species	133	43	31	20	18	11	13	269
Total	157	144	124	112	127	111	82	857

West Africa. The Indian subcontinent had an OR in the same "middle" range as southern Africa. Southeast Asia and South America had similar ORs, at the lower range. The malaria risk in Arab countries did not differ significantly from the risk in Central America and the Caribbean.

Malaria was significantly more often diagnosed in men than in women, as well as in the age-group <1–6 years, after adjustments were made for the various confounders. The calculated malaria risk per 100,000 travelers in 4 countries with alternative sources of travel information is shown in Table 5, with risk data based on the travel database, and the annual malaria incidence reported to WHO (15) as comparisons.

Discussion

These results are based on official reports of malaria, with data from one of the largest ongoing population-based surveys on travel patterns in Europe as denominator. The laboratory method of microscopy of a blood film for malaria is well defined, and the reporting of diagnosed malaria is believed to be relatively complete in Sweden. Since case-patients are reported both by the clinician and the laboratory, the overall sensitivity of the Swedish surveillance system is comparatively high, with >95% of diagnosed diseases being reported (17).

The information in the tourist and travel database and the reporting database were not fully consistent. The travel database did not contain any data on travel-related illnesses, while the official reports did not contain information on length of stay. Previous studies have shown that the duration of stay influences the risk for malaria (5,12,13), but this factor could not be evaluated in this study. Because the tourist and travel database classification sometimes included both malaria-endemic and malaria-nonendemic countries within the same region, some travelers who only visited regions that were not malaria-endemic were included in the denominator for the region. Therefore, the risk for the region may be underestimated, e.g., in east Asia, which includes several malaria-free countries. However, many of these countries are comparatively rare as tourist destinations.

To further evaluate the precision of the estimates from the tourist database, we also used official in-flight and visa data obtained from 4 countries. Risk estimates from the 2 sources for Thailand and The Gambia/West Africa had good agreement. For India and the Indian Subcontinent and South Africa/southern Africa, where we did not have any tourist data per country, most travelers were going to India and South Africa, respectively, while most malaria patients were from other countries in these regions. The risk estimates from the 2 sources were therefore in less agreement with each other in these regions.

We found that men had a significantly higher risk of being reported with malaria compared to women. A predominance of imported malaria infections in male patients has been documented before (2,5,18,19); men are often also less compliant with chemoprophylaxis than women (5). We also found that children <6 years of age had a significantly higher risk of being reported with malaria. To our knowledge, this risk has not been described before. Many of these young children belonged to immigrant families with roots in the country of infection, where they visited friends and relatives; such children are referred to as "VFRs" (20,21). Parents may be unaware of the fact that the children lack the immunity against malaria when returning "home."

Table 3. Reported travel-associated cases of malaria in Sweden by species of malaria, 1997–2003

Region	*Plasmodium falciparum*	*P. malariae*	*P. ovale*	*P. vivax*	Unspecified	Total
Arab countries and Iran	0	0	0	2	2	4
Indian subcontinent	4	0	3	42	25	74
East Asia	16	1	0	64	30	111
West Africa	141	5	14	9	73	242
East Africa	87	5	14	31	79	216
Central Africa	58	4	9	8	28	107
Southern Africa	40	0	6	6	26	78
Central America + Caribbean	1	0	0	4	2	7
South America	1	0	1	12	4	18
Total	348	15	47	178	269	857

Table 4. Estimated number of travelers to malaria-endemic areas, respondents in the tourist database (controls), and reported patients with travel-associated malaria, 1997–2003*

Age/sex/region†	Estimated no. of travelers	Controls	Reported cases	Risk per 100,000	95% CI	Multivariate odds ratio	95% CI	Incidence/ 100,000
Total	3,560,000	881	857	24	22–26	–	–	36,865
≤6 y	70,000	18	38	54	31–95	4.8	1.5–14.8	No data
7–18 y	300,000	74	91	30	22–41	2.7	1.1–6.0	No data
19–45 y	1,630,000	404	506	31	27–35	4.1	1.9–9.0	No data
46–65 y	1,340,000	331	205	15	13–18	2.0	0.9–4.3	No data
>65 y	220,000	54	17	7.7	4–13	1.0	Reference	No data
Men	1,790,000	444	536	30	26–34	1.7	1.3–2.3	No data
Women	1,770,000	437	321	18	16-21	1.0	Reference	No data
Arab countries and Iran‡	220,000	44	4	1.8	0.7–5.1	1.7	0.5–6.4	1,279
Indian subcontinent	120,000	31	74	62	41–94	57.4	23–141	366
East Asia	2,050,000	517	111	5.4	4.4–6.6	5.6	2.5–12.5	205
West Africa	80,000	22	242	302	196–468	277	112–683	13,356
East Africa	90,000	18	216	240	148–388	341	134–866	7,126
Central Africa	30,000	8	107	357	174–732	317	108–930	5,508
Southern Africa	170,000	42	78	46	32–67	49.6	21–119	7,742
Central America + Caribbean	550,000	43	7	1.3	0.6–2.7	1.0	Reference	155
South America	250,000	61	18	7.2	4.3–12.2	7.1	2.7–18.4	1,205

*With a multivariate odds ratios (with 95% confidence intervals [CI]) for the risk factors age, sex, and travel destination from a logistic regression model, and incidence per 100,000 inhabitants as reported to the World Health Organization (15).
†Malaria-free countries as defined by the World Health Organization (16).
‡Arab countries and Iran = Bahrain,† Iraq, Iran, Jordan,† Kuwait,† Lebanon,† Oman, Qatar,† Saudi Arabia, Syria, United Arab Emirates, Yemen; Indian Subcontinent = Afghanistan, Bangladesh, Bhutan, India, Maldives,† Nepal, Pakistan, Sri Lanka; East Asia = Brunei, Burma, Cambodia, China, Hong Kong,† Indonesia, Japan,† Laos, Malaysia, Mongolia,† Philippines, South Korea, Singapore,† Taiwan,† Thailand, Tibet,† Vietnam; West Africa = Benin, Burkina Faso, Cape Verde, Ghana, Guinea, Guinea-Bissau, Ivory Coast, Liberia, Mali, Mauritania, Senegal, Sierra Leone, The Gambia, Togo; East Africa = Burundi, Djibouti, Eritrea, Ethiopia, Kenya, Rwanda, Seychelles,† Somalia, Sudan, Tanzania, Uganda; Central Africa = Cameroon, Central African Republic, Chad, Congo, Democratic Republic of Congo, Equatorial Guinea, Gabon, Niger, Nigeria, São Tomé et Principe; Southern Africa = Angola; Botswana, Lesotho, Madagascar, Malawi, Mauritius,† Mozambique, Namibia, South Africa, Zambia, Zimbabwe; Central America and Carribean = Antigua and Barbuda,† Bahamas,† Barbados,† Belize, Bermuda,† Cayman Islands,† Costa Rica, Cuba,† Dominica,† Dominican Republic, El Salvador, Grenada,† Guadeloupe,† Guatemala, Honduras, Jamaica,† Haiti, Martinique,† Mexico, Netherlands Antilles,† Nicaragua, Panama, Puerto Rico,† St. Christopher and Nevis,† St. Lucia/St. Vincent,† Saint Kitts-Nevis,† The Grenadines,† Trinidad and Tobago,† Virgin Islands;† South America = Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Paraguay, Peru, Suriname, Uruguay,† Venezuela.

According to official statistics from the Swedish Migration Board, >775,000 persons were granted permanent residence permit in Sweden during the period 1980–2002. A large proportion of these persons came from countries where malaria is highly endemic. A survey based on Norwegian surveillance data has shown that the incidence of malaria was higher in VFRs than in people of Norwegian origin (22). In our study, distinguishing native Swedes from persons with an origin in other countries was not possible among controls. However, reporting data, which often includes this information, indicated that a large number of the patients were VFRs who had visited

Table 5. Malaria risk for officially reported travelers compared to incidence (as reported to WHO, 2001*) and estimated rate (based on TDB) in corresponding region†

			Data from in-flight/visa		Data from TDB			
Country	Y	Cases	No. of travelers	Risk/100,000	No. of travelers	Risk/100,000	95% CI	Data from WHO incidence/100,000
Thailand	2001–2002	9	453,000	2.0	435,000	2.1	1.0–4.2	100
India	2001–2003	7	48,687	14.4	–	–	–	192
Indian subcontinent	2001–2003	25‡	–	–	72,000	35	17-71	–
Gambia	1997–2003	79	31,242	253	–	–	–	10.096
West Africa	1997–2003	242§	–	–	80,000	302	196–468	–
South Africa	1997–2001	3	98,886	3	–	–	–	61
Southern Africa	1997–2001	63¶	–	–	128,000	49	32–76	–

*Information from The Gambia was from 1999.
†WHO, World Health Organization (15);TDB, Swedish Travel and Tourist Database; CI, confidence intervals.
‡7 cases from India, 13 cases from Afghanistan, and 5 cases from Pakistan.
§6 cases from Benin, 4 cases from Burkina Faso, 31 cases from Ivory Coast, 79 cases from The Gambia, 65 cases from Ghana, 11 cases from Guinea, 5 cases from Guinea-Bissau, 11 cases from Liberia, 3 cases from Mali, 11 cases from Senegal, 11 cases from Sierra Leone, and 5 cases from Togo.
¶14 cases from Angola, 5 cases in Madagascar, 8 cases from Malawi, 13 cases from Mozambique, 3 cases from Namibia, 3 cases from South Africa, 7 cases from Zambia, and 10 cases from Zimbabwe.

countries outside usual tourist routes, including Somalia, Ethiopia, Uganda, Bangladesh, and Pakistan. Our data thus suggest that VFRs are a risk group requiring special attention. These persons may be less inclined than other travelers to get pretravel advice and to use chemoprophylaxis against malaria (21,23).

P. falciparum malaria is the prime target for chemoprophylaxis to prevent death and severe disease. Although we considered doing a separate risk analysis for this species, reporting at the species level in the first year of the study was not sufficiently complete to allow for meaningful analysis. Furthermore, other malaria species also contribute substantially to malaria illness in travelers (24). Falciparum malaria is a clinically overt disease and will most probably be diagnosed. For other malaria species, a few cases, especially in persons with partial immunity, might be missed, thus underestimating the true risk.

No systematically collected data on chemoprophylaxis are included in this study, an obvious limitation when assessing the malaria risk. However, previously published reporting data from 2003 for *P. falciparum* infection indicate that 12 of 17 Swedish travelers had not taken any prophylaxis, and another 2 had taken drugs with insufficient effect for the country they visited. Of 34 VFRs, only a few had taken prophylaxis (25). Prophylaxis influences the number of cases, as do other factors associated with the behavior of individual travelers, such as use of mosquito-protective measures and the standard of housing visited, on which we do not have any information from reports. Furthermore, local malaria transmission intensity is key to the malaria risk for a traveler. Within several malaria-endemic countries, the risk for malaria varies greatly, reflecting local transmission intensity at the district level (16,26,27). This local variation, together with the different travel patterns within countries, may greatly influence the risk of travelers contracting malaria. For example, in Thailand, most Swedish travelers go to areas of the country in which no malaria occurs, a fact that could partly explain the low incidence in Swedish travelers compared to WHO data on local incidence. This low incidence in Swedish travelers is in line with a previously cited report on malaria risk in Danish residents traveling to Thailand (13), where the risk was estimated to be 2.5 per 100,000 travelers. Our corresponding figure, with denominator data from the embassy, was 2.1 per 100,000. A British study reported a slightly higher risk, 8.2 per 100,000 (6). All 7 Swedish travelers who contracted *P. falciparum* malaria in Thailand from 1997 to 2003 had visited regions outside of the usual charter tourist destinations, such as remote national parks and jungle areas. Another example is The Gambia, where the average Swedish tourist often stays most (or all) of the vacation in beach resorts by the coast, where the malaria transmission intensity is lower than in inland areas (28).

Discrepancies do exist between the calculated risks for Swedish travelers to have malaria diagnosed in Sweden after traveling to malaria-endemic regions, and the incidence rates of patients in the same regions reported to WHO. Several factors could explain these differences, including the small number of Swedish malaria cases, the fact that indigenous malaria is associated with more than just transmission intensity, i.e., poverty (29), duration of exposure, and different sensitivity of the surveillance and reporting systems in different countries. Data from Africa, especially, are incomplete (29).

In general, the order of magnitude of the relative risk for malaria in the different regions was consistent with earlier data on relative malaria risk for travelers (9–12). Based on these risk data, we divided the regions into 3 groups: sub-Saharan Africa (except for southern Africa) exhibited the greatest relative risk for malaria in returning travelers (>250), followed by India and southern Africa (relative risk [RR] ≈50) and at the lower end Southeast Asia, South America, and the Arab countries (RR <10). A number of other studies have shown higher risk for acquiring malaria in West Africa compared to East Africa (5,8). Our data show higher risk in those visiting central Africa and West Africa than East Africa, corresponding to these previous studies. Our results are representative for the overall malaria risk in Swedish travelers and are also likely to reflect the risk in travelers from other European countries.

This study confirms that the risk of a traveler's contracting malaria is highest in Africa, south of the Sahara, and that male travelers and small children constitute groups with increased risks. Furthermore the added complexity of immigrants from malaria-endemic areas needs to be considered when discussing malaria prevention among travelers. All pretravel advice needs to be individualized for each traveler, based on the exact travel route, season, and type of travel.

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Notifiable Disease Surveillance and Practicing Physicians

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Primary care physicians in Germany are essential participants in infectious disease surveillance through mandatory reporting. Feedback on such surveillance should reflect the needs and attitudes of these physicians. These issues were investigated in a questionnaire survey among 8,550 randomly sampled physicians in Germany in 2001. Of the 1,320 respondents, 59.3% claimed not to have received any feedback on infectious disease surveillance, and 3.7% perceived feedback as not important. Logistic regression analysis showed that physicians in the former East Germany were 2.2 times more likely to have received feedback than those in the former West Germany. Physicians preferred to receive occasional reports (e.g., in case of outbreaks, 31.6%) as opposed to actively having to search for constantly updated information on the Internet (7.8%). The preferred formats were fax (31.7%), mail (30.9%), and the official organ of the German Medical Association (Deutsches Ärzteblatt) (30.5%). Feedback of surveillance data to physicians should be delivered through occasional nonelectronic reports on current issues of local public health importance.

In most countries, notifiable disease surveillance systems rely on mandatory reporting of cases by physicians and laboratories. Although primary care physicians are likely to remain the first and most qualified entry post into such an information system, little research is available on the knowledge, attitudes, and needs of these physicians regarding surveillance of reportable diseases.

In 2001, a new infectious disease control law (IfSG) was implemented in Germany. Most diseases are to be reported to the local county (Landkreis) health department from where they are reported by the state (Land) to the national surveillance institute (Robert Koch Institute). The IfSG also introduced national case definitions for notifiable diseases.

The aim of this study was to identify the needs and attitudes of primary care physicians towards public health surveillance. Studies in various countries have concluded that low compliance of physicians with notification systems is partly caused by insufficient feedback of surveillance data to the physicians (1–3). However, practical information is lacking on how this feedback should be organized. This study identified ways to accommodate the needs of physicians regarding surveillance to increase their notification compliance.

Methods

We conducted a survey among primary care physicians in Germany identified by the Green Cross, a non-profit, nongovernmental organization that aims to improve health care. We sent a standardized questionnaire to a random sample (N = 8,550, 14.5%) of 60,280 primary care physicians to be returned by mail on a voluntary and anonymous basis (4).

The questionnaire included the following items: sociodemographic and practice-related characteristics, information about changes in the IfSG, expectations from the surveillance system, and reporting practices. West German States were defined as the States of former West Germany including Berlin. The questionnaire was pilot-tested for usefulness and validity by 70 primary care physicians.

Univariate statistical analysis, chi-square tests, and *t* tests were used as appropriate. We performed multivariate logistic regression to identify predictors for the level of feedback received. Variables were kept in the model according to the likelihood ratio statistic (forward selection $p < 0.05$). SPSS software version 11.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

To assess response bias, the mail survey was followed by a telephone survey. We conducted this survey with 14.5% of the primary care physicians who had originally been sampled for the mail survey. We asked a limited set of questions on the demographic characteristics of the respondent. The data were compared with responses on the

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completed questionnaires to identify possible bias due to nonresponders.

Results

Overall, 1,320 completed questionnaires (response rate 15.4%) were returned and included in the study. Of all participating physicians, 87.6% resided in the former West Germany and 86.0% in urban areas. Other demographic characteristics are shown in Table 1. Two-thirds of the physicians saw 250–750 patients per month (median 500 patients per month). Internet access was available for 67.4% of the responders.

Of the physicians, 47.9% felt sufficiently informed about the new infectious disease law. A specific need for information was expressed for the following items: rights and duties of the physician with respect to reporting (55.5%), criteria for disease notification (43.7%), which diseases are reportable (38.4%), rights and duties of the local health department (36.3%), notification format (33.2%), who in the public health system is responsible for outbreak investigations and control measures (33.1%), confidentiality issues (17.1%), and other aspects (4.5%). The existence of case definitions was unknown to 86.5% of the respondents; 75.2% expressed the desire to have case definitions available.

Only 40.7% of the physicians had received any feedback on surveillance data. In the former East Germany, a higher proportion of the physicians received feedback, compared with those in the former West Germany (56.7% vs. 38.8%; $p < 0.001$). Multivariate logistic regression showed that physicians in the former East Germany were 2.19 times more likely to have received feedback on surveillance data than their colleagues in the former West Germany (95% confidence interval 1.54–3.10).

The sources from which the physicians had received feedback (537 responders) were scientific literature (58.1%), daily press (20.9%), the weekly Epidemiological Bulletin of the Robert Koch Institute (18.4%), reports of the state health departments (11.9%), and other sources (15.3%). Table 2 shows the results of the questions related to how the physicians would like to receive feedback.

The average monthly time invested in disease reporting was 1.17 hours (median 1, range 0–48) under the new law, compared with 1.02 hours (median 1 hour, range 0–48, $p < 0.001$) under the old surveillance system. Primary care physicians in the former East Germany invested more hours (median 1, mean 1.92, range 0–48) compared with their colleagues in the former West Germany (median 1, mean 1.08, range 0–30), but the difference was not significant ($p = 0.07$). Half (50.2%) of the participants stated that under the current system, the obligation to report diseases is likely to influence their diagnostic approach compared with 41.1% under the old surveillance system ($p < 0.001$).

Of the physicians, 73.9% expressed their willingness to participate in voluntary infectious disease sentinel projects (75.5% in the former West Germany vs. 66.2% in the former East Germany, $p < 0.05$). The following criteria were chosen when asked about the 2 most important conditions for voluntary participation: easy handling (77.7%), feedback of results (43.1%), and financial compensation (38.9%).

For the nonresponder analysis, a sample of 1,241 physicians (14.5%) was chosen from the 8,550 to whom the questionnaires had been originally sent. Of those 1,049 physicians who could be reached by telephone, 656 (62.6%) agreed to participate. Responders differed significantly from nonresponders for the following items: responders were younger (mean age 49.4 years vs. 51.6 years among the nonresponders; $p < 0.001$), more often specialists of various medical practices rather than general

Table 1. Demographic characteristics of primary care physicians in Germany and the study participants, 2001

Characteristic	All physicians in Germany* (N = 59,610), %	Study population (N = 8,550), %	Survey (questionnaire) (N = 1,320), %	Survey (telephone) (N = 656), %
Age, y				
≤39	13.7	–	10.6	6.1
40–49	40.3	–	38.7	32.5
50–59	35.0	–	40.9	43.5
≥60	11.0	–	9.8	18.0
Sex				
Male	68.3	70.6	73.9	72.8
Female	31.7	29.4	26.1	27.2
Specialization				
General medicine	73.2	70.7	72.6†	75.6
Internal medicine	26.8	28.0	24.9	22.9
Other		1.3	2.5	1.5
Region				
West†	83.3	88.6	83.9	84.0
East	16.7	11.4	16.1	16.0

*According to the National Association of Statutory Health Insurance Physicians.
†Including Berlin.

Table 2. Feedback preferences for epidemiologic notifiable disease surveillance data for primary care physicians in Germany, 2001 (N = 1,320)

Preference	%
Preferred time for feedback (maximum of 1 response)	
Quarterly	33.4
Semiannually	27.2
Upon an occasion (e.g., outbreak)	31.6
Access through the Internet*	7.8
Preferred region on which to deliver feedback (maximum of 2 responses)	
Own county (Kreis)	41.1
Own state (Bundesland)	29.3
Germany	34.8
Europe	28.8
Worldwide	37.3
Preferred medium by which to deliver feedback (maximum of 2 responses)	
Fax letter	31.7
Fax call	2.0
Mail	30.9
Official organ of the German Medical Association (Deutsches Arzteblatt)	30.5
E-mail	20.9
Internet	16.2
Weekly national epidemiologic bulletin	16.5
Preferred issues for feedback (multiple responses)	
Outbreak of infectious diseases	85.2
Trends	47.6
Control of vaccination programs	31.5
Revelation of imported or travel-associated diseases	60.4
Recommendations on preventive measures	65.3
Other	1.4

*Constant availability of information for active retrieval through the Internet by user.

practitioners (11.8% vs. 5.6%, $p < 0.001$), and were less likely to know about the special rules that exclude costs for infectious disease laboratory diagnosis from budgetary limitations (74.5% vs. 79.0%, $p = 0.03$). No differences existed between responders and nonresponders for any other variables.

Discussion

The survey revealed some unexpected findings about attitudes and expectations of primary care physicians toward the notifiable infectious disease surveillance system. Only half of the respondents felt sufficiently informed about the new law. Studies in southern Africa (5), Australia (1), and the United Kingdom (6) have also found that the list of notifiable diseases is not well known by physicians. This underscores the need to repeatedly inform physicians about the notifiable disease surveillance system.

The survey showed that physicians request information on the occurrence of outbreaks in their county or other acute issues with direct implications and that they are hesitant to actively retrieve the information on the Internet or through faxing (where the user dials the fax server number of a provider to receive a fax). This finding is compatible with findings in the United Kingdom and Australia that general practitioners make limited use of computers (7–9). A possible consequence may be that local health departments maintain a fax mailing list of all relevant physicians in their county and distribute concise warnings about important public health events as they occur. During a major measles outbreak in northern Germany in 2001, this method was implemented very successfully (10).

The finding that physicians in the former East Germany spend more time on notification and receive feedback more often than their colleagues in the former West Germany is consistent with the results of a survey among health departments, in which health departments in the former East Germany would write their own local surveillance reports significantly more often than health departments in the former West Germany (11). These observations suggest that the public health system in the East German Democratic Republic had a stronger emphasis on infectious disease surveillance and reporting than in the West German Federal Republic. These differences have been major enough to remain detectable >10 years after unification of West and East Germany. Whether similar differences will be detected in the European Union between western European member states and eastern member states that have recently joined the Union should be determined.

One major goal of the new infectious disease control law was to drastically reduce the number of diseases reportable by the physician to increase notification compliance (12). However, this goal may be wishful thinking since the physicians claimed that time invested in notifications had increased slightly in the new system.

Whether the obligation to report certain diseases influences the diagnostic behavior of physicians has rarely been addressed. Half of the respondents in our survey stated that this was indeed the case. This influence may depend on the situation and the disease. Clinical diagnoses of diseases perceived by physicians to be of little importance may be less likely to require microbiologic testing to avoid the administrative task of notification (13). Conversely, diseases may be considered important because they are notifiable, thus resulting in a higher probability of microbiologic testing of samples.

The physicians related their willingness to participate in sentinel systems not so much on the financial compensation or the feedback of such activities but rather on the easy handling of the reporting procedure. In this context, it seems to be a priority to generate compatible interfaces between the software systems increasingly used by general practitioners that allow easy generation of disease

reports to health departments. Within a national initiative for electronic-government, the Robert Koch Institute is currently developing a concept for highly automated Internet-based reporting of infectious diseases for physicians and laboratories.

Although the response rate of 15% is not unusually low for a survey among physicians, those who participated may have been particularly interested in infectious disease and public health surveillance, thus leading to a bias towards a greater willingness to comply with the system and to be interested in feedback. However, analysis of the nonresponders did not find any evidence for a relevant selection bias. Physicians' use of media may differ between countries and also change over time, and the situation may be different for participants of research networks or sentinel surveillance systems.

This study showed that the feedback of surveillance data to primary care physicians should use conventional, nonelectronic formats and concentrate on current outbreaks or other public health issues directly relevant to the physician. Low physician compliance with reporting has led to intensification of laboratory-based reporting systems in which electronic notification systems appear to be easier to implement (1,9,14–17). However, laboratory-based surveillance systems only include diseases that are confirmed by a complete laboratory diagnosis. Physician notification should take place at a much earlier stage of the diagnostic process, enabling local health departments to rapidly initiate control activities. However, at times of increasing cost awareness in healthcare systems, the proportion of infectious diseases confirmed by laboratory diagnosis may decrease further. Parallel to implementing syndromic systems to achieve early and sensitive surveillance, improving classic disease notification systems for physicians (18) will also be important.

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SARS-associated Coronavirus Transmitted from Human to Pig

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Severe acute respiratory syndrome–associated coronavirus (SARS-CoV) was isolated from a pig during a survey for possible routes of viral transmission after a SARS epidemic. Sequence and epidemiology analyses suggested that the pig was infected by a SARS-CoV of human origin.

Severe acute respiratory syndrome (SARS) was first identified in Guangdong Province, China, in November 2002 (1). A novel coronavirus, SARS-CoV, was identified as the pathogen; several possible origins of the coronavirus were suggested from wild animal reservoirs, such as Himalayan palm civets and raccoon dogs (2–8). The virus infects many other wild and domesticated animals, such as *Mustela furo*, *Felis domesticus*, and *Nyctereutes procyonoides* (9,10), but infection of domesticated pigs has not been previously reported.

The Study

We surveyed 6 major domestic animal species that are in close contact with humans and could be infected by SARS-CoV if transmission were possible. The survey was conducted in a suburban area and its extended farming villages, Xiqing County of Tianjin, China, where a SARS outbreak occurred in late spring of 2003. Animal samples, blood and fecal swab specimens, for antibody and RNA detection were collected from the sites and transported on ice to a biosafety level 3 laboratory within 24 hours. We used 2 types of assays for the initial viral screen, immunologic assays to identify antibodies and reverse transcription–polymerase chain reaction (RT-PCR) to detect the viral genome. The immunoassays were carried out by the double-antigen sandwich method with a recombinant N protein and a partial S protein of SARS-CoV, and results were confirmed by Western blot (11). RT-PCR with virus-specific primers was used to detect viral genome RNA, which was extracted from blood samples with a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) and from fecal swabs with Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was then reverse transcribed with random hexamers, and cDNA was amplified with a nested PCR method (12). We also isolated viruses from Vero E6 cultures, performed a cross-neutralization test, and sequenced the viral genome (13).

Of 242 animals surveyed, we identified 2 antibody-positive samples from 2 pigs; test results for the other 240 animals were negative (Table 1 and Figure 1). Of 93 blood specimens and 15 fecal swabs on which we performed RT-PCR, 1 of the same 2 pigs tested positive. We subsequently obtained 2 viral isolates from its blood and fecal samples, designated TJB and TJF, respectively. We also performed follow-up studies for 4 weeks on the infected pig until its blood tested negative with our RT-PCR assay. The animal later died giving birth. We also tested serum samples from the swineherd on the farm and a few persons who may have had contact with the swineherd. All were negative by the tests that we conducted.

Using a viral isolate, TJF, we conducted cross-neutralization experiments with antisera and an early viral isolate, BJ01 (8), to prove their equivalent virulence (Table 2). We then sequenced TJF completely (GenBank accession no. AY654624) and compared its sequence to that of BJ01. Eighteen nucleotide (nt) substitutions are between the TJF and BJ01 sequences, and 4 of them are nonsynonymous over the entire length (29,708 bp). Two pieces of evidence strongly suggested a human origin for the TJF strain. First, it is only distantly related to SZ16, which was isolated from Himalayan palm civets of southern China, in which 64 substitutions over a length of 29,731 bp were found, 3.6 times more than were identified between TJF and BJ01. Second, a sequence signature (a 29-nt insertion [246 nt upstream of the N gene, from residues 27869 to 27897]) found only in an early isolate, GD01 (from Guangdong Province), but absent from all the SARS-CoV isolates so far discovered, is also absent in the TJF sequence (14). This sequence has been found in all coronavirus isolates of animal origin except from the pig identified in this study. Therefore, direct viral transmission of SARS-CoV from a human host to the pig bearing TJF is most likely. To further elucidate our point, we constructed a phylogenetic tree based on S-gene sequences; it shows that TJF is more closely related to human SARS-CoV isolates than to animal coronaviruses (Figure 2).

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Table 1. Animal surveys for antibodies to SARS-CoV and viral RNA*

	Antibodies in serum samples				RT-PCR				Virus isolation			
	ELISA		WB		Blood		Fecal swab		Blood		Fecal swab	
Animals	N	P	N	P	N	P	N	P	N	P	N	P
Pigs	108	2	5	2	14	1	14	1	6	1	6	1
Cattle	60	0	0	0	22	0	0	0	0	0	0	0
Dogs	20	0	0	0	14	0	0	0	0	0	0	0
Cats	11	0	0	0	11	0	0	0	0	0	0	0
Chickens	11	0	0	0	11	0	0	0	0	0	0	0
Ducks	30	0	0	0	20	0	0	0	0	0	0	0

*Numbers in table refer to number of animals tested with the assays. SARS-CoV, severe acute respiratory syndrome–associated coronavirus; RT-PCR, reverse transcriptase–polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; WB, Western blot; N, negative; P, positive.

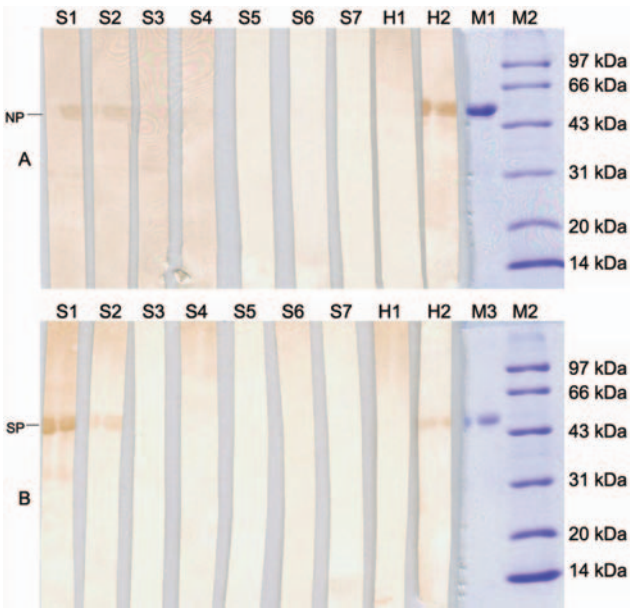

Figure 1. Detection of antibodies against severe acute respiratory syndrome (SARS)–associated coronavirus recombinant proteins in animal sera by Western blotting. Recombinant nuleocapsid protein in panel A (NP, 54 kilodaltons [kDa]) and partial spike protein in panel B (SP, 57 kDa) were used as antigens. Goat antiswine immunoglobulin G horseradish peroxidase was used as a secondary antibody. Serum samples from a convalescent SARS patient and healthy persons were used as positive and negative controls, respectively. Swine (S1 to S8) and human (H1 and H2) samples are sera collected during the survey. M1, M2, and M3 are purified NP, SP, and molecular weight markers, respectively. Positive bands at the corresponding molecular weight of the 2 proteins are indicated with arrows.

Conclusions

We have shown that human SARS-CoV can infect domesticated mammals, in particular, the pig. The direct source of SARS-CoV transmission to the identified infected pigs was most likely virus-contaminated animal feed because the farm where the infected pig was identified is rather remote, >1 km from the nearest village. The only person routinely in close contact with the animals is the swineherd, whose serum samples were negative for SARS-CoV on all tests. Swineherds in rural areas often obtain leftovers from restaurants in the cities for use as hogwash (without thoroughly fermenting it). Thus, even if no direct evidence for human-to-swine SARS-CoV transmission exists, a strong warning should be issued to prevent such a practice, or regulatory procedures should be instituted to block this route of disease propagation (15). Whether or not other domesticated (such as dogs and cats) and wild animals that are common in and around human settlements can easily contract and pass on SARS-CoV remains to be seen in future studies. Intensive surveillance and investigations on animals, especially during and after an outbreak of SARS, will lead to a better understanding and ability to control this disease's natural animal reservoirs and to prevent interspecies transmission events.

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Table 2. Cross-neutralization tests for severe acute respiratory syndrome (SARS)–associated coronavirus*

	Sera (ND_{50})					
Virus strains	S1	S2	S3	H1	H2	H3
TJF	1:160	1:640	<1:10	1:1,280	1:640–1,280	<1:10
BJ01	1:160–320	1:640	<1:10	1:640–1,280	1:320–640	<1:10

*S1 and S2 were sera from swine that were positive by enzyme-linked immunosorbent assay (ELISA). H1 and H2 were sera from SARS patients. H3 and S3 were controls from sera of a normal human and an ELISA-negative pig, respectively. ND_{50}, 50% neutralization dose.

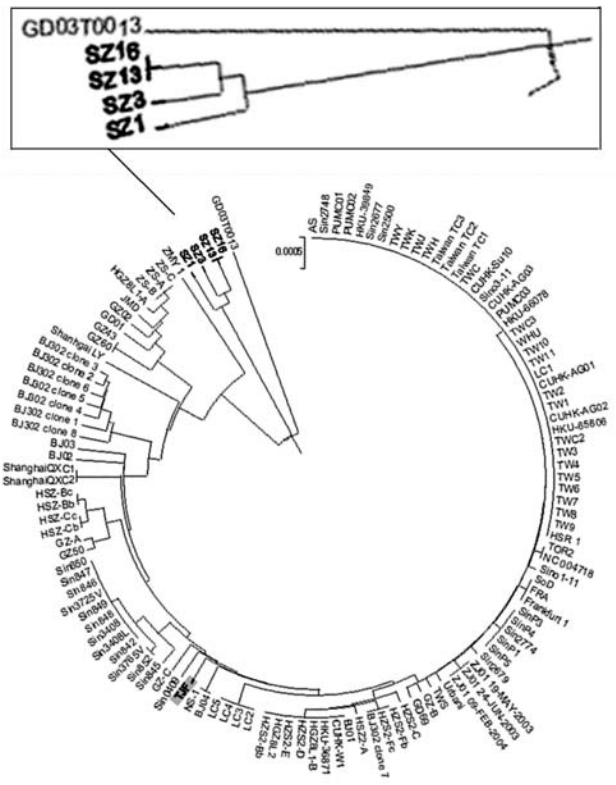

Figure 2. Phylogenetic analysis of severe acute respiratory syndrome-associated coronavirus S-gene. Nucleotide sequences of S genes (from 21491 to 25258 and 3768 bp in length) were compared. The result was displayed with MEGA-2 program and based on 125 complete S-gene sequences from GenBank.

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Mimivirus in Pneumonia Patients

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Mimivirus, the largest virus known to date, is an amebal pathogen–like *Legionella* sp. When Mimivirus was used as an antigen in a migration inhibition factor assay, seroconversion was found in patients with both community- and hospital-acquired pneumonia. Mimivirus DNA was found in respiratory samples of a patient with hospital-acquired pneumonia.

The causative agent of pneumonia, the leading cause of infection-related death throughout the world, is unknown in 20% to 50% of cases (1). Therefore, identifying new causative agents of both community- and hospital-acquired pneumonia is a major public health goal. Aquatic bacteria such as *Legionella* spp., *Pseudomonas* spp., *Stenotrophomonas* spp., *Burkholderia* spp., and *Acinetobacter* spp. colonize hospital water supplies. These bacteria, such as *L. pneumophila*, have been causally associated with both hospital- and community-acquired pneumonia. *Legionella* spp. and other bacteria are associated with free-living amebas in natural and hospital aquatic environments (2). Bacteria that resist phagocytic destruction by amebas and are found in aerosolized water are potential agents of pneumonia (3). Ameba-associated bacteria other than *L. pneumophila*, including other *Legionella* spp., new α-proteobacteria belonging to the Bradyrhizobiaceae (*Bosea massiliensis*) family, and members of the genus *Parachlamydia* might be implicated in hospital-acquired pneumonia (4–6).

In strict intraamebal bacteria, we found *Legionella*-like amebal pathogens (7), *Parachlamydia acanthamebae* (8), and a giant virus resembling gram-positive cocci that we named Mimivirus (9). The Mimivirus's genome is larger than that of *Mycoplasma* and genome sequencing is finished (10). Because we found antibodies against several ameba-associated bacteria in patients with community- and hospital-acquired pneumonia (3,4) in previous studies, we tested for antibodies to Mimivirus by using a microimmunofluorescence assay on serum samples from patients with community- and hospital-acquired pneumonia. DNA of Mimivirus was also found in the bronchoalveolar lavage specimens of patients with hospital-acquired pneumonia.

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The Study

We studied serum samples from 376 Canadian patients with community-acquired pneumonia (121 ambulatory and 255 hospitalized) and from 511 healthy control subjects. Extensive clinical data were available for 104 patients with community-acquired pneumonia. All of these samples were previously tested for other pneumonia agents (4). To prepare antigen for microimmunofluorescence study, Mimivirus was grown in *Acanthameba polyphaga* strain Linc AP-1 in 75-cm^2 cell culture flasks with peptone yeast extract glucose medium as previously described (11). After amebal lysis occurred, unlysed amebas were removed by low speed centrifugation at 100 g for 15 min. Mimivirus particles present in supernatant were centrifuged at 4,000 g for 30 min and washed 3 times in phosphate-buffered saline (PBS). The pellet obtained after the last washing was then resuspended in PBS at 2 mg/mL concentration of protein and used as antigen in microimmunofluorescence assay under previously described conditions (5). Evidence of serologic reaction to Mimivirus was defined as: 1) seroconversion from <1:50 to ≥1:100 between acute-phase and convalescent-phase serum samples or a 4-fold rise in antibody titer between acute-phase and convalescent-phase serum samples, or 2) a single or stable titer of ≥1:400. The cut-off titer for single serum was chosen to have <2.5% positive rate in control subjects. We also tested paired serum samples from 26 patients with intensive care unit (ICU)-acquired pneumonia for a 1-year period and 50 paired serum samples from patients in our institution to determine antibodies to *Rickettsia* spp. as controls.

To verify that antibodies against Mimivirus in patients with pneumonia recognize Mimivirus particles specifically, a serum sample of 1 of these patients was used to detect Mimivirus particles by immunogold technique as previously described (12). Two serum samples of patients who did not have detectable antibodies against Mimivirus were used as controls. Grids were incubated briefly twice in incubation buffer (PBS with 0.2% bovine serum albumin) for 5 min, then for 15 min in lysine buffer (PBS with 0.05 mol lysin). Grids were washed twice in incubation buffer for 5 min, then incubated for 3 h at 37°C in patients' diluted samples (diluted 1/1,000 in incubation buffer with 3% nonfat dry milk). Grids were washed 6 times for 5 min in incubation buffer, then incubated for 2 h at 37°C in goat antihuman immunoglobulin (IgG)-gold conjugate (Aurion Biovalley, Marne la Vallee, France) diluted 1/20 in incubation buffer with 3% nonfat dry milk. Grids were washed 6 times for 5 min in incubation buffer, then twice in PBS for 5 min. Grids were then immersed twice in gutaraldehyde (2% in PBS) for 5 min, rinsed 3 times in distilled water for 5 min, treated by R-GENT SE-EM (Aurion Biovalley) for 25 min, then rinsed 3 times in distilled water for 5 min before being stained with uranyl acetate before examination.

Genomic DNA of Mimivirus was found in bronchoalveolar lavage specimens from patients in the ICU of the Ste. Marguerite Hospital, Marseilles, France; serologic reaction was studied in serum samples of the patients for 1 year. The study was retrospective; samples were tested anonymously from 12 to 18 months after sampling. Mimivirus was found in bronchoalveolar lavage specimens from 1 of 32 patients with ICU-acquired pneumonia and none in specimens from 21 intubated control patients in ICU who did not have pneumonia (5). We designed 4 primer pairs chosen from the genome sequence of Mimivirus. To avoid any contamination, we used a nested polymerase chain reaction (PCR), previously described as "suicide-PCR," that incorporates 2 primer pairs used only once without positive control, followed by sequencing and comparing to the targeted sequence (13). DNA from patient and control BAL specimens, 3 water samples, and a suspension of *A. polyphaga* were extracted by using the QIAmp Tissue kit (QIAGEN GmbH, Hilden, Germany). DNA extracts from selected pathogens, including agents that are most commonly encountered in cases of hospital-acquired pneumonia (14), were tested: *Enterobacter aerogenes*, *Proteus mirabilis*, *Citrobacter freundii*, *Escherichia coli*, *Citrobacter koseri*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Serratia marcescens*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Acinetobacter baumanii*, *Stenotrophomonas maltophila*, *Pseudomonas aeruginosa*, *Bacteroides fragilis*, *Prevotella intermedia*, *Streptococcus pneumoniae*, *Streptococcus oralis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans*, CMV, HSV1, and adenovirus. Extracted DNA was used as template with primers BCFE (5′-TTATTGGTCCCAATGCTACTC-3′) and BCRE (5′-TAATTACCATACGCAATTCCTG-3′) as external primers and BCFI (5′-TGTCATTCCAAATGTTAACGAAAC-3′) and BCRI (5′-GCCATAGCATTTAGTCCGAAAG-3′) as internal primers. A minimum of 1 negative control was used for every 2 samples from patients with pneumonia; testing was conducted in a blinded manner.

Conclusions

In the study of 511 healthy Canadian controls, 12 (2.3%) exhibited a substantial titer of antibodies to Mimivirus. Patients with community-acquired pneumonia were positive more frequently than controls, as 36 (9.66%) were found positive (chi-square test, $p < 0.01$). Although the typical morphologic traits of Mimivirus make its confusion with ameba organelles unlikely (Figure), positive serum samples tested on noninfected intact amebas suspended in PBS by using the same migration inhibition factor protocol did not show any reactivity. Immunoelectron microscopic examination showed that antibodies of positive patients recognize mature Mimivirus particles specifically (Figure), whereas antibody fixation was not found in serum samples from 2 patients who were negative for Mimivirus (data not shown). We compared selected features of 14 patients with community-acquired pneumonia who had serologic evidence of infection with Mimivirus with those of 90 patients with community-acquired pneumonia who were seronegative for Mimivirus (Table). Only hospitalization from a nursing home (3/14 vs. 3/90) and rehospitalization after discharge (6/14 vs. 16/90) were significantly associated with Mimivirus antibodies ($p < 0.05$). Older age and diabetes mellitus were more common (both 6/14 versus 18/90) in patients with Mimivirus antibodies but not significantly so ($p = 0.07$). In patients with community-acquired pneumonia, more frequent rehospitalization after discharge in patients with serologic evidence of Mimivirus is likely explained by the poor efficacy of antimicrobial agents against viruses (15). Seropositive patients with community-acquired pneumonia were more likely to be admitted from a nursing home; this factor suggests that Mimivirus is a particularly good candidate as an etiologic agent of pneumonia acquired in institutions, as is *L. pneumophila* (16). The seroprevalence of Mimivirus

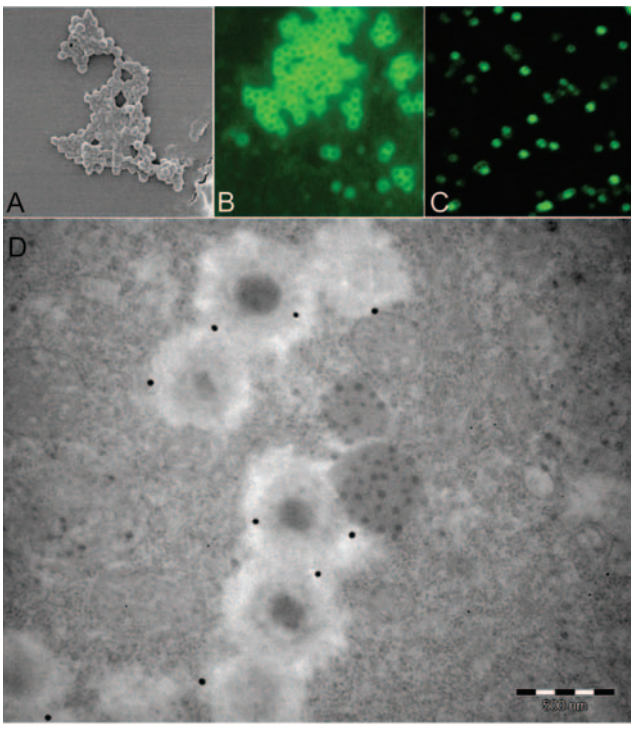

Figure. As observed by scanning electronic microscopy, Mimivirus antigen (A) is recognized by antibodies in our microimmunofluorescence assay using conventional fluorescence microscope (B) and confocal microscope (C). Mature particles within amebas are also recognized by antibodies seen with transmission electronic microscopy immunogold technique (D) (Mimivirus particle size 400 nm).

Table. Comparison of selected characteristics from patients with CAP who had serologic evidence of infection with Mimivirus with those of seronegative patients with CAP*

Characteristic	Positive (N = 14)	Negative (N = 90)	p
Age ≥80 y	6	18	0.07
Male	8	51	0.60
Length of hospital stay (d)	10.6	16.9	0.27
Days between onset and admission	6.9	5.2	0.77
Admission from nursing home	3	3	0.03
Retired	10	50	0.26
Smoked for >1 y	11	70	0.62
History of COPD	4	28	0.56
History of asthma	0	13	0.13
History of bronchiectasis	1	10	0.55
Diabetes mellitus	6	18	0.07
Hemodialysis	2	3	0.13
Rehospitalization after discharge	6	16	0.03
Death	2	2	0.08

*CAP, community-acquired pneumonia; COPD, chronic obstructive pulmonary disease.

was significantly higher than that of ameba–associated bacteria tested on the same samples (4).

Serologic evidence of infection was observed in 5 (19.2%) of 26 ICU patients. None of the 50 control patients was positive for Mimivirus ($p < 0.01$). Mimivirus DNA was detected in bronchoalveolar lavage specimen from a 60-year-old comatose patient who had 2 episodes of hospital-acquired pneumonia during hospitalization in ICU. Mimivirus DNA was amplified from the second episode sample only. The sequenced amplified fragment was 100% homologous to the target DNA (GenBank accession no. AY026860). No serum sample was available from this patient. None of the DNA extracts from control microorganism showed a positive PCR reaction. The finding of Mimivirus DNA in the bronchoalveolar lavage specimens from an ICU patient with nosocomial pneumonia confirms that Mimivirus may reach the respiratory tract of these patients. Because of the procedure used for suicide PCR amplification, contamination appears highly unlikely. In this patient, however, we cannot distinguish colonization and infection, but this feature is common to most microorganisms isolated from respiratory samples.

This high rate of seroconversion observed in patients with pneumonia from our seroepidemiologic study suggests that community-acquired pneumonia and hospital-acquired pneumonia patients may have contact with Mimivirus or a cross-reacting agent. As we do not report direct evidence of infection by Mimivirus, these results have to be interpreted with caution. Viruses usually have a broad range of hosts, but the extraordinary size of the Mimivirus genome (1.2 Mb), comparable to that of small bacteria such as *Mycoplasma* (17), suggests a possible adaptation to an extended range of hosts. We propose that Mimivirus be tested as a possible novel human pathogen among ameba-resisting microorganisms. These results are preliminary, but raise the question of the pathogenic potential of the biggest identified virus to date. Mimivirus is an agent easy to cultivate and is freely available from our laboratory on request to researchers working on pneumonia who wish to introduce Mimivirus antigen in serologic tests to confirm our results.

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Methicillin-resistant *Staphylococcus aureus* in Neonatal Intensive Care Unit

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A neonatal intensive care unit outbreak was caused by a strain of methicillin-resistant *Staphylococcus aureus* previously found in the community (ST45-MRSA-IV). Fifteen infected neonates were identified, 2 of whom died. This outbreak illustrates how a rare community pathogen can rapidly spread through nosocomial transmission.

Since 1998, strains of highly virulent, community-associated, methicillin-resistant *Staphylococcus aureus* (CA-MRSA), which are distinct from the typical nosocomial MRSA (NA-MRSA), have been reported (1,2). CA-MRSA is susceptible to numerous antimicrobial agents, in contrast to the multidrug-resistant (MDR) NA-MRSA phenotype, because it carries the staphylococcal cassette chromosome *mec* (SCC*mec*) type IV or V, rather than type I, II or III (3,4). The high virulence of CA-MRSA has been linked to Panton-Valentine leukocidin (PVL), a virulence factor found in most of these strains (2,5). We report a nosocomial MRSA outbreak in a neonatal intensive-care unit (NICU), by a non-MDR MRSA strain that carries the SCC*mec* type IV.

The Outbreak

Sheba Medical Center is a 1,500-bed, tertiary care hospital in which ≈10,000 infants are delivered annually. MDR MRSA is endemic in the hospital, constituting 50%–60% of all *S. aureus* isolates, while non-MDR MRSA has been observed in only 2 cases in the last 5 years. The premature neonatal department admits 500 premature neonates annually, nearly 100% of whom are born in the hospital. The department contains ≈45 beds: 12–14 level 3 NICU beds in a single large space, 15 intermediate-intensive beds in a separate room, and 18 additional beds in 2 additional rooms. The NICU is separated by a corridor from the rest of the department. The nurse/patient ratio in the NICU is 1:3–1:5.

During October 2003, the microbiology laboratory identified MRSA blood isolates from 2 neonates in the NICU that were atypically susceptible to erythromycin, clindamycin, gentamicin, rifampicin (rifampin), trimethoprim-sulfamethoxazole (TMP-SMX), and ofloxacin (therefore designated non-MDR MRSA). Because of the rarity of such a phenotype in this institution, a retrospective search for all *S. aureus* clinical isolates from the premature neonatal department since 1998 was undertaken by using computerized laboratory data.

A total of 14 neonatal infections with non-MDR MRSA were discovered during this period. A cluster of 12 cases was observed from October 2002 to December 2003 (designated the outbreak period), while 2 sporadic cases were isolated from January 1, 1998, to September 31, 2002, the preoutbreak period (Figure 1). The incidence of all *S. aureus* infections was 15.2 per 1,000 hospitalized neonates in the preoutbreak period, while during the outbreak period, it increased to 27.7 ($p = 0.032$). The incidence of non-MDR MRSA was 1.4 cases per 1,000 hospitalized neonates in the preoutbreak period; the rate increased to 18.5 cases per 1,000 hospitalized neonates ($p < 0.0001$) during the outbreak period (Table).

The first case of the cluster occurred 15 months after the second sporadic case. This case was in a 690-g female neonate of 25 weeks' gestational age, hospitalized in the NICU since birth. A non-MDR MRSA was isolated from eye discharge on her 22nd day of life, and she recovered with no antimicrobial drug treatment. The next 11 case-patients (Figure 2A) were also hospitalized in the NICU since birth; 9 were bacteremic and had signs of sepsis, 4 had sputum isolates (2 of these patients had pneumonia), 2 died, and 1 death was attributable to the infection. Thirteen of 14 neonates were treated with vancomycin for periods ranging from 6 to 48 days; 2 were treated with vancomycin and

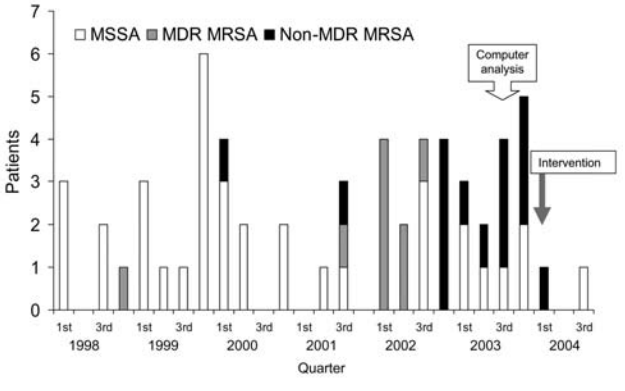

Figure 1. Incidence of *Staphylococcus aureus* infections in premature neonatal ward, 1998–2004. MSSA, methicillin-susceptible *Staphylococcus aureus*; MDR, multidrug resistant; MRSA, methicillin resistant *S. aureus*.

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Table. Incidence rates of non–multidrug-resistant (MDR) methicillin-resistant *Staphylococcus aureus* (MRSA) and *S. aureus* infections: preoutbreak versus outbreak periods*

Infection	No. infected patients per 1,000 hospitalized patients	p value
Non-MDR MRSA, preoutbreak period	1.4	<0.0001
Non-MDR MRSA, outbreak period	18.5	
All *S. aureus*, preoutbreak period	15.2	0.031
All *S. aureus*, outbreak period	27.7	
Other *S. aureus* (methicillin-susceptible and MDR MRSA), preoutbreak period	13.8	0.38
Other *S. aureus* (methicillin-susceptible and MDR MRSA), outbreak period	9.2	

*Preoutbreak period, January 2000–September 2002; outbreak period, October 2002–December 2003.

rifampicin. None of the patients had perinatal infections, and the median age when infection was diagnosed was 30 days (range 11–115 days). Eleven patients had extremely low birth weight (range 568–2,440 g, median 810 g), and 12 had low gestational age (range 23–35 weeks, median 25 weeks). All patients had indwelling devices (mechanical ventilation for 11 to 74 days, central lines and total parenteral nutrition for 7–38 days). Four neonates had previous major surgery, and all neonates had at least 1 of several complications of prematurity (respiratory distress syndrome, bronchopulmonary dysplasia, intraventricular hemorrhage, retinopathy of prematurity).

An infection control intervention was initiated on December 5, 2003. Screening for *S. aureus* carriage was performed by nasal and umbilical sampling of all hospitalized neonates (N = 30; all were sampled on day 1) and nasal sampling of all the department healthcare workers (N = 114 [47 nurses, 30 physicians, 37 other healthcare workers]; 85% were sampled within 5 days). Swabs were streaked onto a differential media (CHROMagar plates, HyLabs, Rehovot, Israel) and incubated for 24 to 48 hours at 35°C. Suspicious colonies were then conventionally identified. Oxacillin resistance was determined according to National Committee for Clinical Laboratory Standards recommendations (6) and was verified by polymerase chain reaction (PCR) for the presence of *mecA* (1). Eight patient-unique, non-MDR *S. aureus* blood isolates from the previous year were also available for analyses.

In this point-prevalence surveillance, a non-MDR MRSA was isolated from 3 neonates with previous clinical isolates and from 4 newly recognized carriers. Thus, 7 (23.3%) of 30 hospitalized neonates were carriers. In addition, 2 (1.7%) healthcare workers, both nurses (4.3% of nurses), carried this strain; MDR MRSA was not isolated. Methicillin-susceptible *S. aureus* was carried by 28.1% of healthcare workers and was not isolated from any of the neonates.

Pulsed-field gel electrophoresis (PFGE) was performed, as previously described (7), on 13 unique isolates from 11 neonates and 2 nurses. A single identical outbreak strain was identified by the PFGE pattern (Figure 2B). All non-MDR MRSA were found to be SCC*mec* type IV by a modification of the method described by Oliveira (8). By using multilocus sequence typing (MLST) (9), the outbreak strain was classified as ST45-MRSA-IV. PCR screening of the outbreak strain for 25 virulence factor genes was performed as described by Jarraud et al. (10). MLST and PCR of virulence factors were performed on 8 outbreak isolates. Virulence factors commonly found in CA-MRSA, *lukS*-PV-*lukF*-PV encoding PVL components S and F, γ-hemolysin variant (*hlg-v*), and *agr* type 3, were

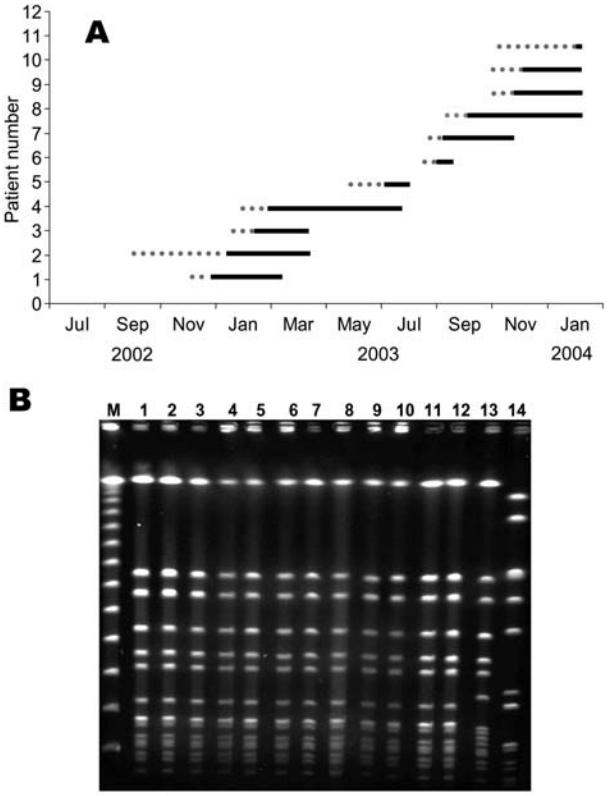

Figure 2. A) Nosocomial transmission of non-multidrug-resistant (MDR) methicillin-resistant *Staphylococcus aureus* (MRSA) in the premature neonatal ward. Dotted lines represent hospitalization until first non-MDR MRSA isolation. Solid lines represent hospitalization after first non-MDR MRSA isolation until discharge. B) pulsed-field gel electrophoresis of MRSA outbreak isolates; lanes 1–10: ST45-MRSA-IV outbreak strains isolated from neonates; lanes 11–12: ST45-MRSA-IV outbreak strains isolated from nurses; lane 13: ST45 methicillin-susceptible *S. aureus* strain from a community survey; lane 14: common nosocomial MRSA strain ST5-MRSA-II.

not detected in the outbreak strain. The outbreak strain was positive for enterotoxin gene cluster (*egc*), γ-hemolysin (*hlg*), and *agr* type 1 and had similar PFGE and MLST results as the major methicillin-susceptible *S. aureus* (MSSA) strain found in the community served by this hospital. The outbreak strain was identical to the CA-MRSA that is rarely isolated in healthy carriers in Israel (11). Moreover, this strain differed from the MDR MRSA strains that are commonly isolated in this medical center (Figure 2B).

After initial surveillance, all case-patients were isolated and cohorted, appropriate hand hygiene practices were reinforced for healthcare workers, and all neonates were bathed with diluted (1:10) chlorhexidine gluconate 4% once daily for 3 consecutive days. Nasal mupirocin was implemented 3 times per day for 5 consecutive days for all carriers. These regimens were well tolerated with no adverse events.

Three weeks after the first surveillance and intervention, a second surveillance was conducted in the NICU and intermediate neonatal care unit. No new cases were identified. Of the 7 carriers, only 4 were still hospitalized. Of these, 2 continued to carry the outbreak strain, and 2 were successfully decolonized. The 2 carriers were decolonized after a second course of a similar regimen (chlorhexidine bath followed by mupirocin administration).

The 2 colonized nurses were sampled twice, 1 week apart, and were found to be persistent carriers of the outbreak strain. They were instructed about good hand hygiene practices, and nasal mupirocin was recommended for 5 days. One nurse cleared her nasal non-MDR MRSA after mupirocin treatment and acquired a new strain of MSSA 2 weeks later. The other refused mupirocin treatment and persistently carried the outbreak strain for 8 weeks. During this period, despite our instructions, she continued to work until she went on maternity leave. Before she returned to work 12 weeks later, she was decolonized by using nasal mupirocin. In a 7-month follow-up, no new cases of non-MDR MRSA were identified (Figure 1).

Conclusions

This report documents a nosocomial outbreak of a non-MDR, PVL-negative MRSA strain, ST45-IV, in a NICU. This strain is clearly distinct from the NA-MRSA strains in this medical center, but it is identical to a CA-MRSA strain previously isolated in the community (in 0.5% of *S. aureus* carriers) and is nearly identical to the major MSSA strain circulating in this community (11). We hypothesize that the outbreak strain evolved in the community and penetrated into the NICU. Two previous reports of possible CA-MRSA in NICUs have been reported; however, neither characterized the alleged pathogen genetically (12,13).

The outbreak strain reported here is similar to CA-MRSA strains described in Europe, the United States, and Australia in that it is susceptible to many antimicrobial drugs and carries the SCC*mec* type IV (2,5). However, this strain is distinct from those strains because it lacks the PVL components S and F, as well as other virulence factors. Indeed, skin and soft-tissue infections typically described as caused by CA-MRSA (1,2) were not observed in any of the neonates in this outbreak.

MRSA outbreaks in NICUs have been reported to be difficult to contain (12,14). Only implementation of aggressive infection control measures, frequently combined with mupirocin treatment, has been successful in controlling such outbreaks. The outbreak described here was similarly contained by implementing a multifaceted infection control intervention. Since all the measures were undertaken simultaneously, we cannot define which of the measures was the most important.

We assume that the primary source of this outbreak was either a parent of the index patient or a carrier nurse. Because of a low ratio of nurses to neonates in the NICU and the high contact rate each nurse had with neonates in that facility, we could not trace specific contact patterns or perform a case-control study to further investigate this outbreak.

The difficulties encountered in implementing proper infection control measures are demonstrated by the fact that 1 healthcare worker continued to be engaged in patient care for 8 weeks, despite continued colonization by the outbreak strain, against our advice to her supervisors. Fortunately, this action did not result in persistence of the outbreak.

This outbreak illustrates the penetration of a community pathogen into the hospital, where nosocomial transmission, particularly in an intensive care setting, may rapidly spread the pathogen. The susceptibility of newborns, coupled with insufficient infection control measures and inadequate nurse-to-patient ratio, contributed to this outbreak. We call for specific attention to the possibility of "reverse penetration" of community MRSA strains becoming nosocomial pathogens.

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Inquilinus limosus in Patients with Cystic Fibrosis, Germany

Nele Wellinghausen,* Andreas Essig,* and Olaf Sommerburg*

We identified *Inquilinus limosus*, a recently described α-proteobacterium, in sputum of 2 patients with cystic fibrosis whose respiratory tracts were persistently colonized for >9 months. We present data on the epidemiology, antimicrobial susceptibility, and molecular characteristics of *I. limosus*.

Chronic microbial colonization of the respiratory tract, leading to exacerbations of pulmonary infection, is the major cause of disease and death in patients with cystic fibrosis (CF). Typical pathogens in respiratory secretions of patients with CF include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Burkholderia cepacia* complex (1–3). Other gram-negative glucose nonfermenters, such as *Achromobacter xylosoxidans*, *B. gladioli*, *Ralstonia pickettii*, and *Stenotrophomonas maltophilia* are also occasionally recovered from respiratory samples from CF patients, but their pathogenic importance remains to be clarified (2–4).

Determining the clinical relevance of nonfermentative microbes is hampered by the difficulty in identifying these pathogens by conventional laboratory techniques. Recent studies that applied molecular approaches to identify unusual pathogens in patients with CF showed various infrequently encountered and novel species (4–7). In 1 of these studies, the gram-negative nonfermentative species *Inquilinus limosus* was newly described in respiratory secretions of 8 patients with CF in the United States (8). *I. limosus* belongs to the α-proteobacteria and is, thus, not closely related to *B. cepacia* complex or *P. aeruginosa* (8).

To detect unusual gram-negative microbes in respiratory samples, we screened all patients attending the CF clinic of the University Hospital, Ulm, Germany, from May 2002 to September 2004 (N = 85 patients). Respiratory samples (N = 459 samples) were plated with a 10-μL loop on sheep blood agar (Heipha, Heidelberg, Germany), MacConkey agar (Heipha), and *B. cepacia* complex selective agar (containing 100 mg/L ticarcillin and 300,000 IU/L polymyxin B, MAST Diagnostica, Reinfeld, Germany). All plates were incubated for 48 h at 36°C under ambient air, and the *B. cepacia* complex selective agar was incubated another 5 days at room temperature. *I. limosus* was recovered from sputum samples of 2 patients (0.9%).

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Case Reports

Patient 1

Strain A was isolated from a 17-year-old male patient with CF with persistent colonization of the respiratory tract since childhood with *S. aureus*, *P. aeruginosa*, including the mucoid variant, and *H. influenzae*. In the initial sputum sample, apart from ≈10^6 CFU/mL of *S. aureus*, 10^5 CFU/mL of mucoid *P. aeruginosa*, and 10^3 CFU/mL of *Candida albicans*, 10^4 CFU/mL of a mucoid gram-negative rod was isolated from *B. cepacia* complex selective agar after 6 days of incubation. The isolate had positive oxidase and catalase reaction, but failed to grow on MacConkey agar. It was identified by using Api 20NE as *Sphingomonas paucimobilis* with questionable profile (Code 0427544). Final identification of *I. limosus* was achieved by sequencing the 16S rRNA gene with the primers 16Sfor and 16Srev (9) and a Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, United Kingdom) on a 310 Genetic Analyser (Abi Prism). The isolate showed 99.8% sequence homology to the 16S rDNA sequence of the *I. limosus* type strain (LMG 20952^T) by using the BLAST algorithm.

During sequence analysis, we discovered a mistake in the *I. limosus* type strain 16S rDNA sequence deposited in GenBank (accession no. AY043374): the CGGGTC motif, repeated twice from base 956 to 967 in AY043374, is only found once in the type strain's 16S rRNA gene, such as in the *Inquilinus* sp. strain AU1979 (accession no. AY043375 [8]) and in our isolates. We performed susceptibility testing by using Etest (VIVA Diagnostics, Solna, Sweden) on Mueller-Hinton agar, incubated at 36°C for 48 h. In addition, susceptibility testing against colistin was performed by disk diffusion with 10-μg disks (BD, Heidelberg, Germany) on Mueller-Hinton agar (McFarland 0.5, 48 h incubation). All results are shown in the Table. At the time of sputum sampling, the patient was clinically well, with normal values of C-reactive protein, leukocytes, and erythrocyte sedimentation rate. He regularly played soccer. A lung function test was not done. Two weeks after his visit, an elective 14-day course of antimicrobial therapy was initiated consisting of intravenous (IV) ceftazidime (3 g 3 times daily) and IV tobramycin (500 mg once daily) because of *P. aeruginosa* colonization.

Five and a half months later, the patient was seen in the CF clinic again. He was still in very good clinical condition. Results of lung function tests conducted 6 weeks after his first visit and at his second visit were the following: vital capacity, 3.27l/3.34l (77%/74% of predicted vital capacity); and forced expiratory volume, 1 s 2.81l/2.89l

Table. Antimicrobial susceptibility of *Inquilinus limosus* strains

Antimicrobial agent	MIC (μg/mL)				
	Isolate A-1	Isolate A-2	Isolate A-3	Isolate B	LMG 20952^T
Trimethoprim/sulfamethoxazole	>32	>32	>32	>32	>32
Amikacin	8	32	32	>256	>256
Gentamicin	8	16	12	>256	12
Tobramycin	>256	>256	>256	>256	>256
Ampicillin	>256	>256	>256	>256	>256
Piperacillin/tazobactam	>256	>256	>256	>256	>256
Cefotaxime	>32	>32	>32	>32	>32
Ceftazidime	4	4	4	0.5	32
Imipenem	0.012	0.012	0.016	0.006	0.016
Ciprofloxacin	0.032	0.032	0.032	0.500	0.064
Colistin*	Resistant	Resistant	Resistant	Resistant	Resistant

*Susceptibility testing done by disk diffusion (see text); no zone of inhibition was seen.

(79%/77% of predicted forced expiratory volume). Sputum culture showed ≈10^6 CFU/mL of *S. aureus*, 10^4 CFU/mL of mucoid *P. aeruginosa*, 10^3 CFU/mL of *Aspergillus fumigatus*, and again 10^3 CFU/mL of *I. limosus*. We performed pulsed-field gel electrophoresis (PFGE) of the isolates with the CHEF DRIII equipment (BioRad, Munich,Germany) in 1% agarose at 14°C and a constant voltage of 200 V (10), with the restriction enzyme *XbaI* (11). Results showed that the strain (A-2) was identical to the former isolate of the same patient (A-1) (Figure). A 14-day course of oral ciprofloxacin (750 mg twice daily) was initiated because of *P. aeruginosa* colonization.

Four months later, the strain was still detected in his sputum. The isolate (A-3) grew in low quantities (10^3 CFU/mL) and was accompanied by 10^6 CFU/mL of *S. aureus*, 10^6 CFU/mL of mucoid *P. aeruginosa*, 10^3 CFU/mL of *A. fumigatus*, and 10^4 CFU/mL of *C. albicans*. PFGE showed identity with the former strains (Figure), and antimicrobial susceptibility was unchanged (Table). Lung function test showed a vital capacity of 3.81l (68% of predicted vital capacity) and a forced expiratory volume of 3.25l (71% of predicted forced expiratory volume), and the patient was in good health. More than 2 months later, the *I. limosus* strain was no longer cultured from his sputum, while *P. aeruginosa* (10^3 CFU/mL), *S. aureus* (10^6 CFU/mL), and *C. albicans* (10^3 CFU/mL) were still found.

Patient 2

Strain B was isolated from a sputum sample of a 14-year-old female patient with CF with respiratory colonization since childhood with *P. aeruginosa*, including the mucoid variant, and *H. influenzae*. The mucoid isolate of *I. limosus* grew in large quantities (≈10^5 CFU/mL) on *B. cepacia* complex selective agar after 5 days of incubation. In addition, ≈10^5 CFU/mL mucoid of *P. aeruginosa*, 10^3 CFU/mL of *A. fumigatus* and *C. albicans* were found in the sputum sample. Colonies were oxidase- and catalase-positive and failed to grow on MacConkey agar. Api 20NE showed *Sphingomonas paucimobilis* with questionable profile (Code 0424744), and identification was achieved by 16S rDNA sequencing, as described above. The isolate showed 99.3% sequence homology to the *I. limosus* type strain (LMG 20952^T). PFGE showed a different band pattern, which suggests that this strain was different from strain A and, thus, excluded cross-infection between both patients (Figure).

The antimicrobial susceptibility of strain B was comparable to that of strain A, apart from higher MIC values for amikacin and gentamicin and lower values for ceftazidime (Table). Like the first patient, this patient was also in good health and active. At the time of sputum sampling, pulmonary function and laboratory tests were not done, but a pulmonary function test conducted 2 weeks before showed a vital capacity of 2.96l (74% of predicted vital capacity) and a forced expiratory volume 1 of 1.95l (77% of predicted forced expiratory volume 1), leukocyte count and erythrocyte sedimentation rate were normal, and C-reactive protein was slightly elevated to 5.8 mg/L. During the following 2 months, 6 sputum samples were investigated, but

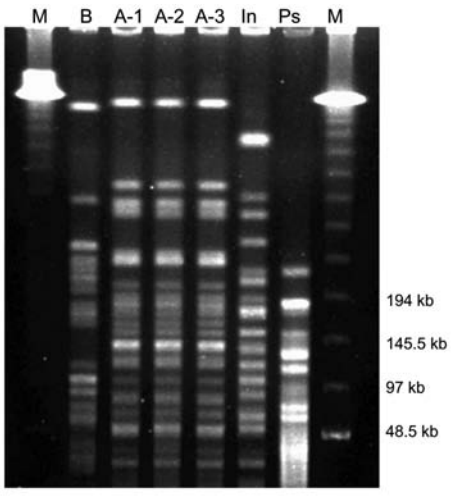

Figure. Pulsed-field gel electrophoresis of *Inquilinus limosus*. M, molecular weight marker 48.5 to 970 kbp (BioRad 170-3635); In, *Inquilinus limosus* type strain LMG 20952; Ps, *Pseudomonas aeruginosa* ATCC 27853.

Inquilinus was not detected again, while *P. aeruginosa* was still present in a concentration of 10^5 CFU/mL. The patient moved to another CF clinic and was lost to follow-up.

Conclusions

To our knowledge, isolation of *I. limosus* from clinical samples has only been described in 1 study (8). The prevalence of this species in our CF clinic between May 2002 and September 2004 was 2.4%. The natural reservoir of *I. limosus* has yet to be discovered, but its relatedness to other nonfermentative rods suggests environmental sources. *Inquilinus* might be overlooked in clinical samples because of its rather slow growth and failure to grow on MacConkey agar. Recovery of *Inquilinus* can be improved by using selective media containing polymyxin B or colistin and ticarcillin, such as *B. cepacia* complex selective agar, and prolonged incubation at 36°C. The necessary duration of incubation has yet to be determined since our isolates grew better at 36°C than at room temperature. Identifying the species is difficult since it is not contained in the databases of commercial identification kits and its mucoid appearance may lead to confusion with mucoid *P. aeruginosa* strains. This species' failure to grow on MacConkey agar, positive oxidase reaction, and typical antimicrobial susceptibility profile (see below) in respiratory samples of CF patients should arouse suspicion. Identification of *I. limosus* can be confirmed by 16S rRNA gene sequencing (8). *I. limosus* is able to persist in the respiratory tract of CF patients for several months. As with *P. aeruginosa*, abundant amounts of mucus with *I. limosus* infection may favor persistence and chronic infection. However, the pathogenic role of *I. limosus* in the patients described here is unclear. The stepwise deterioration of pulmonary function seen in patient 1 may also be attributed to irregular intervals of inhalation and of elective antimicrobial therapy. The patient had finished his education and had started his first employment.

I. limosus shows a distinct antimicrobial susceptibility profile with high MICs for cotrimoxazole, most aminoglycosides, ampicillin, cefotaxime, and piperacillin/tazobactam. Although ceftazidime and ciprofloxacin would be interpreted as susceptible applying the NCCLS interpretation criteria for *P. aeruginosa* (12), strain A persisted in the respiratory tract of the patient for several months after therapy with these substances. *Inquilinus* may be effectively protected from the action of antimicrobial agents by mucus production, and local host factors may also contribute to colonization and persistency. Further studies are necessary to evaluate the epidemiology and clinical importance of *I. limosus* as well as the therapeutic options in CF patients and in other patient groups. For instance, screening large CF patient groups by selective culture methods or molecular methods, like the use of specific fluorescence in situ hybridization probes or polymerase chain reaction assays, are desirable for assessing the epidemiology of the species. Longitudinal studies of infected patients are valuable in evaluating the clinical relevance and the factors influencing persistency of *Inquilinus*.

Acknowledgments

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Dr. Wellinghausen is a staff microbiologist and senior researcher at the University Hospital of Ulm, Germany. Her research interests focus on molecular techniques in diagnostic microbiology.

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Fever Screening at Airports and Imported Dengue

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Airport fever screening in Taiwan, July 2003–June 2004, identified 40 confirmed dengue cases. Results obtained by capture immunoglobulin (Ig) M and IgG enzyme-linked immunoassay, real time 1-step polymerase chain reaction, and virus isolation showed that 33 (82.5%) of 40 patients were viremic. Airport fever screening can thus quickly identify imported dengue cases.

Dengue viruses are arboviruses that cause substantial human disease in tropical and subtropical regions of the world, especially in urban and semiurban areas. Because of its high endemicity in many countries in the Western Pacific, Southeast Asia, and South American regions, dengue has become an important public health problem in most nations in these areas (1). Dengue is not considered endemic in Taiwan, however, and the constant importation of dengue viruses from the neighboring Southeast Asian countries through close commercial links and air travel is believed to cause local outbreaks (2,3). Until now, local outbreaks, which are most frequent in the summer and fall, have each been caused by a single imported dengue virus strain that disappears when each outbreak ends. Because waves of relatively cold temperature of ≈10°C cause low mosquito density in winter, winter outbreaks are rare, with the exceptions of large outbreaks in 1915–1916, 1942–1943, 1987–1988, and 2001–2002. Outbreaks occur mainly in southern Taiwan, where *Aedes aegypti* and *A. albopictus* coexist, and rarely occur in central and northern Taiwan, where only *A. albopictus* exist. The dengue hemorrhagic fever cases in Taiwan are highly correlated with increasing age and secondary dengue virus infection (4; J-H Huang, unpub data).

The Study

To identify imported dengue cases and reduce the local spread of newly introduced dengue viruses, the health authority, now Center for Disease Control, Taiwan, has established an integrated dengue control program that includes various surveillance systems, a network of rapid diagnostic laboratories, and mechanisms of rapid response to implement control measures (3). The primary objective is to prevent the introduction of new dengue viruses into Taiwan by travel and subsequent local spread. Dengue is classified as a reportable infectious disease, and suspected cases must be reported within 24 hours of clinical diagnosis in Taiwan. For effective surveillance, both passive (hospital-based reporting system) and active (such as health statement of inbound passengers, self-report, expanded screening for contacts of confirmed cases, patients with fever of unknown origin, school-based reporting, community screening) surveillance systems were established in central and local health departments.

For rapid diagnosis, 2 central dengue diagnostic laboratories were set up in Center for Disease Control–Taiwan, at Kun-Yan Laboratory in northern Taiwan and at a fourth branch laboratory in southern Taiwan. Serum samples from suspected dengue patients were sent to the diagnostic laboratories, and the results were reported within 24 to 48 hours. To avoid delays, the laboratory was scheduled to perform the tests on a daily basis without vacations. A rapid diagnostic system was developed to detect and differentiate various flavivirus infections on the basis of the results of 1-step real-time polymerase chain reaction (PCR) and envelope membrane (E/M)–specific capture immunoglobulin (Ig) M and IgG enzyme-linked immunosorbent assay (ELISA) (5–7). Analysis of a total of 959 acute- and convalescent-phase serum specimens from 799 confirmed dengue patients showed that 95% of acute-phase serum specimens could be identified as being from confirmed or probable case-patients based on these 2 assays (8).

In 2003, Taiwan was one of the countries heavily affected by the multinational epidemics of severe acute respiratory syndrome (SARS) (9). During the SARS epidemic, the body temperature of all inbound and outbound passengers at the 2 international airports was screened to prevent international spread of SARS. Since July 14, 2003, all inbound passengers have been required to complete the "SARS Survey Form" before landing and to have their body temperature taken by an infrared thermal camera. Any passenger showing body temperature >37°C is rechecked by ear temperature, and serum samples are collected and sent for SARS diagnosis if the ear temperature is >37.5°C. After July 5, 2003, the world was largely considered to be SARS free, and other causes of fever had to be considered; therefore, a panel of diagnostic tests, including tests for pathogens of dengue, malaria, enteric bacteria, and other diseases (such as yellow fever, plague), was performed for selected fever patients. Since dengue fever is among the top yearly imported reportable diseases in Taiwan, we began a trial fever screening program for dengue along with SARS screening at the airports.

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We report our findings on early identification of dengue fever through fever screening at the 2 international airports, C.K.S. and Kaohsiung Airports, Taiwan. More than 8,000,000 inbound travelers passed through the 2 airports from July 2003 to June 2004. Among these, ≈22,000 passengers were identified as fever patients by an infrared thermal camera and rechecked by ear temperature. Diagnostic testing algorithms for screened fever patients were based on evaluation by airport clinicians. After clinical diagnosis, 3,011 serum samples were sent for laboratory diagnosis of dengue virus infection. Forty (1.33%) of 3,011 serum samples were confirmed to be positive on the basis of the results of real-time PCR and E/M-specific capture IgM and IgG ELISA. During the same period, 6,005 dengue cases were reported in Taiwan (both indigenous and imported cases), which includes 935 cases from the passive surveillance system and 5,070 cases from active surveillance systems (3,011 fever patients were identified by fever screening and 2,059 cases were identified from other systems). Among these, 73 were confirmed to be imported dengue cases, including 25 cases reported from hospitals through passive surveillance and 48 cases identified by active surveillance, such as airport screening and self-report by patients. Airport fever screening alone identified 40 (83.3%) of 48 of all imported cases identified by the active surveillance system. Thus, 8 imported cases were identified by other active surveillance methods. The average length between the onsets of dengue symptoms to the time of diagnosis was 4.15 days for the 40 case-patients who were identified at the airport, as opposed to 11 days for those who were reported from the hospital. Whether the shorter length required to diagnose conditions identified by airport fever screening contributed to the low indigenous dengue in the season warrants further investigation.

Fever screening at the airports has also dramatically increased the proportion of imported dengue cases identified by active surveillance, 48 (65.8%), of 73 which is significantly higher than the number identified during years before fever screening were implemented ($p < 0.0001$ by chi-square test) (Table 1). The countries of origin of imported dengue fever from July 2003 to June 2004 were all located in the Western Pacific and Southeast Asia (Table 2). The distribution of the countries of origin accurately reflected the frequency of air travel between Taiwan and these nations, as well as the intensity of massive dengue outbreaks during the same period in the country of origin. Analyses of dengue virus serotypes showed that various serotypes were circulating in each of these countries during this period.

Most of the confirmed cases (33 of 40) identified by airport fever screening were viremic (real-time PCR positive, IgM and IgG negative). The other 7 case-patients tested positive for dengue-specific IgM or IgG antibody, although they were febrile at the time of testing (data not shown). Estimating how many patients might have been viremic but were not picked up by the system is difficult, since persons infected with dengue virus are usually viremic from 2 to 3 days before onset of symptoms until defervescence.

Conclusions

Our results demonstrated that fever screening at airports is an effective means of identifying imported dengue cases, whereas the health statements of inbound passengers, which have been required for years, are ineffective. Although fever screening with infrared temperature screening was implemented in an attempt to avoid SARS transmission, it proved to be effective in active surveillance of dengue. This approach seems promising for dengue and perhaps for other diseases and should be further evaluated.

The cost of identifying dengue virus infections with airport fever screening is similar to that of other surveillance methods. The airport fever screening method requires an infrared thermal camera, which costs approximately U.S. $43,000 for each set of instruments. In addition, 1 additional worker is needed to monitor this alarm system. The reporting procedure and clinical and laboratory diagnoses are similar to those of surveillance methods. Therefore, the method is a cost-effective means of identifying imported dengue cases.

Although febrile passengers suspected of having dengue virus infection were not detained at the airport, and an epidemiologic investigation was not conducted, they were provided with a mosquito net to avoid mosquito bites and instructed to report to the local health department if they felt ill. Laboratory diagnoses were performed on a

Table 1. Summary of imported dengue cases identified by passive and active surveillance systems in Taiwan from 1998 to June 2004

Year	Total	Passive surveillance, no. cases (%)	Active surveillance, no. cases (%)
1998	110	96 (87.3)	14 (12.7)
1999	29	24 (82.8)	5 (17.2)
2000	27	23 (85.2)	4 (14.8)
2001	56	46 (82.1)	10 (17.9)
2002	52	42 (80.8)	10 (19.2)
Jan 2003–June 2003	20	18 (90.0)	2 (10.0)
Jul 2003–June 2004	73	25 (34.2)	48 (65.8)

Table 2. Countries of origin and dengue virus serotype of imported dengue cases in Taiwan, July 2003–June 2004

Country of origin	No. imported cases		Serotype*				
	Total	Fever screening	D-1	D-2	D-3	D-4	Unknown
Vietnam	21	13	0	11	3	3	4
Indonesia	15	8	3	6	2	1	3
The Philippines	15	7	5	2	0	5	3
Thailand	11	7	3	3	1	3	1
India	3	1	0	2	0	0	1
Malaysia	2	2	0	2	0	0	0
Myanmar	1	0	0	0	0	0	1
Cambodia	4	2	1	2	0	0	1
Sri Lanka	1	0	0	0	0	0	1
Total	73	40	12	28	6	12	15

*Dengue virus serotypes were identified by real time 1-step polymerase chain reaction, virus isolation, or both, for all imported cases.

daily basis, and results were reported within 24 to 48 hours. Control measures were implemented as soon as possible if probable or confirmed dengue cases were identified. Since viremic persons, going about their normal activities for a mean interval of 2 to 3 days before diagnosis, could have transmitted dengue, the laboratory detection method on its own will not be effective in preventing transmission. Therefore, developing an integrated program that includes various surveillance systems, rapid diagnostic laboratories, and emergency control measures is necessary to prevent the introduction and spread of new dengue viruses into a region. Control measures should consist of epidemiologic investigation, health education, analysis of mosquito density, source reduction, and insecticide application. As part of an integrated dengue control program, fever screening at the airport has become one of the most important active surveillance systems in Taiwan since its introduction in July 2003. We believed that this active surveillance system could also be successfully applied to screen febrile patients and reduce the introduction of many potential infectious diseases.

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Rumor Surveillance and Avian Influenza H5N1

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We describe the enhanced rumor surveillance during the avian influenza H5N1 outbreak in 2004. The World Health Organization's Western Pacific Regional Office identified 40 rumors; 9 were verified to be true. Rumor surveillance informed immediate public health action and prevented unnecessary and costly responses.

In January 2004, 14 persons in Vietnam were admitted to provincial hospitals with severe respiratory illness (1). Avian influenza H5N1 was detected in samples from 3 of these patients. Health officials and the World Health Organization (WHO) were concerned, as these were sporadic cases of an influenza strain that normally infects birds exclusively (2). Furthermore, little was known about the extent of the outbreak, its potential for international spread, and the possible evolution of a pandemic influenza strain. WHO issued an international public health alert on January 13, 2004, to inform the world about the outbreak (1).

News of the outbreak led to international anxiety and the propagation of unofficial outbreak reports or disease rumors (3). These rumors could have led countries to impose trade and travel restrictions with negative social, economic, and health consequences (3,4). To protect both the international community and the affected countries, WHO introduced enhanced rumor surveillance for reports of avian influenza H5N1, a process of investigating unofficial reports of disease events to determine their veracity. Rumor surveillance aims to decrease the potential for misinformation and misunderstanding and to inform the public and health officials about disease outbreaks, facilitate a rapid response, and promote public health preparedness (3).

Rumor surveillance is a passive process, where rumors are identified from media reports, professional groups, the public, and persons in the WHO network, which is made up of WHO headquarters, country offices, and WHO Collaborating Centers. In an enhanced system, rumor surveillance is intensified by actively seeking out rumors and undertaking more rigorous follow up. This surveillance includes analyzing more media sources and regularly requesting information from the WHO network about outbreak events. Previous studies have examined the role of enhanced rumor surveillance during public health emergencies, such as the Chernobyl nuclear accident in 1986 and the outbreak of Ebola in Uganda in 2000 (5,6). However, research has not examined the role of rumor surveillance in multicountry or regional outbreaks.

The importance of rumor surveillance is likely to increase as the international community considers the revised draft of the International Health Regulations (IHR). Article 8 of the IHR Working Paper (7) states, "WHO, in consultation with the health administration of the State concerned, shall verify rumors of public health risks which may involve or result in international spread of disease."

During the avian influenza outbreak, WHO's Western Pacific Regional Office (WPRO) was the focal point for identifying rumors and coordinating their investigations in the region (8). WPRO covers 37 nations and stretches from China in the north and west, to New Zealand in the south, and to French Polynesia in the east (9). This study examines whether the enhanced rumor surveillance undertaken by WPRO during the first 40 days of the outbreak achieved its aims of: 1) offering timely assistance to potentially affected nations, 2) prompting countries to undertake preparedness measures appropriate to their level of risk of being affected, and 3) informing the public and the international community about relevant events.

The Study

WPRO designated a rumor surveillance officer to develop and implement the rumor surveillance system for avian influenza in animals and humans. This officer actively assessed media sources and email-based public health discussion and regularly contacted the WHO network to identify rumors. Media sources included journalists visiting WPRO and Web sites for television networks and newspapers. Most were English-based media sources; however, some were also in Japanese and Arabic. To increase the scope of the active media search, this officer also accessed the Global Public Health Intelligence Network (10), an electronic surveillance system that continuously monitors >600 media sources and biomedical journals in a number of languages, including Chinese, Spanish, English, and French.

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Each rumor was followed up by an email or a telephone request to the relevant WHO country office to investigate its veracity. The WHO country office in turn sought verification from the country's health authorities. Overall, the onus of the verification process was in the hands of the affected countries' health authorities. The authorities had to demonstrate to WHO that appropriate investigations were conducted to deem rumors correct or incorrect. To ensure this process, WHO sometimes supported rumor verification by assisting in laboratory testing or shipment of isolates.

Once available, the outcome of the investigation was disseminated to WHO stakeholders, including the outbreak response team. For events reported in the media, WPRO's media officers made information publicly available through press releases and media interviews, as well as providing up-to-date information on the WHO Web site (http://www.who.int).

From January 20 to February 26, 2004, a total of 40 rumors were identified, most within 4 weeks of the outbreak alert (Figure). The rumors concerned 12 countries and 1 special administrative region. Of the total rumors received, 19 (48%) were received from the media, 18 (45%) from the WHO network, 2 (5%) from embassy staff living in affected countries, and 1 (2%) from ProMED Digest with a media source as the origin. Nine (23%) rumors were confirmed to be true events: 5 in China and 1 each in Cambodia, Japan, Laos, and South Korea. Of the incorrect rumors, 6 were in China, 6 in Laos, 4 in Vietnam, 4 in Hong Kong, 3 in Cambodia, 2 in Germany, and 1 each in Bangladesh, Indonesia, Japan, Malaysia, Saudi Arabia, and Singapore.

The average period for verification of true events was 2.7 days (range 1–5 days). The average period to verify that a rumor was incorrect was 9.3 days (range 1–26 days). Sixty percent of the rumors related to human outbreaks, of which 1 was true, and 40% to animal outbreaks, of which 8 were true. The Table provides examples of rumors received during the 40-day study, the outcomes of the investigation, and the public health action taken. (An expanded version of this table is available online from http://www.cdc.gov/ncidod/eid/04-0657.htm#table.) The remaining 32 rumors are not shown for reasons of brevity and privacy; however, not all rumors resulted in public health action after the verification process. This finding was expected because the high sensitivity of the system decreased the predictive value positive.

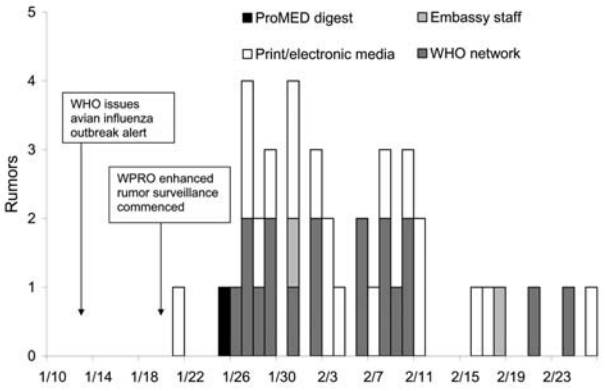

Figure. Number of rumors received from January 20 to February 26 by source of rumor, Western Pacific Regional Office (WPRO) of the World Health Organization (WHO), 2004.

Conclusions

WPRO's enhanced rumor surveillance system identified many rumors. Most were identified in the first few weeks after the public health alert. A similar pattern was also observed during the 2003 SARS outbreak, when most rumors were received within the first 7 weeks of the public health alert (11). The decreased rate of rumor detection later in the outbreak is consistent with Allport and Postman's basic law of rumor (12). According to this law, the amount of rumors in circulation is roughly equal to the importance of the rumor multiplied by the uncertainty surrounding the rumor. We found that, as more information became available about the outbreaks and about the H5N1 virus, fewer rumors circulated. This decrease was despite the fact that the importance of the disease remained high because of the ongoing risk for evolution of a pandemic influenza strain.

Through rumor surveillance, WHO assisted affected countries by issuing guidelines, providing technical expertise, and mobilizing supplies. Unaffected countries also took action by banning the importation of poultry from affected countries. This action was crucial in preventing the further spread of avian influenza.

An important part of rumor surveillance is the timely dissemination of accurate information to reduce misunderstanding and unwarranted concern, especially for rumors reported in the media. One example was the need to address the international concern that arose about the rumor that pigs were infected with avian influenza (13). If the rumor had not been reported to be incorrect publicly after the verification process, health authorities may have heightened avian influenza surveillance to include the investigation of persons with symptoms of influenza and a history of contact with pigs.

The literature lacks guidance on how to establish and operate enhanced rumor surveillance during large outbreaks. Based on our experience and drawing on the recommendations in standard texts on public health surveillance (14,15), we suggest the following criteria for developing rumor surveillance: 1) Define the goals of surveillance as part of an early warning system in which each

Table. Avian influenza H5N1 rumors, Western Pacific Regional Office, World Health Organization (WHO), 2004

Rumor (source, date)	Verification (outcome, date)	Public health action
500 chicken deaths outside Phnom Penh (Dow Jones International News, 1/21/04).	True (avian influenza H5N1, 1/24/04).	Thailand banned importation of poultry from Cambodia (1/24/04). Japan supplied stocks of oseltamivir for prophylaxis. WHO supplied personal protective equipment for culling poultry.
Duck deaths, unknown cause, Guangxi, China (WHO network, 1/26/04).	True (avian influenza H5N1, 1/28/04).	48 countries banned importation of poultry from China (South China Morning Post, 1/29/04). WHO invited 2 Dutch experts to assist China contain the outbreak.
14-year-old boy, tracked to Guangdong, China, died in Hong Kong (Wenhui Newspaper, 8/2/04).	Incorrect (2/21/04).	Hong Kong reported investigation outcome in media. No public health action taken (2/21/04).
Persons in 2 Laotian provinces ate chicken, died of natural causes (WHO Network, 2/6/04).	True (2/11/04).	WHO released draft guidelines on food safety (2/12/04) posted at http://www.who.int/foodsafety/micro/avian2/en/.
Four pigs tested positive for bird flu, Vietnam (Reuters Health Online, 2/6/04).	Incorrect (2/6/04).	WHO and Food and Agricultural Organization publicized investigation outcomes (2/7/04).
Bird flu outbreak in poultry farm, south of Seoul, Korea (South China Morning Post, 1/27/04).	True (1/28/04).	Virus strains demonstrated in vitro susceptibility to oseltamivir (http://www.who.int/csr/don/2004_02_12a/en/).
Bird flu in German tourist returning from Asia (Washington Times, 1/22/04).	Incorrect (1/24/04).	WHO issued a press release that there was no need to shift into the Influenza Pandemic Plan Phase 1 (1/24/04 and 1/26/04).
48 children with respiratory illness, Nam Dinh Province, Vietnam (WHO network, 8/2/04).	Incorrect (9/2/04).	No public health action taken.

rumor deserves investigation to determine its veracity; 2) Apply a case definition that will have a high level of sensitivity (and therefore a relatively lower specificity) to identify the event of interest early in the outbreak; 3) Articulate clearly the steps to be undertaken to assess the veracity of the rumor, the criteria for deeming the verification process complete, and the ethics and confidentiality in conducting investigations; 4) Clarify the actions to be taken if the rumored events are true, or incorrect, or if the response of the verifying authority lacks credibility; 5) Delegate responsibility for data collection, management of the rumor database, and verification to a person trained in surveillance. This person must have access to relevant national and international networks and appropriate negotiation skills to investigate the veracity of the rumors. In selected instances, multilingual staff may be essential; 6) Include among the data sources print and electronic media, the Global Public Health Intelligence Network, national health authorities, and professional bodies and networks. Consider mechanisms for the public to report rumors through a hotline or an email address; 7) Develop mechanisms to provide regular updates on current verification activities, the number of rumors investigated, and their outcomes to the outbreak response team; 8) Provide regular feedback on the outcomes of investigations to those who provided data, and where appropriate, to the international community; and 9) Evaluate the efficiency and effectiveness of the investigations and upgrade the rumor surveillance system through a process of continuous quality improvement.

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Human Metapneumovirus RNA in Encephalitis Patient

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We describe a fatal case of encephalitis that might be correlated with primary human metapneumovirus (HMPV) encephalitis. Postmortem HMPV RNA was detected in brain and lung tissue samples from the patient. Furthermore, HMPV RNA was found in culture fluids from cells coincubated with lung tissue.

Human metapneumovirus (HMPV), a new member of the paramyxovirus family closely related to respiratory syncytial virus (RSV), was first described in 2001 (1). Subsequent reports demonstrated that HMPV is distributed worldwide (2–5), causing mild-to-severe acute infections of the nasopharyngeal tract (6,7). Although neurologic symptoms have been described for infections caused by other paramyxoviruses, such as Hendra, Nipah, mumps, and measles (8–12), no such symptoms have been associated with HMPV infection. We describe a child who died from edema caused by encephalitis probably induced or triggered by HMPV.

The Case

A 14-month-old boy who was unresponsive to verbal and tactile stimulation with high fever (temperature 39°C) was admitted to a primary care hospital ≈30 minutes after the onset of generalized febrile convulsions that did not respond to 2 doses of rectal diazepam (5 mg). His weight was 8 kg, all extremities were warm and well perfused, his heart rate was 140 beats per minute (bpm), and oxygen saturation was 93% in ambient air. Initial clinical examination showed no purpuric or petechial lesions, no heart murmurs or pulmonary rales, and no palpable enlargement of the liver or spleen. The boy had been healthy until 2 days earlier, when rhinorrhea, mild pharyngitis, and cough developed without signs of lower respiratory tract involvement. On the day of admission, he vomited once in the morning and than refused to drink. In the afternoon of the same day, a high fever developed, and seizures began, with extension and struggling of all extremities but without opisthotonos. The patient initially turned both eyes upwards but later stared straight ahead with no spontaneous eye movements and fixed pupils of 3 to 4 mm in diameter.

The boy had been born at 34 weeks' gestational age (weight 2,300 g), and he was treated for a few days with antimicrobial drugs after prolonged rupture of amniotic sac membranes, even though early-onset infection had not been confirmed. His neonatal period was uneventful, with the exception of moderate withdrawal symptoms (irritability, frequent bowel movements) probably due to tobacco use by his mother during pregnancy. The primary pediatrician documented a subtle, generalized muscular hypotension at the age of 9 months but did not consider this finding remarkable enough to investigate further (sonographic examination of the central nervous system showed normal results immediately after birth and 2 weeks later).

Clinical seizure activity subsided ≈2 hours after the intravenous administration of diazepam, clonazepam, phenobarbital, and lorazepam. Blood pressure was stable without high volume infusion or vasosuppressor support, and oxygen saturation (pulse oximetry) was 100% with 2 L of supplemental oxygen. Glasgow coma scale (GCS) was 10 at the end of the seizure. Cerebrospinal fluid (CSF) drawn by lumbar puncture showed no pleocytosis, glucose level of 4.6 mmol/L, and a slightly elevated protein content of 116 mg/dL (normal value <45mg/dL) (Table 1). Although leukocyte count and serum C-reactive protein did not suggest inflammation, empiric antimicrobial chemotherapy with ceftriaxone, ampicillin, gentamicin, and acyclovir was started immediately. An electroencephalogram showed generalized slow waves but no seizure activity.

On the basis of abnormal findings on a magnetic resonance imaging scan (MRI) performed 12 hours after admission (Figure 1A and 1B), attending physicians diagnosed meningoencephalitis of unknown origin. During the next few hours, the GCS of the patient deteriorated to 5. Both pupils were 8 mm in diameter and not reactive to light. The patient was intubated (no gag reflex observed during the procedure) and mechanically ventilated. He was sent to the pediatric intensive care unit of a tertiary-care hospital 24 hours after admission to have an external ventricular drain inserted. Upon arrival, the patient was deeply comatose (GCS 5) without response to painful stimuli; he had rectal temperature of 32°C, arrhythmic heart rate (90 bpm), and a mean arterial pressure of 29 mm Hg.

Corneal reflexes and gag reflex were silent. No spontaneous movements were observed; pupils were 5 mm in

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Table 1. Overview of laboratory parameters tested before the patient was admitted to the primary care hospital

Parameter	Measured	Reference range
Hemoglobin	11.2 g/dL	11–14.4 g/dL
Thrombocytes	264/mm^3	286–509/mm^3
Leukocytes	5,700/mm^3	6,000–17,500/mm^3
Neutrophils	47%	55%–75%
Band forms	8%	2%–8%
Lymphocytes	37%	20%–40%
Monocytes	7%	0%–12%
Eosinophils	1%	0%–7%
C-reactive protein	1 mg/L	0–3 mg/L
Ammonium	43 μmol/L (<94)	15–55 μmol/L

diameter and fixed. A chest radiograph excluded a pneumonic infection and confirmed the correct position of the ventilation tube and the central venous catheter tip. After stabilization of vital signs, the patient's condition did not change. The abnormal findings on a computed tomographic (CT) scan are shown in Figure 1C. Intracranial pressure measured after inserting an external ventricular drain and repeatedly afterwards was constantly elevated to 90 cm H_2O. Brain tissue extruded locally, and the drain was removed after 2 days. Repeated cultures of blood, urine, CSF, brain tissue, and the tip of the external drainage did not show any bacterial (culture) or viral pathogen (culture and polymerase chain reaction [PCR]) (Table 2). The patient did not exclusively display symptoms typical of measles or mumps, and neither of these viruses could be isolated from brain tissue.

In addition, extensive acute investigations of serum and urine specimens did not confirm any underlying inborn or acquired metabolic illness. A battered-child syndrome was excluded by a normal ophthalmologic examination and radiographs of all extremities; no sign of fresh or old fractures was found. After 10 days of supportive intensive care without clinical improvement, in accordance to the current regulations of German federal law, the child was considered to be dead and extubated after receiving informed consent from his legal guardian. He died shortly thereafter, and an autopsy was performed.

Conclusions

The acute symptoms were managed sufficiently by the primary intensive care team, particularly in terms of oxygenation, and no signs of dehydration were seen at admission. Thus, extensive discussion with the attending intensive care, neurosurgery, neuroradiology, and pediatric neurology team led to the conclusion that the time course of the illness suggested encephalitis as the primary reason for the child's symptoms and the adverse outcome. Other differential diagnoses of the MRI and CT findings such as postictal edema after a status epilepticus, edema due to prolonged hyperpyrexia and dehydration, or hypoxemia were considered highly improbable.

Tissue specimens from brain, lung, liver, kidney, and heart as well as serum and CSF were intensively screened for bacterial and viral infections by using standard techniques (Table 2). No bacterial or viral pathogens, except HMPV, were detected. RNA of this virus was identified by reverse transcription (RT)–PCR in both brain and lung tissue specimens. Moreover, all tissue specimens were mounted routinely on different cell lines susceptible to viruses commonly known to cause encephalitis (Table 3), but HMPV RNA was detected by PCR only in the cell culture supernatant of a Vero culture mounted with lung tissue. Although no cytopathic effect was observed in any of the

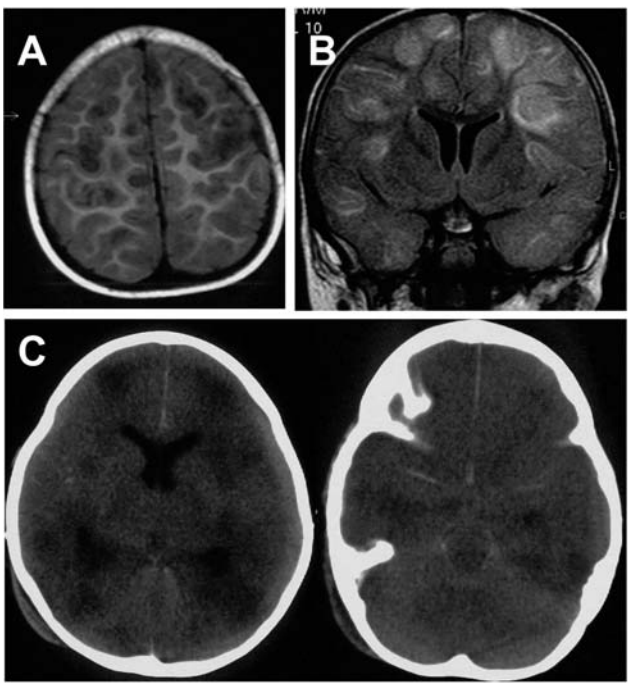

Figure 1. Axial T1-weighted magnetic resonance imaging (MRI) scan (A) and coronal fluid attenuated inversion recovery (FLAIR) (B) show multifocal, mainly cortical and subcortical lesions of high signal intensity, which are most probably caused by multifocal encephalitis. Nonenhanced axial computed tomographic (CT) scan (C) performed 2 days after the MRI shows multiple, hypodense lesions and signs of general edema. Additionally, it shows a hyperdense arachnoid collection that was not yet visible on the MRI 2 days before (panels A and B).

Table 2. Virologic tests performed on patient specimens*†

Virus	CSF	Brain	Liver	Spleen	Kidney	Lung	Heart	Diagnostic methods	Tested before death?
HSV (PCR)	–	–	–	ND	–	–	–	In-house PCR (nested), cell culture	Yes (from CSF and brain biopsy)
VZV (PCR)	–	–	ND	ND	ND	–	ND	In-house PCR (nested), cell culture	Yes (from CSF and brain biopsy)
Adenovirus (PCR)		–	–	–	–	–	–	In-house PCR (nested)	–
HHV-6 (PCR)		–	–	–	–	–	–	In-house PCR (nested)	–
HBV (PCR)		–	–	–	–	–	–	In-house PCR (nested)	–
HCV (PCR)		–	–	–	–	–	–	In-house PCR (nested)	–
ParvoB19 (PCR)		–	–	–	–	–	–	In-house PCR (nested)	–
CMV (PCR)		–	–	–	–	–	–	In-house PCR (nested), cell culture	–
Enterovirus (PCR)		–	–	–	ND	–	–	In-house RT-PCR, cell culture	–
RSV (Antigen)		ND	ND	ND	ND	–	ND	Antigen ELISA, Directigen RSV (Becton Dickinison, Heidelberg, Germany)	–
RSV (cell culture)		–	–	–	–	–	–	Cell culture	–
Influenza A+B		–	–	–	–	–	–	Cell culture	–
Mumps		–	–	–	–	–	–	Cell culture	Measles excluded, no symptoms of mumps†
Measles		–	–	–	–	–	–	Cell culture	
HMPV (PCR)	–	+	ND	ND	ND	+	ND	In-house RT-PCR (5,13)	–
HMPV (PCR from cell culture supernatant)	–	+	ND	ND	ND	+	ND	In-house RT-PCR (5,13) from Vero-cell culture supernatant	–

*CSF, cerebrospinal fluid; HSV, herpes simplex virus; PCR, polymerase chain reaction; ND, not done; VZV, varicella–zoster virus; HHV, human herpesvirus; HBV, hepatitis B virus; HCV, hepatitis C virus; CMV, cytomegalovirus; RT, reverse transcriptase; RSV, respiratory syncytial virus; ELISA, enzyme-linked immunosorbent assay; HMPV, human metapneumovirus.
†Before death, viral encephalitis induced by HSV and VZV was excluded by nested PCR from the CSF and also from a biopsy. Measles and mumps were excluded by the primary anamnestic protocol (no exanthema or other clinical symptoms). The anamnestic protocol also showed a successful vaccination against those pathogens. Furthermore, no clinical symptoms of mumps were observed. Postmortem specimens from brain, lung, kidney, liver, spleen, and heart were mounted on different cell lines. Detailed PCR protocols are available on request.

cell cultures, the fluid from the Vero cells mounted with lung tissue was repeatedly positive for HMPV RNA. None of the other viruses was detected by PCR, and no cytopathic effect was seen in any of the cell cultures.

Amplified fragments of HMPV DNA derived from the Vero cell culture supernatant were subjected to direct sequencing. The alignment of the resulting sequences showed close relatedness of HMPV RNA sequences present in brain and lung tissues and those obtained from the supernatant of infected cell culture (Figure 2). Upon phylogenetic analysis, the identified HMPV sequences were clearly separated from the sequences of other HMPV isolates obtained by our laboratory (13). These results allowed us to exclude the possibility of contamination of the brain and lung tissues under investigation with HMPV sequences from another source.

Virologic data on the detection of HMPV were complemented by results of histologic and immunochemical investigations. Thus, both the lung and the brain tissues showed evidence of an active inflammation. The alveolar lumina were partly or completely filled with fluid mixed with inflammatory cells, and the alveolar walls were thickened. The alveoli in half of the specimens were atelectatic. Some bronchioles had segmental loss of epithelium; meninges were diffusely thickened by many neutrophils and a few macrophages mixed with fibrin and a few erythrocytes.

Unfortunately, specific immune staining of paraffin-embedded tissues did not allow us to detect HMPV

Table 3. Cell lines routinely used for isolation of individual viral pathogens*†

Cell line	HSV	VZV	CMV	RSV	Mumps	Measles	Enterovirus	Influenza	HMPV
Vero	+	–		–	+	+	+	–	+
LLC-MK2	–	–	–	–	–	–	–	–	+
MS	–	–	–	+	–	–	–	–	+
MDCK	–	–	–	–	–	–	–	+	–
RD	–	–	–	–	–	–	+	–	–
HEF	+	+	+	–	–	–	–	–	–

*The + symbol indicates that the cell line is susceptible to the virus and was used for diagnostic procedures.
†HSV, herpes simplex virus; VZV, varicella zoster virus; CMV, cytomegalovirus; RSV, respiratory syncytial virus; HMPV, human metapneumovirus; LLC-MK2, kidney cell line from rhesus monkey; MS, monkey stable; MDCK, Madin-Darbin canine kidney; RD, human Caucasian rhabdomyosarcoma; HEF, human embryonic fibroblasts.

Figure 2. Alignment of 302 nucleotide human metapneumovirus (HMPV) sequences amplified from brain and lung tissues of the patient and from the supernatant of infected Vero cell culture. Sequence of the HMPV strain NLD00-1 was used as a prototype sequence. Conditions of the reverse transcription-polymerase chain reaction were described elsewhere (5).

antigens in the brain or lung of the patient. Several reasons may explain these negative results, including low concentration of viral antigens in the infected tissues, relatively low sensitivity of the immunochemical procedures, and a high sensitivity of the RT-PCR assay. Similar observations may be noted, namely, the positive RT-PCR findings of viral RNA and the inflammatory response in investigated tissues in the case of Nipah virus infection of the brain (11).

To our knowledge, this case report is the first of fatal encephalitis that might be associated with HMPV infection. We base a possible etiologic relationship between HMPV and the observed neurologic manifestations on the detection of HMPV RNA in the brain and lung tissues. Some clinical observations might serve as circumstantial evidence to support this hypothesis. The clinical course and MRI data for our patient are very similar to those observed in several patients with fatal encephalitis associated with Nipah virus infection, another member of the paramyxovirus family (8,11). Definite conclusions on the possible involvement of HMPV in neurologic disorders might be drawn only after additional studies. At this stage, however, we recommend HMPV screening for patients, especially young children, with symptoms of encephalitis of unknown origin. These investigations might extend our knowledge of the clinical manifestations and consequences of HMPV infection.

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Japanese Encephalitis Virus in Meningitis Patients, Japan

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Cerebrospinal fluid specimens from 57 patients diagnosed with meningitis were tested for Japanese encephalitis virus. Total RNA was extracted from the specimens and amplified. Two products had highest homology with Nakayama strain and 2 with Ishikawa strain. Results suggest that Japanese encephalitis virus causes some aseptic meningitis in Japan.

Japanese encephalitis virus is one of the leading causes of epidemic encephalitis worldwide; 35,000–50,000 cases are reported each year with 10,000 deaths (1). Before 1960, >1,000 Japanese encephalitis cases were reported annually in Japan. Because the Japanese encephalitis immunization program was introduced ≈30 years ago, and because of changes in rice farming, the annual number of Japanese encephalitis cases has decreased dramatically. Fewer than 10 Japanese encephalitis cases have been reported annually since 1990 (2). During the past 10 years, only 3 Japanese encephalitis cases have been reported in Hiroshima prefecture, the western part of the main island of Japan (Figure 1); all of these cases were reported in 2002 (2,3).

Japanese encephalitis virus causes meningitis as well as encephalitis (4). However, physicians tend not to list Japanese encephalitis virus as a cause of meningitis. In this study, we examined cerebrospinal fluid (CSF) specimens from patients with aseptic meningitis for Japanese encephalitis virus genome.

The Study

CSF specimens were obtained for diagnostic purposes from 170 patients with a clinical diagnosis of aseptic meningitis from August to October in each year from 1999 to 2002, in Hiroshima prefecture. These CSF specimens were sent to the Division of Microbiology II, Hiroshima Prefectural Institute of Health and Environment, for virologic examination. Viruses were isolated from 112 of 170 CSF specimens in cell cultures by using Vero cells, BGM cells, FL cells, Hep2 cells, and RD18S cells. Enteroviruses and mumps viruses were isolated from 96 and 16 CSF specimens, respectively (see online Appendix Table, available from http://www.cdc.gov/ncidod/eid/04-0285_app.htm). Thus, etiologic agents were not determined for 58 meningitis cases. CSF specimens from 57 of these 58 cases were used to detect Japanese encephalitis virus genome. Whether the 57 patients had been vaccinated for Japanese encephalitis virus was not known.

Total RNA was extracted from CSF by using ISOGEN-LS (Nippon Gene, Tokyo, Japan). The RNA pellet was resuspended in DEPC Treated Water (Invitrogen Corp., Carlsbad, CA, USA). The RNA was reverse transcribed and amplified by using polymerase chain reaction (PCR) with AMV Reverse Transcriptase XL (Life Sciences Inc., St. Petersburg, Florida, USA.) and Tth DNA Polymerase (Toyobo Co., Ltd., Osaka, Japan) with the primer pair reported by Morita et al. (5). The PCR product was nested PCR-amplified by TaKaRa EX Taq (Takara Bio Inc., Otsu, Japan), with the primer pairs for E gene region reported by Kimura et al. (6); JEN_ATC GTG GTT GGG AGG GGA GA(1147-1166)_JENR_AGC ACA CCT CCT GTG GCT AA(1472-1453). The DNA amplicons were separated by electrophoresis on 1.5% (wt/vol) agarose gel, followed by staining with ethidium bromide (1 μg/mL). The target band detected by electrophoresis was purified by using the Qiagen gel extraction kit (QIAGEN Inc., Valencia, CA,

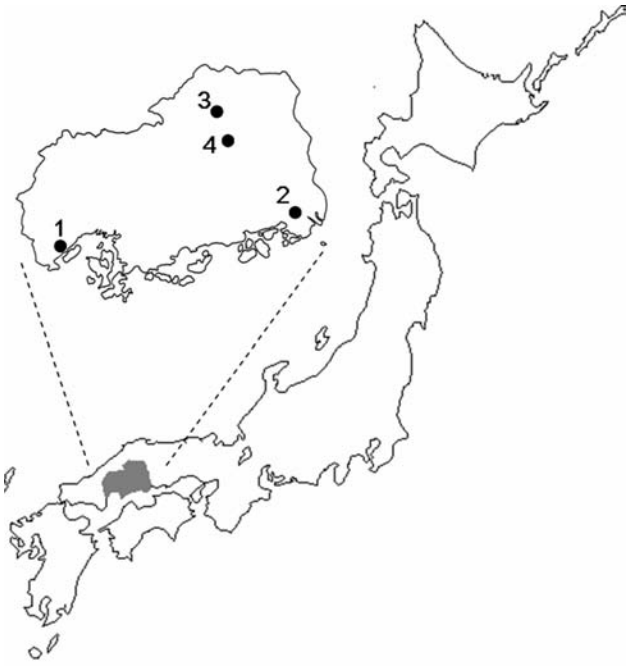

Figure 1. Location of Hiroshima prefecture in Japan and the areas where patients 1-4 resided. Numbers 1-4 correspond to the patients' numbers.

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USA). Purified cDNA was directly sequenced in both directions by using the sense and the antisense primers JEN and JENR. The samples that could not be sequenced using these primers were reverse transcribed and PCR-amplified, nested PCR-amplified, and directly sequenced by using other primers designed to amplify the Japanese encephalitis virus E gene region; RT-PCR JEen37s-first: AAG GAG CCA GTG GAG CCA CTT, JEen329c-first: TTC CCG AAA AGT CCA CAT CC, nested PCR and sequence JEen98s-second: CAT GGC AAA CGA CAA ACC AAC, JEen301c-second: CAG TRA AGC CTT GTT TGC ACA C. The samples that could not be directly sequenced by using the second primer sets were amplified with nested PCR and sequenced by using JEen98s-second and JEen271r-inner (RGT RAA GCC TTG TTT GCA CAC).

Electrophoresis demonstrated the band with expected size of 326 bp in 4 of 57 PCR products (Figure 1). Two (numbers 1 and 2) of these products were sequenced on 326 bp and 247 bp, respectively, and the highest homology was with Japanese encephalitis virus, Nakayama strain (genotype III). PCR products 3 and 4 were sequenced on 121 bp and 187 bp by using other primers, and the highest homology was with Japanese encephalitis virus, Ishikawa strain (genotype I) (Table 1).

Patients 1–4 lived in Hiroshima prefecture (Figure 2). Patient 1 was a 3-year-old boy and patient 4 was a 6-year-old girl (Table 2). They became sick in early August 2000. Patient 2 was a 2-year-old girl who became sick in late August 2000. Patient 3 was a 4-year-old boy who got sick in mid-September 2000. CSF samples were obtained 2–3 days after the onset of illness. All 4 patients had symptoms characteristic of meningitis and were clinically diagnosed with meningitis.

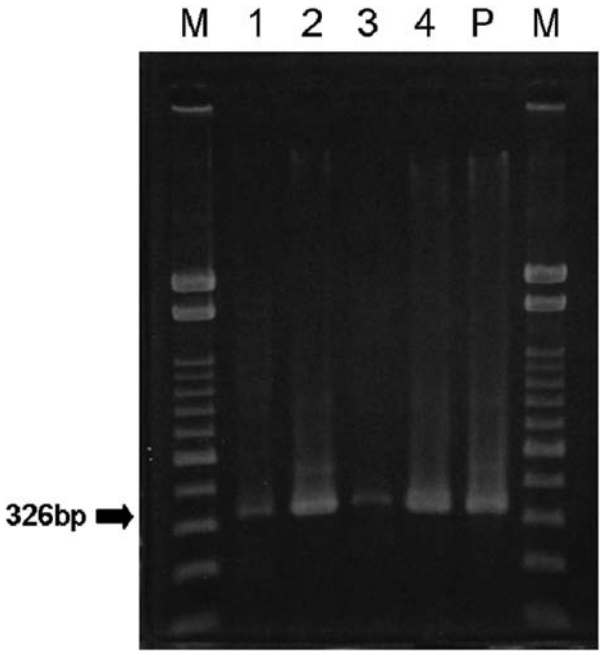

Figure 2. Detection of the Japanese encephalitis virus E gene in cerebrospinal fluid from patients with meningitis. Lanes 1–4, patients 1–4; lane P, positive control (JaGAr#01); lane M, marker (100-bp ladder)

Conclusions

Enteroviruses are the most frequent cause of aseptic meningitis in summer and autumn in Japan (online Appendix Table). In the CSF samples from these 4 patients, however, enteroviruses were not found. Watt et al. reported that in Thailand, 14% of adult patients with acute, undifferentiated fever had a diagnosis of Japanese encephalitis virus infection because anti–Japanese encephalitis virus immunoglobulin (Ig) M antibodies were found (10). Many physicians in Japan consider Japanese encephalitis virus to be mainly associated with encephalitis, and examination for the virus is not usually conducted for patients with meningitis or undifferentiated fevers.

Three Japanese encephalitis cases occurred in 2002 for the first time in 12 years, but anti–Japanese encephalitis virus IgM antibodies were detected in porcine serum samples every year in Hiroshima prefecture (data not shown). So we expected that Japanese encephalitis virus might be associated with aseptic meningitis, and CSF specimens from 57 cases during the past 5 years (1998–2002) had been stored at –30°C in Hiroshima Prefectural Institute of Health and Environment. Those specimens were used to detect Japanese encephalitis virus genome retrospectively. Four of 57 CSF samples were Japanese encephalitis virus genome–positive by nested PCR. We may find more

Table 1. Comparisons of the nucleotide sequences of Japanese encephalitis virus E gene detected in cerebrospinal fluid (CSF) with reference Japanese encephalitis virus strains

	Identity of nucleotide (%)				
Patient number and length of sequence	Ishikawa* Genotype I (Ref. 5)	Shizuoka33* Genotype I (Ref. 6)	JaGAr#01* Genotype III(8) (Ref. 7)	Nakayama* Genotype III(8) (Ref. 8)	JKT7003* Genotype IV(9) (Ref. 9)
1 (326 bp)	87.5	88.7	95.4	96.3	80.4
2 (247 bp)	86.2	87.4	96.0	96.4	81.3
3 (21 bp)	98.3	97.5	89.3	89.3	90.1
4 (187 bp)	99.5	98.9	89.8	89.8	87.7

*GenBank accession numbers of these strains are AB051292, AB112703, U44964, U44966, and U70408.

Table 2. Summary of the meningitis patients from whom Japanese encephalitis virus genome was detected by reverse transcription–polymerase chain reaction

Number	Age (y)	Sex	Date of onset	Sampling date	Fever (°C)
1	3	Male	Aug. 5, 2000	Aug. 7, 2000	40
2	2	Female	Aug. 27, 2000	Aug. 7, 2000	Unknown
3	4	Male	Sep. 14, 2000	Sep. 14, 2000	39.5
4	6	Female	Aug. 4, 2000	Aug. 7, 2000	37

febrile or meningitis cases caused by Japanese encephalitis virus if we routinely conduct serologic or molecular diagnostic tests for Japanese encephalitis virus. CSF samples were from infants and children. Most Japanese encephalitis patients in Japan are ≥55 years of age. Japanese encephalitis meningitis may also occur among elderly meningitis patients. The Japanese encephalitis vaccination coverage of children has been decreasing in Japan recently; <10 Japanese encephalitis cases are reported annually. If the general population recognizes that Japanese encephalitis virus causes aseptic meningitis at a higher rate than expected, the percentage of Japanese encephalitis vaccination may increase, and the emergence of Japanese encephalitis will be prevented. In 2000, anti–Japanese encephalitis virus IgM antibody was first detected in porcine serum samples in late July, in Hiroshima prefecture (data not shown). This finding suggests that Japanese encephalitis virus was active when these 4 meningitis cases occurred. Further, we found evidence that Japanese encephalitis viruses belonging to genotypes I and III were active in Hiroshima prefecture (data not shown). The results of this study suggest that Japanese encephalitis virus should be considered in the differential diagnosis of aseptic meningitis in areas where Japanese encephalitis is endemic or epidemic.

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Identifying Relapsing Fever *Borrelia*, Senegal

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Danielle Postic,† Guy Baranton,†
and Ronan Jambou*

We describe a nested polymerase chain reaction for the identification of *Borrelia* species from serum of patients with unidentified fevers. This technique, based on single nucleotide polymorphisms of the 16S ribosomal RNA gene, was used to test blood samples from 7,750 patients, 33 of whom were diagnosed with spirochete infections. *Borrelia crocidurae* was the only species identified.

Tickborne relapsing fevers, caused by spirochetes of the genus *Borrelia*, vary in severity and frequency of recurrence. The prevalence of various *Borrelia* species depends on the locality. In West Africa, *Borrelia crocidurae* is the primary cause of relapsing fevers (1–3), although *B. duttonii and B. recurrentis* are also found in Senegal. Tickborne relapsing fever is difficult to diagnose and is often confused in its early stages with malaria or relapsing malaria. Diagnosis is routinely made by conventional microscopy (Giemsa-stained thick blood smear) or fluorescence microscopy after concentration of blood in capillary tubes and staining with acridine orange (quantitative buffy coat [QBC] analysis) (3). However, these methods require technical expertise and do not identify *Borrelia* species. Another method, culture ex vivo, is difficult to perform and rarely used in African medical laboratories. In contrast, the polymerase chain reaction (PCR) is a sensitive diagnostic tool that is widely used in developing countries in AIDS, malaria, and entomology-related disease control programs.

The Study

The objective of our study was to develop a PCR method to identify the main *Borrelia* species in uncultured serum from patients with relapsing fevers in Senegal. This study was conducted using primers for single nucleotide polymorphisms (SNPs) in the 16S ribosomal RNA (*rrs*) gene of *Borrelia* sp. Sequencing of the *rrs* gene has shown that *B. crocidurae*, *B. duttonii*, *B. recurrentis*, and *B. hispanica* belong to the same species cluster (4). *B. crocidurae* differs from *B. duttonii* in this gene by only 1 nt (G63A polymorphism, GenBank accession no. M88329) and from *B. recurrentis* by 2 nt (G63A and C211T, GenBank accession no. M88329) (4). These SNPs have also been identified in *Borrelia* species found outside sub-Saharan Africa.

We used this method to analyze blood samples collected from patients treated at the medical laboratory of the Institut Pasteur in Dakar, Senegal, for suspected malaria from October 1999 to October 2003. In addition to routine laboratory analysis, PCR was performed on frozen serum. Spirochetes were first detected in fresh blood samples by microscopic examination with a QBC kit (Makromed, Johannesburg, South Africa). Five hundred microliters of serum from spirochete-positive patients was then used for extraction of DNA. Extraction was performed according to the method of Wilson (5), with slight modifications. Serum was diluted in distilled water (final volume 1.5 mL) and centrifuged at 15,000 rpm for 30 min at 4°C. DNA was extracted from the pellet by incubation for 2 h at 37°C in extraction buffer (1mol/L Tris, 0.5mol/L EDTA, proteinase K [20 mg/mL], and 10% sodium dodecyl sulfate), purified by a standard phenol/chloroform procedure (6), and precipitated with ethanol. Thermolysates provided by the National Borrelia Reference Center (CNRB) (Pasteur Institute, Paris, France) served as controls.

The primers used in this study are listed in the Table. *Borrelia* species were first identified by using the nested PCR reported by Ras et al. (4). It consisted of amplification of a 523-bp region of the *rrs* gene using primers sets fD3-T50 and Rec4-Rec9. In a second PCR, the presumptive identification of the *Borrelia* species was made using primer set Fd3-595R for the first amplification and specific primer sets (BcroF-255R for *B. crocidurae*, BdutF-255R for *B. duttonii*, and BrecF-500R for *B. recurrentis)* for the second amplification. These 3 specific primers sets were used in 3 separate reactions for the second PCR. Species identification was systematically confirmed by sequencing the *rrs* gene. The 3 species-specific primer sets include the SNPs up to their last 3′ base. Based on the alignment of sequences of *Borrelia* found in GenBank, these 3 primer sets can also hybridize with other sequences from the Eurasian *B. burgdorferi* sensu lato group and the North America relapsing fever group.

The reaction conditions were adapted to optimize species-specific amplification by using thermolysates of strains obtained from CNRL as positive controls. Serum samples negative for *Borrelia* and DNA extracted from *Escherichia coli*, *Plasmodium*, *Mycoplasma*, and *Candida* obtained from our medical laboratory were used as negative controls.

All PCR amplifications were performed in a final volume of 25 μL containing approximately 3 ng of DNA, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L $MgCl_2$, 200 mmol/L of each deoxynucleotide triphosphate, 1 U of *Taq*

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Table. Primers used for amplification of the *Borrelia* 16S ribosomal RNA gene based on GenBank accession no. M88329 for *Crocidurae*

Primer (reference)	Sequence	Tm, °C*	Nucleotide position
Fd3 (4)	AGAGTTTGATCCTGGCTTAG	58	8–27
T50 (4)	GTTACGACTTCACCCCCCT	60	1478–1499
Rec4 (4)	ATGCTAGAAACTGCATGA	50	659–675
Rec9 (4)	TCGTCTGAGTCCCCATCT	56	1191–1174
Fd4	GGCTTAGAACTAACGCTGGCAG	68	21–42
595R	CTTGCATATCCGCCTACTCA	60	621–602
500R (4)	CTGCTGGCACGTAATTAGCC	64	548–529
BcroF	CGTCTTAAGCATGCAAGTCAG	62	45–65 (U42283)
BdutF	CGTCTTAAGCATGCAAGTCAA	60	45–65 (AF107364)
BrecF	GAAAGGAAGCCTTTAAAGCTTT	60	193–214 (AF10362)
255R	CCCTACCAACTAGCTAATAAGACGC	74	255–231

*Tm, melting temperature.

polymerase (Amersham Biosciences, Piscataway, NJ, USA), and 1 mmol/L of each primer. Amplification in all PCRs was carried out for 35 cycles with denaturation at 93°C for 1 min, annealing at 4°C below the melting temperature of the primer (Table) used for 1 min, and extension at 72°C for 2 min. For PCR species typing, annealing was performed at 70°C for 40 s and extension at 72°C for 40 s. Final PCR products were detected by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. PCR analyses were performed according to Good Laboratory Practice to avoid cross-contamination between samples during extraction or amplification. Under the PCR conditions defined, the 3 West African *Borrelia* species were amplified only with their respective primers.

We tested potential cross-amplification of Eurasian and North American strains by using strains provided by CNRB. Typical bands were found in the thermolysates of *B. hermsii* (strainHS1), *B. parkeri* (strain M3001), and *B. turicatae* (strain 2007) when the specific primer for *B. crocidurae* was used. The thermolysates of *B. duttonii* (strain *Ly*), *B. garinii* (strain 20047), and *B. burgdorferi* sensu stricto (strain B31) were amplified with the specific primer for *B. duttonii*. However, since the geographic distribution of these pathogens does not overlap, species identification is relatively simple. For example, *B. hispanica* and *B. burgdorferi* sensu lato have not been previously found in sub-Saharan Africa.

Conclusions

Most patients treated at the medical laboratory of the Institut Pasteur in Dakar, Senegal, during the study period were residents of the Dakar area. Of the 7,750 patients included in this study, 3,300 were females and 4,450 were males; their mean age was 44.5 years. A total of 605 (7.8%) patients tested positive for malaria; most infections were diagnosed as *Plasmodium falciparum*. Spirochetes were found in the blood of 33 patients by microscopic examination. Only 1 patient was infected with both spirochetes and *P. falciparum*. In contrast to malaria, detection of spirochetes does not exhibit a seasonal variation in prevalence rates. Sera were available for 25 of 33 spirochete-positive patients. Using our species-specific nested PCR, we found that serum from 25 of these patients contained *B. crocidurae*. As described earlier, the presence of this species was confirmed by gene sequencing.

This new nested PCR is an efficient method for identifying tickborne relapsing fevers in sub-Saharan Africa. The procedures were conducted over a 2-day period with standard PCR equipment and could also be useful in epidemiologic studies. This study also confirmed that *B. crocidurae* is the most prevalent *Borrelia* species in the study area.

Mr. Brahim is a PhD candidate in the Immunology Department of the Institut Pasteur in Dakar. His research interests include the study of relapsing fever in urban Senegal.

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Babesia microti, Upstate New York

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Five cases of human babesiosis were reported in the Lower Hudson Valley Region of New York State in 2001. An investigation to determine if *Babesia microti* was present in local *Ixodes scapularis* ticks yielded 5 positive pools in 123 pools tested, the first detection of *B. microti* from field-collected *I. scapularis* in upstate New York.

Babesiosis is a malarialike infection often caused in humans by the bite of an infected tick (1,2). Currently, most cases of human babesiosis in the United States occur in the northeastern and northern Midwest portions of the country and may be attributed to infection with *Babesia microti* (2,3). *B. microti* is maintained naturally through the same reservoir (the White-footed mouse, *Peromyscus leucopus*) and vector (the Black-legged tick, *Ixodes scapularis*) as *Borrelia burgdorferi*, the etiologic agent of Lyme disease in the United States (1,4,5).

Human cases of babesiosis caused by *B. microti* were first identified in the United States in coastal areas of the Northeast, including several islands off the coast of Cape Cod, Massachusetts; in Rhode Island; and on Long Island, New York (1). Studies in Connecticut, Maine, and New Jersey have detected *B. microti* in other northeastern areas. In New Jersey, human cases have been reported from various inland locations across the state (6,7). In addition, *B. microti* has been identified in local populations of *I. scapularis* from the western portion of the state (8). *B. microti* has recently been found in local populations of White-footed mice collected in Connecticut (9,10). Detection of *B. microti* in Maine has been reported from the Southern Red-backed Vole (*Clethrionomys gapperi*), the Masked Shrew (*Sorex cinereus*), and the Northern Short-tail Shrew (*Blarina brevicauda*) (11), as well as from questing *I. scapularis* (12).

The first reported case of human babesiosis in New York was from Long Island in 1975 (13). Previously, detection of *B. microti* in New York has been limited to small mammals from Shelter Island (off the eastern end of Long Island) (13), except for 1 study in 1958 that identified *B. microti* in blood smears taken from a local population of Meadow Voles (*Microtus pennsylvanicus*) in the central portion of the state, near Ithaca (14). From 1986, when babesiosis officially became a reportable disease in New York, to 2001, a total of 560 human cases have been reported. Before 2000, most human babesiosis cases were reported from residents of Long Island. Cases reported from residents of upstate New York (north of New York City) are limited; most patients reported travel to locations with a known risk of potential exposure to *B. microti* (15). In 2001, 5 confirmed cases of human babesiosis were reported from residents of the Lower Hudson Valley who lived and worked north of recognized risk areas and for whom acquisition of the pathogen by blood transfusion or travel was ruled out (New York State Department of Health, unpub. data). These presumably locally acquired human cases were reported from 4 counties: Columbia (n = 1), Dutchess (n = 2), Putnam (n = 1), and Westchester (n = 1) (Figure).

The Study

In response to these presumably locally acquired human cases, an investigation was initiated to detect *B. microti* in local host-seeking populations of *I. scapularis*. Using the limited epidemiologic information available, we chose sites near the residences of suspected locally acquired human case-patients. Typical sites included parks and recreational areas run by state, county, or town governments. All sites were locations in which the possibility of human exposure to potentially infected ticks was considered high. Ticks were collected for 1 hour from each site during spring and fall of 2002 by using a combination of standard techniques, including walking and flagging using a 1-m^2 piece of white cloth (16). All ticks encountered were collected and kept alive until returned to the laboratory, where they were maintained at 4°C until they were sorted by life stage and identified to species (17). Specimens were stored in 80% ethanol *I. scapularis* were pooled by location and life stage for testing purposes. Pools consisted of 1 to 10 ticks and were tested for *B. microti* by polymerase chain reaction (PCR) in a blinded fashion. All samples were treated and processed alike, in addition to undergoing the same PCR conditions and analysis.

Briefly, each pool of ticks was homogenized with 125 µL of 5% Chelex-100 resin (BioRad, Richmond, CA, USA) to extract the DNA (the Chelex-100 DNA extraction procedure is the subject of a manuscript in preparation). The primers Bab 1 (5′-CTTAGTATAAGCTTTTAT-ACAGC-3′) and Bab 4 (5′-ATAGGTCAGAAACTTGAA-TGATACA-3′), targeting the 16S-like small subunit gene (3), amplified a product 238 bp in size. Each reaction

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consisted of 5 μL of 10x PCR buffer (Roche, Indianapolis, IN, USA), 30 pmol of each primer, 1 μL of 2.5 mmol/L deoxynucleoside triphosphate mixture (Roche), 5 U of *Taq* DNA polymerase, and 5 μL of sample. A negative control consisting of 5 μL of nuclease-free H_2O was included with each run (nuclease-free, reagent quality H_2O was used throughout to dilute reagents). Known negative tick controls included *Amblyomma americanum*, which do not harbor *B. microti*, and *I. scapularis* from areas where babesiosis is unknown. A positive control consisting of DNA (5 μL) extracted from whole blood of a *B. microti*–infected C3H/HeN mouse (PureGene DNA Blood Isolation Kit, Gentra Systems, Minneapolis, MN, USA) was also included. The PCR was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) under the following conditions: 5 min of initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C (20 s), annealing at 55°C (30 s), and extension at 72°C (30 s). Electrophoresis was carried out on 2% agarose gels, followed by staining with ethidium bromide.

A total of 1,139 *I. scapularis* was collected from 5 locations in the Lower Hudson Valley (Figure). Of the 123 pools tested, evidence of *B. microti* was found in 5 pools of female ticks collected from 3 locations (Table). None of the pools of New York nymphs was positive for *B. microti*. The positive pools collected from Columbia and Westchester Counties each contained 10 females, while the single positive pool from Dutchess County contained 7 female ticks.

To confirm the identity of each positive PCR product, amplimers were sequenced by using primers Bab 1 and Bab 4. Initial database (GenBank, EMBL, DDBJ) searches for each PCR positive sequence by using MacVector 7.1.1 (Accelrys, San Diego, CA, USA) software (BLASTN, National Institutes of Health, Bethesda, MD, USA) showed high homology with the *B. microti* strain GI 16S-like small subunit rRNA gene. For further confirmation, the sequences were aligned and compared to the *B. microti* 16S-like gene from strain GI reported by Persing et al. (3). Homology between the documented 16S-like gene sequence and all 5 PCR products was 100% (not shown).

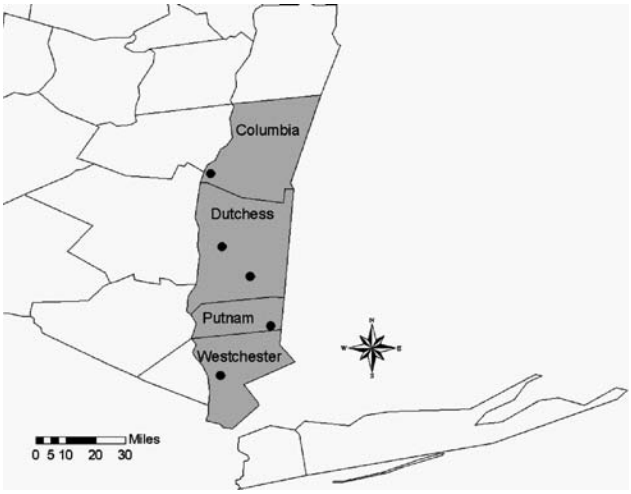

Figure. New York State Hudson Valley region. Circles denote generalized locations of tick collection sites in close proximity to locally acquired human babesiosis cases.

A 60-bp sequence segment, representing all 5 positive specimens, was deposited in GenBank (accession no. AY724679).

Conclusions

Borrelia burgdorferi and *Anaplasma phagocytophila*, the causative agents of Lyme disease and human granulocytic ehrlichiosis, respectively, have been studied more frequently in this region of New York than has *B. microti*. With the discovery of a cluster of human babesiosis cases in the Hudson Valley region, we focused on detecting *B. microti* in vector populations. Finding *B. microti* in local populations of *I. scapularis* provides evidence of locally acquired human babesiosis in the Hudson Valley Region. Since *B. microti* is maintained through the same reservoir and vector species as the causative agent of Lyme disease (5), human cases of babesiosis in areas of this state considered endemic for Lyme disease would not be unexpected. The 5 cases represent the first reports of locally acquired babesiosis in residents of New York not living in New York City or on Long Island.

Table. *Ixodes scapularis* collected in the Lower Hudson Valley Region of New York State and tested for *Babesia microti*

County	Site	Month	Nymphs	Adult	Total	Pools tested	Positive pools
		Jun	52	0	52	6	0
Columbia	A	Oct	1	177	178	19	2
		Jun	67	2	69	8	0
Dutchess	B	Nov	0	72	72	8	0
		Jun	52	2	54	8	0
Dutchess	C	Nov	0	192	192	20	1
		Jun	80	0	80	8	0
Putnam	D	Nov	0	120	120	12	0
		Jul	103	0	103	12	0
Westchester	E	Nov	0	219	219	22	2
Totals			355	784	1,139	123	5

As passive and active surveillance of human disease and tick distribution have demonstrated the continual expansion of Lyme disease and *I. scapularis* throughout New York (18), public health authorities should be aware of the potential for an increase in the geographic range of other human diseases transmitted by *I. scapularis*. Accordingly, the New York State Department of Health sent a letter alerting New York physicians to the possibility of patients' acquiring babesiosis in the lower Hudson Valley. Precautions to prevent tick bites should be adhered to, especially as more information becomes available with regard to the variety of pathogens being transmitted by a single tick species. Further studies to determine the prevalence and distribution of *B. microti*–infected ticks, as well as investigations of simultaneous infection by multiple pathogens such as *B. burgdorferi* and *A. phagocytophila*, are necessary to more readily define the expanding range of *I. scapularis* and the disease agents it harbors.

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Pythiosis in Africa

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We report the first case of pythiosis from Africa in an 8-month-old dog with a chronic and ulcerative cutaneous lesion. The etiologic agent belonged to the genus *Pythium*. Phylogenetic analysis placed the isolate in a sister group to the other *P. insidiosum* strains. However, the isolate may belong to a new *Pythium* species.

Members of the genus *Pythium* are soil- or water-dwelling organisms that belong to the kingdom Stramenopila (1,2). More than 200 species of this genus have been described. They usually live as saprophytes, but several species have been reported to cause disease in plants and fish, whereas *Pythium insidiosum* is the only species that has been recognized as a mammalian opportunistic pathogen. *P. insidiosum*–related infection was considered solely an animal disease until 1987, when De Cock et al. reported 2 cases in humans (3). In mammals, pythiosis is characterized by the development of cutaneous and subcutaneous abscesses and intestinal necrotic lesions. The bones and lungs may be affected less frequently (1). In the absence of specific treatment, pythiosis progresses rapidly, leading to the death of the affected animal or person. To date, the disease has been reported in horses, cattle, dogs, cats, polar bears, and humans (1).

The geographic distribution of the disease is very large. Clinical cases have been observed in tropical and subtropical areas of South America (Argentina, Brazil, and Colombia), Central America and the Caribbean islands (Costa Rica, Guatemala, Haiti, Panama, and Nicaragua), North America (the United States, especially in Florida, Louisiana, Mississippi, and Texas), and Asia (India, Indonesia, Japan, New Guinea, New Zealand, North Korea, and Thailand). Recently, evidence for molecular intraspecific variability was demonstrated, according to the geographic origin of 29 *P. insidiosum* isolates (4). Pythiosis has not been reported in Europe. The presence of *P. insidiosum* in Africa is likely. In a recent review, Mendoza (1) pointed out that "the geographical location and tropical climate of Africa seemingly would make it an ideal region for pythiosis." Nevertheless, human or animal cases from Africa have not been reported previously.

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The Case

We recently diagnosed a case of subcutaneous pythiosis in an 8-month-old German Shepherd dog originally from Bamako, Mali, a country in northwestern Africa. The dog was born in that region and had lived in Mali until his infection required special treatment. When the cutaneous lesion became chronic and unresponsive to surgical and medical treatments, the owners brought the dog to France for further examination and diagnosis. At the physical examination, the dog had a large (15 cm in diameter), ulcerative and draining, single, cutaneous lesion on the right side of the hip (Figure 1). The animal was otherwise in good health. The cutaneous lesion had appeared in May 2003, and despite several surgical excisions and oral administration of antimicrobial agents, the lesion progressed. In September 2003, several skin biopsy specimens were excised from the lesions. Histopathologic examination showed a pyogranulomatous inflammation with numerous broad (3–9 μm), irregular, septate hyphae (Figure 2). The hyphae were easily observed on sections stained with Gomori methenamine silver and periodic-acid Schiff, but cultures from biopsy samples failed to grow on Sabouraud dextrose agar.

To obtain a specific identification, genomic DNA was extracted from biopsy specimens collected from the infected tissues (Dneasy Tissue kit, Qiagen, Valencia, CA, USA) and was further subjected to internal transcribed spacer (ITS) and 5.8S rRNA gene sequencing by using primers ITS1 and ITS4 (5). Sequencing reaction was performed in a 10-μL volume containing 50 ng of sample DNA, 4 pmol of primers, and 4 μL of BigDye Mix (Applied Biosystems, Foster City, CA, USA). The unique polymerase chain

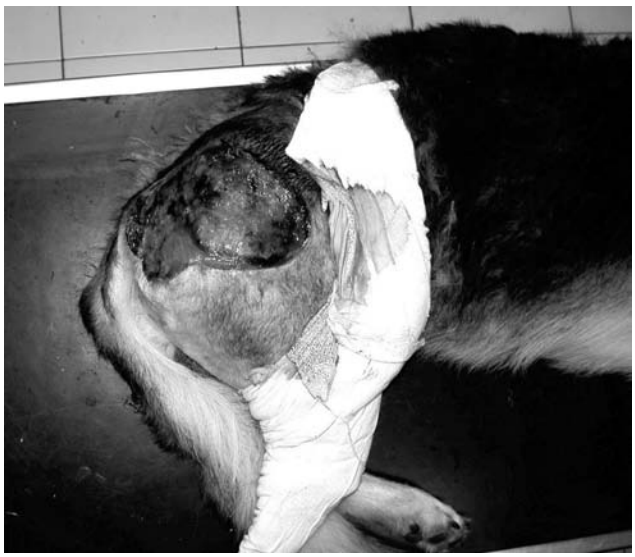

Figure 1. Unique cutaneous lesion on the right side of the hip in an 8 month-old German Shepherd. The lesion had appeared 4 months before this image was taken and had rapidly evolved into a large ulcer with draining tracts.

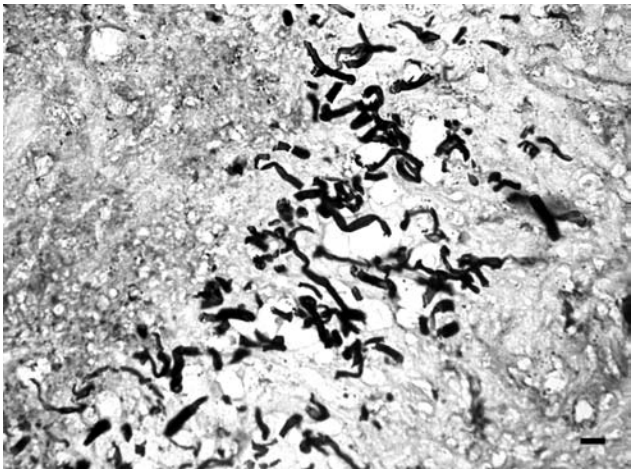

Figure 2. Presence of numerous, broad (3–9 μm in diameter), irregular, septate hyphae in a pyogranulomatous dermatitis (Gomori methenamine silver stain). Bar = 15 μm.

reaction product was analyzed on an ABI Prism genetic analyzer (Applied Biosystems). We obtained a sequence of 785 bp (GenBank accession no. AY683444), which was closely related to ITS and 5.8S rRNA sequences of *P. insidiosum* (GenBank accession no. AY151157-79) (BLASTn, National Center for Biotechnology Information, Bethesda, MD, USA). The dog was treated with oral ketoconazole (10 mg/kg/day) for 5 weeks. However, antimicrobial therapy was not sufficient to shrink the cutaneous lesion to a size that could permit surgery, and the dog was finally killed in December 2003. A necropsy was not performed.

Pythiosis most commonly affects young and large breed (>20 kg) dogs (6,7). The disease usually occurs in the cutaneous tissue and the gastrointestinal tract. Outdoor and hunting dogs, which are likely to be in contact with swampy water, are at higher risk of contracting pythiosis. The dog in this report was living in a large park and had no access to a swamp. The dog had swum in the Niger River a few weeks before the cutaneous lesion appeared. However, the owners did not recall any trauma, puncture, or wound at the site of the infection.

The climate in Mali is subtropical to arid. Bamako is located in the Sudanese climatic region with an average annual rainfall of ≈55 inches. In that region, the year is divided into 2 major seasons: a cool and dry season from November to February and a rainy season from June to September. The cutaneous lesion of the dog appeared at the beginning of the rainy season.

Conclusions

To identify more precisely the etiologic agent of the disease, we conducted a complete phylogenetic analysis of the ITS sequence obtained from the dog's infected tissues and other *Pythium* spp. sequences. Representative ITS and 5.8S rRNA sequences were obtained from GenBank and initially aligned with Clustal X version 1.63b (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France) and then by visual optimization. To infer phylogenetic relationships among *Pythium* isolates of our dataset, we conducted neighbor-joining and maximum parsimony (8) analyses using PAUP 4.0b9 software (9). Maximum parsimony analysis was performed by using heuristic searches. Evaluation of statistical confidence in nodes was based on 1,000 bootstrap replicates (10). ITS and 5.8S rRNA sequences from other oomycetes (*Lagenidium giganteum*, GenBank accession no. AY151183; *Saprolegnia parasitica*, GenBank accession no. AY310504; and *S. salmonis*, GenBank accession no. AY647193) were chosen as outgroups. The phylogenetic analyses were performed by taking into account 842 characters, including gaps. The total number of 264 characters was constant, 214 were variable but parsimony uninformative, and 364 were parsimony informative. Congruent phylogenetic trees in terms of branching and clustering of taxa were generated with the neighbor-joining (online Figure 3 available from http://www.cdc.gov/ncidod/eid/04-0697-G3.htm) and maximum parsimony methods. In each of these topologies, ITS and 5.8S rRNA sequences from *P. insidiosum* isolates were aligned into 3 clusters previously described by Schurko et al. (4). Inside and between these clades, the genetic distances were very low (0.1%–2.8%). The sequence AY683444, corresponding to the canine case, was very different from all other oomycetes sequences (genetic distances varied from 25.0% to 48.0%). Although the dog's ITS sequence clearly could be grouped with the other *P. insidiosum* clusters, the branching associated this sequence with the *P. insidiosum* strains was supported with only 60% bootstrap values. Sequences from other *Pythium* species formed 2 distinct groups. In the first group (*P. dissotocum*, *P. myriotylum*, *P. volutum*, *P. vanterpoolii*, and *P. porphyrae*), genetic distances varied from 7.1% to 19.9%. In the second group (*P. acanthicum*, *P. hydnosporum*, *P. oligandrum*, and *P. periplocum*), genetic distances varied from 1.8% to 6.2%. This finding suggests that the causative agent of the disease represents a new species within the genus *Pythium*. Because the isolation in pure culture of the etiologic agent was not successful, its ecologic and other characteristics remain to be determined. Specific nutritional requirements might account for the failure of the isolation of this particular strain. This report indicates that more cases of pythiosis in animals or humans from Africa could be expected in the near future.

Dr. Rivierre is a veterinary practioner in southern France.

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Hepatitis E Infections, Victoria, Australia

Benjamin C. Cowie,*‡ Jim Adamopoulos,† Karen Carter,† and Heath Kelly‡

In the first half of 2004, acute hepatitis E virus infections diagnosed in Victoria, Australia, increased 7-fold. Of the interviewed patients with highly reactive serologic results, 90% reported recent clinically compatible illness and overseas travel. The increase is compared with a background of exposure in countries in which hepatitis E is endemic.

Hepatitis E virus (HEV) is a major cause of enterically transmitted hepatitis worldwide. It is an important pathogen in Asia, the Middle East, and parts of Africa and Central America (1,2). Epidemic HEV is manifested in waterborne outbreaks, often involving thousands of people, which predominantly occur in areas where environmental sanitation facilities are inadequate. However, endemic (sporadic) HEV accounts for the majority of infections (2). In India, HEV is responsible for 50% to 70% of all cases of sporadic viral hepatitis (1).

HEV seroprevalence in disease-nonendemic areas such as Australia is low, ≈1% to 2% (3–5), in contrast to disease-endemic areas where seroprevalence increases as residents increase in age from 10% to 40% in adults (1,3,5,6). Some of this seropositivity is explained by subclinical infection and by persistence of immunoglobulin (Ig) G against HEV, which has been detected ≤14 years after infection (7).

The incubation period for HEV is 2 to 9 weeks, and the spectrum of disease ranges from subclinical infection to fulminant hepatitis. Clinical features can include fever, chills, jaundice, dark urine, anorexia, nausea, vomiting, abdominal pain, headache, myalgia, and arthralgia (1–3).

In general, HEV infection is more likely to be subclinical in children (1). Although usually a self-limiting disease with death rate ≤1%, a high incidence of fulminant hepatitis is seen in pregnant women, in whom the death rate can exceed 20% in the third trimester (2,3). HEV does not lead to persistent infection or chronic hepatitis (1,2). No vaccine is currently available.

In disease-nonendemic countries such as Australia, almost all patients with HEV infection report recent travel to areas where the disease was endemic (1,2,5). New evidence, however, suggests that HEV is more prevalent in industrialized countries than previously thought (8) and that zoonotic transmission may be implicated (1,2,5).

As in many jurisdictions, hepatitis E must be reported as an infectious disease throughout Australia. In Victoria, the Department of Human Services must be notified by both the treating doctor and the testing laboratory. In the first 6 months of 2004, an increase in positive hepatitis E serologic results was observed at the Victorian Infectious Diseases Reference Laboratory, a state reference and public health laboratory. Investigations to determine whether these serologic results represented true acute hepatitis E infections were conducted.

The Study

To fulfill the Victorian hepatitis E case definition for surveillance purposes, one must demonstrate seroconversion, a 4-fold rise in paired serum specimens or detect highly reactive IgG in a single specimen in the presence of a clinically compatible illness (9). Two commercial tests have been used for HEV serologic testing at the Victorian Infectious Diseases Reference Laboratory, the Abbott HEV enzyme immunoassay (EIA) (Abbott GmbH Diagnostika, Wiesbaden-Denkenheim, Germany) before March 2004 and the Genelabs HEV enzyme-linked immunosorbent assay (ELISA) (Genelabs Diagnostics Pte Ltd., Singapore) after March 2004. These tests detect anti-HEV IgG in the patient's serum by using recombinant antigens from the structural region of the HEV genome (3,4). Internal Victorian Infectious Diseases Reference Laboratory validation confirmed concordance of results using these tests, and both produced highly reactive results in this case series. Specimens are referred to the Victorian Infectious Diseases Reference Laboratory from private pathology laboratories, hospitals, and general practitioners throughout Victoria and also from other states and countries.

The EIA result is expressed as the ratio of the absorbance of the patient sample to the assay cut-off absorbance (s/co). A sample with a ratio of >1.0 is considered positive. A high s/co ratio indicates highly reactive patient serum, which suggests recent infection (2,6,10). Anti-HEV Ig G titers peak from 2 to 4 weeks after disease onset (11,12) and diminish relatively rapidly thereafter (2,8,10–12). For the purposes of this study, we have defined highly reactive results as those with an s/co ratio of ≥5.0, which has been associated with recent infection (12) and the presence of anti-HEV Ig M (13). Testing for antihepatitis E Ig M was not performed.

For the first 2 quarters of 2004, 7 and 10 highly reactive (s/co >5.0) hepatitis E EIA results were found, respectively. Both figures were the highest to date, and well above 1.2, the quarterly mean number of highly reactive results for the previous 5 years ($p < 0.0001$, χ^2 test)

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(Figure 1). Ten highly reactive specimens were from Victorian patients, compared to an expected number of 1.1 for the 6-month period ($p < 0.0001$). Total positive results (all s/co > 1.0) for the first 2 quarters of 2004 were also significantly increased at 7 ($p = 0.0103$) and 12 ($p < 0.0001$) compared to 2.75, the quarterly mean for the previous 5 years.

All highly reactive HEV EIA results detected at the Victorian Infectious Diseases Reference Laboratory are reported to the Department of Human Services. Victorian case-patients (those whose specimens are referred for testing by a Victorian doctor) are investigated by means of a standard questionnaire administered through telephone contact with both the patient and his or her doctor to determine clinical details, travel history, and other information. For patients outside Victoria, the appropriate authorities are notified wherever possible.

The 17 highly reactive samples tested at the Victorian Infectious Diseases Reference Laboratory during the first 2 quarters of 2004 accounted for all hepatitis E notifications in the state during this period. However, only 2 of these patients were also notified by the treating doctor, as required by legislation.

Nine of the 10 Victorian patients reported having been in a disease-endemic area within the incubation period and also having experienced an illness clinically compatible with HEV infection. (For further clinical and epidemiologic data, refer to Online Table, available from http://www.cdc.gov/ncidod/eid/04-706.htm#table.) The remaining patient reported no compatible illness and, therefore did not meet the Victorian case definition for surveillance. He last travelled in a disease-endemic area (Pakistan) in 2001.

Except for 1 patient whose serum tested positive for antibodies to hepatitis B core antigen, no positive serologic results for other hepatitis viruses (including hepatitis A) were reported in the patients from Victoria. Four of the 7 specimens from non-Victorian residents had been collected from patients from India and sent by a private laboratory to the Victorian Infectious Diseases Reference Laboratory, and 3 specimens were from patients from other states.

Conclusions

We found a 7-fold increase in the number of serum samples that were highly reactive for anti-HEV Ig G tested at the Victorian Infectious Diseases Reference Laboratory in the first half of 2004, from a mean for the last 5 years of 2.4, to 17 ($p < 0.0001$). Ten of these specimens were from patients in Victoria, a notable increase from the mean number for the previous 5 years of 1.1 for the 6-month period ($p < 0.0001$). As is characteristic in a disease-nonendemic region, 9 of the 10 highly reactive Victoria serum samples

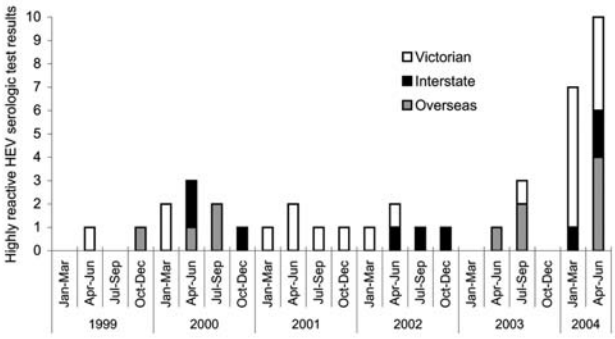

Figure 1. Highly reactive hepatitis E virus (HEV) enzyme-linked immunosorbent assay results at the Victorian Infectious Diseases Reference Laboratory per quarter, January 1, 1999, to June 30, 2004.

tested in this period were from patients who had recently traveled in disease-endemic countries, namely, India, Sri Lanka, Thailand, and Vietnam.

Why HEV infections in Victoria have recently increased cannot be established with certainty. Possible explanations include an increase in the number of tests performed, an increase in HEV activity in the countries visited by the travelers, an increase in the number of travelers to or from disease-endemic areas, and changes in behavior among the travelers. The last 2 possibilities are beyond the scope of this article but merit further consideration. The first 2 are explored further.

The mean number of hepatitis E serologic tests performed at the Victorian Infectious Diseases Reference Laboratory per quarter over the previous 5 years was 51.6 (Figure 2). In the first 2 quarters of 2004, respectively, 57 and 59 tests were performed, which is not significantly different from what was seen in previous quarters ($p = 0.452$ and 0.303).

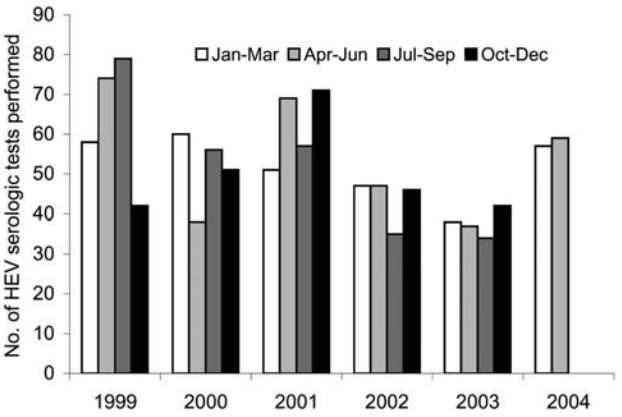

Figure 2. Total number of hepatitis E virus (HEV) enzyme-linked immunosorbent assays performed at the Victorian Infectious Diseases Reference Laboratory per quarter, January 1, 1999, to June 30, 2004.

Overseas hepatitis E activity does appear to have increased during this period. ProMED-mail, the global electronic reporting program for emerging diseases hosted by the International Society for Infectious Diseases, reported >800 cases of hepatitis E infection in eastern Calcutta, India, on April 23, 2004 (14). Contaminated water pipelines were implicated. Other areas with hepatitis E outbreaks reported by ProMED-mail in the first half of 2004 included Bangui, Central African Republic (6/19/2004), Punjab, Pakistan (6/6/2004), Sadr, Iraq (6/2/2004), and Gujarat, India (5/5/2004).

The first confirmed outbreak of hepatitis E with ≈29,000 cases of hepatitis occurred in Delhi in 1955–1956 when raw sewage contaminated drinking water during heavy flooding (2,11,14). Other epidemics include Kashmir in 1978, with an estimated 52,000 cases of hepatitis and 1,560 deaths (8), and the largest epidemic on record in northwest China in 1986–1988 with >100,000 cases (15).

Increased diagnoses of hepatitis E in Victorian travelers may have provided "early warning" of an evolving outbreak in an HEV-endemic area, particularly if a similar increase is reported in other non–HEV-endemic areas. Residents of resource-rich, non–disease-endemic countries such as Australia likely have greater access to hepatitis E testing than those living in resource-poor, HEV-endemic areas where the greatest incidence of this disease occurs.

Persons traveling to developing countries must be advised of preventive measures they should take against hepatitis E and other enterically transmitted diseases. Hepatitis E infection should be considered in any febrile person who has recently traveled in a disease-endemic area, particularly if jaundice or abnormal liver function tests are found, and especially in pregnant women due to the risk of fulminant hepatitis. Cases should be reported to public health authorities according to local legislation.

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Concomitant Tickborne Encephalitis and Human Granulocytic Ehrlichiosis

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We report a patient with febrile illness and epidemiologic and clinical findings consistent with human granulocytic ehrlichiosis and tickborne encephalitis, in whom infection with *Anaplasma phagocytophilum* was demonstrated by polymerase chain reaction and seroconversion. Tickborne encephalitis virus infection was established by serum immunoglobulin (Ig) M and IgG antibodies.

Ticks transmit several bacteria, viruses, and parasites capable of infecting and causing diseases in humans. In general, only a small proportion of bites from infected ticks result in infection, and only a fraction of these infections result in clinical illnesses such as Lyme borreliosis, ehrlichioses, rickettsioses, tickborne encephalitis (TBE), and babesiosis. Infection does not inevitably indicate the presence of the illness nor does it always allow for a reliable explanation of all the signs and symptoms a patient may have. Many distinct causative agents transmitted by the same vector make coinfection and, consequently, the simultaneous presence of more than 1 tickborne disease possible. Human coinfection may occur from a bite of a single tick that transmits multiple pathogens or from multiple tick bites; sequential infections can occur from bites taking place at different times.

Although several reports of coinfection (some of them most probably sequential infections) with tick-transmitted agents have been made, rather limited information exists on the simultaneous clinical features of corresponding illnesses (1). Reports from the United States involve cases with concurrent Lyme disease, babesiosis, or human granulocytic ehrlichiosis (HGE). In addition, U.S. studies indicate that the frequency of simultaneous diseases caused by infection with >1 tickborne pathogen is usually low and varies among geographic locations (1,2). Data from Europe are limited to the reports on coinfections with TBE virus and *Borrelia burgdorferi* sensu lato (s.l.) in patients with acute meningitis (3–7). Other combinations that could have also been possible in European populations, including infections with *Anaplasma phagocytophilum*, *B. burgdorferi* s.l., or TBE virus, have been so far indicated only by the findings of serosurvey studies (8–11).

Slovenia is a small central European country where TBE, Lyme borreliosis, and HGE are known to be endemic. Residents of Slovenia are often exposed to ticks and thus at risk of acquiring infection with multiple tickborne pathogens (7). We report on a patient with TBE and HGE in whom infection with *A. phagocytophilum* was demonstrated by polymerase chain reaction (PCR) and seroconversion, and TBE virus infection was established by the presence of immunoglobin (Ig)M and IgG antibodies to TBE virus in serum.

Case Report

On May 2, 2003, a 47-year-old-woman, who lived in northwestern Slovenia, was admitted to the Department of Infectious Diseases, University Medical Centre Ljubljana, Slovenia, with a 7-day history of fever ≤40.0°C, severe headache, nausea, dry cough, malaise, intense myalgia, and arthralgia. She recalled having sustained 3 tick bites on her abdomen during the previous month. The last bite occurred 14 days before onset of her illness while she was walking in the woods near her home; no skin lesions appeared at the site of the bites. Her previous medical history was unremarkable. She had not been vaccinated against TBE, nor had she traveled outside Slovenia during the last few years.

When she sought treatment, her body temperature was 38.5°C, pulse rate was 90 beats/min, and blood pressure was 110/70 mm Hg. With the exception of fever, the physical examination did not show any notable abnormality; rash and meningeal signs were not present. Routine laboratory tests showed leukopenia, thrombocytopenia, abnormal liver function test results, elevated concentration of serum C-reactive protein, and elevated procalcitonin levels (Table 1). A chest radiograph showed no abnormalities. Amoxicillin–clavulanic acid was prescribed. The fever subsided in 3 days (on day 10 after the onset of her illness), and the patient was discharged from the hospital. Her condition improved, but the headache persisted and intensified. On May 13, she was reexamined in our department, and lumbar puncture was performed. Cerebrospinal fluid examination showed normal protein and glucose concentrations but a mildly elevated number of leukocytes (7 x 10^6/L). At subsequent evaluations, the patient reported feeling better. On day 20 after onset, the control laboratory test results, including liver function test results, were within the normal range.

Several microbiologic procedures, including those for determining infections with *A. phagocytophilum*, *Ehrlichia chaffeensis*, *B. burgdorferi* s.l., and TBE virus,

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Table 1. Hematologic and blood chemistry values

Variable	Day of illness		
	8	9	12
Leukocytes (normal, 4,000–10,000/mm^3)	1,900	1,800	4,800
Platelets (normal, 150,000–350,000/mm^3)	22,000	16,000	43,000
Aspartate aminotransferase (normal, 0–36 U/L)	NT*	49	75
Alanine aminotransferase (normal, 0–42 U/L)	NT	72	108
Lactate dehydrogenase (normal, 140–290 U/L)	NT	499	354
C-reactive protein (normal, <5 mg/L)	277	230	61
Procalcitonin level (normal, <0.50 μg/L)	NT	2.6	0.13

*NT, not tested.

were performed to elucidate the cause of the illness. Giemsa-stained peripheral blood smear examination by light microscopy for the presence of ehrlichial morulae within leukocytes was negative. Serum samples were tested by an indirect immunofluorescence assay for the presence of specific IgG antibodies to *A. phagocytophilum* (strain USG3 propagated in HL60 promyelocyte cells), IgM and IgG antibodies to *E. chaffeensis* antigens (MRL Diagnostics, Cypress, California, USA), and IgM and IgG antibodies to *B. burgdorferi* s.l (whole cells of a local isolate of *B. afzelii* were used as an antigen). The presence of serum TBE virus IgM and IgG antibodies was assessed by enzyme-linked immunosorbent assay (ELISA) (Dade Behring Marburg GmbH, Marburg, Germany). The results of serologic tests indicating recent infection with *A. phagocytophilum* and TBE virus are depicted in Table 2. Primers Ehr521 and Ehr790, which amplified the 16S rRNA gene of *A. phagocytophilum*, produced a fragment of the expected size (293 bp) in the acute-phase blood specimen (Figure) (12). No nucleic acids were amplified with primers specific for *E. chaffeensis* HE1 and HE3 (Table 2) (13).

Conclusions

Few reports have been published on serologic evidence of coinfection with TBE virus and *A. phagocytophilum* in Europe. The results on groups of persons representing different risk categories for tick exposure in Switzerland provided serologic evidence of coinfection with *A. phagocytophilum* and TBE virus (9). Weber et al., who retrospectively tested serum specimens of patients with Lyme borreliosis or TBE for antibodies to *A. phagocytophilum*, reported similar findings (10). Although serologic data suggested coinfection with *A. phagocytophilum* and TBE virus, no confirmation of acute HGE was obtained among the residents of Switzerland. In the Czech Republic, where TBE is endemic, among 67 patients hospitalized for TBE, 6 (9%) were seropositive to *A. phagocytophilum* (11). In Slovenia, the background seroprevalence of HGE in children and young adults (15%) was found to be similar to that of Lyme borreliosis (15%) and TBE (13%) (14). In addition, a prospective study was performed to establish the etiologic agents of acute febrile illnesses that occurred within 6 weeks after a tick bite in residents of Slovenia, by using a combination of microbiologic and clinical criteria. Out of 130 adult patients, 36 (28%) had laboratory evidence of TBE virus infection (all had clinically confirmed disease), whereas 4 of 22 (17%) patients with the evidence of *A. phagocytophilum* infection, had confirmed HGE. Infection by multiple organisms (>1) was found in 19 (15%) of 130 patients. Four of them had confirmed TBE and also met the study criteria for probable HGE but not for confirmed HGE (7).

The clinical signs and symptoms of HGE are unspecific and usually consist of fever, headache, chills, malaise, myalgia, or arthralgia, which often occur after a tick bite. Laboratory analysis shows leukopenia, thrombocytopenia, lymphopenia, elevated activity of hepatic enzymes, and an elevated concentration of C-reactive protein (7). However, leukopenia and thrombocytopenia are common not only in

Table 2. Results of PCR analysis and serum antibody titers to different tickborne pathogens tested at different times after the onset of illness*

Days after onset of illness	PCR† 16S RNA gene		ELISA IgM/IgG‡ TBEV	IFA IgG *A. phagocytophilum*	IFA IgM/IgG *E. chaffeensis*
	Anaplasma phagocytophilum	*Ehrlichia chaffeensis*			
12	Pos	Neg	0.614/129	Neg	Neg/neg
14	Neg	Neg	1.369/251	512	Neg/neg
20	–	–	1.187/382	2,048	Neg/neg
45	–	–	0.419/220	512	Neg/neg
144	–	–	0.400/179	512	Neg/neg

*PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; IFA, indirect immunofluorescence assay; Ig, immunoglobulin; Neg, negative; Pos, positive; TBEV, tickborne encephalitis virus.
†Primers Ehr521 and Ehr790 specific to *A. phagocytophilum* and primers HE1 and HE3 specific to *E. chaffeensis*.
‡Cut-off value for TBEV IgM is 0.272–0.372 and for IgG is 7.5 U/mL, according to the manufacturer's procedure.

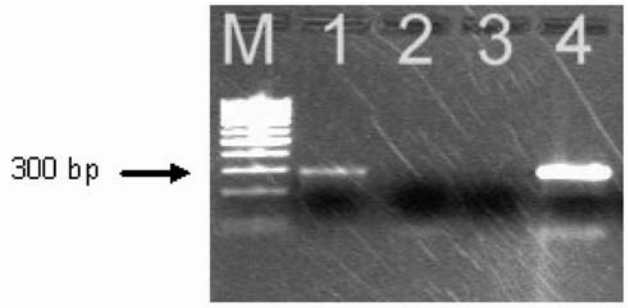

Figure. Polymerase chain reaction amplification of *Anaplasma phagocytophilum* DNA from the patient's acute-phase blood sample. Amplified DNA was separated by electrophoresis through the 2% agarose gel stained with ethidium bromide. Lane 1, patient sample (note the presence of the band at ≈293 bp); lane 2, negative sample; lane 3, negative control (no-DNA template control); lane 4 positive control (DNA extracted from the cultured isolate of *A. phagocytophilum*). Lane M represents a 100-bp DNA ladder for estimation of molecular sizes.

HGE patients but also during the initial phase of TBE, in which they were found in 67% and 71% of patients, respectively (15). For most patients in whom TBE develops, the disease has a biphasic course. Our patient, however, had a monophasic course of febrile illness, which was observed in 27% of patients with TBE in Slovenia (16).

In our report, the diagnosis of TBE was established by the presence of a febrile illness after a tick bite with headache, mild lymphocytic pleocytosis, and demonstration of serum IgM and IgG antibodies to TBE virus by ELISA, the serologic method of choice with the specificity 99.9% for IgM and 99.5% for IgG and the sensitivity 99.8% and 96.8% for IgM and IgG, respectively (17). The presence of TBE IgM antibodies 144 days after the onset of illness in our patient (Table 2) is not surprising because the IgM antibodies as a rule persist for several months after acute infection (17). However, changes in antibody titers as demonstrated in our patient attest for recent infection. The febrile illness associated with leukopenia, thrombocytopenia, an elevated concentration of serum C-reactive protein, and the demonstration of *A. phagocytophilum* infection by seroconversion as well as a positive PCR result also met the criteria for confirmed HGE. To our knowledge, the patient reported herein represents the first case of concurrent confirmed TBE and confirmed HGE.

Some reports suggest that coinfection with more than 1 tickborne pathogen may influence the natural history of each of the corresponding diseases, making the clinical course more severe and the outcome less favorable (1). In spite of proven coinfection and fulfillment of criteria for confirmed TBE and HGE (and the absence of treatment with doxycycline, which could have influenced the natural course of HGE), our patient had a relatively mild illness with uneventful recovery. However, the information obtained from a single patient does not allow for a reliable conclusion on the potential influence of TBE virus and *A. phagocytophilum* coinfection on the clinical features and course of the combined illness.

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Noroviruses in Archival Samples

Sylvain Skraber,* Ronald Italiaander,* Willemijn J. Lodder,* and Ana Maria de Roda Husman*

Application of recent techniques to detect current pathogens in archival effluent samples collected and concentrated in 1987 lead to the characterization of norovirus GGII.6 Seacroft, unrecognized until 1990 in a clinical sample. Retrospective studies will likely increase our knowledge about waterborne transmission of emerging pathogens.

Noroviruses, previously designated as small round-structured viruses or Norwalk-like caliciviruses, are enteric viruses that cause large outbreaks of gastroenteritis (1). Besides person-to-person transmission, these viruses may spread by water. Noroviruses cannot be propagated by cell culture (2), and detecting them by using immunologic or electron microscopic techniques is painstaking and time-consuming. When molecular techniques were developed in the early 1990s, norovirus detection in water and subsequent genotyping became feasible (3). Noroviruses are, therefore, more frequently identified as the causative agent in waterborne outbreaks (1,4). Though humans are frequently infected with 1 specific norovirus strain, many different strains are found in sewage and surface water (5). Based on the comparison of open reading frame 2 sequences, GGI and GGII comprise 5 and 10 genotypes, respectively; all are associated with infections in humans (6). Recently, other regions have been used for norovirus classification, such as the capsid VP1 region, leading to 7 GGI and 12 GGII genotypes (7). This classification may evolve further, as a recent study proposed to define 3 new human genogroups, IV, VI, and VII (8). Season-novel variants may have characteristics that enable them to replace the predominant strain circulating in the population (4). Primer pairs and probes used in norovirus detection need to be optimized to include such novel strains (9). In that context, previously screened water samples may have been falsely negative, or some noroviruses may have been missed.

The Study

We conducted a retrospective study on 4 archival effluent samples collected and concentrated in 1987, analyzed for phages and enteroviruses but not noroviruses and kept frozen at –70°C. We analyzed these samples for noroviruses in 2003 by using JV12Y and JV13I, a recently optimized primer set that allows detection of a broad range of noroviruses by targeting the RNA-dependent RNA polymerase (9).

A sample of effluent waters from the sewage treatment plant situated in Leerdam, the Netherlands, was taken on July 22, August 5, August 26, and September 9, 1987. Each of the 4 samples was concentrated by using a conventional filter adsorption-elution method (10), and the resulting eluates were reconcentrated by ultrafiltration. The 4 ultrafiltrates were analyzed for somatic phages, F-specific phages, and enteroviruses, and each sample was found positive for these viruses. Samples were stored at –70°C. A norovirus reverse transcription–polymerase chain reaction (RT-PCR)-positive stool sample, obtained in 1997 and kept at 4°C, was used as a positive control for cloning and sequencing.

The RT-PCR was conducted as described previously (5). Briefly, 7-mL effluent samples were clarified by centrifugation for 10 min at 3,000 *g*, whereas 10 µL of stool sample was diluted in 3 mL of sterile water. RNA was extracted from the resulting supernatant of the effluent sample and the total volume of diluted stool sample by binding to silica beads in the presence of guanidinium isothiocyanate (11). Five microliters of the extracted RNA was reverse transcribed for 60 min at 42°C after annealing with JV13I (9) at 0.3 µmol/mL in 15 µL of 10 mmol Tris–HCl pH 8.3, 50 mmol KCl, 3 mmol $MgCl_2$, 1 mmol deoxynucleoside triphosphate, 40 U/mL RNAguard, and 5 U AMV-RT (Promega, Leiden, the Netherlands). Five microliters of the RT mix was added to 45 µL of a PCR-mix containing 10 mmol Tris–HCl pH 9.2, 50 mmol KCl, 1.2 mmol $MgCl_2$ (final concentration 1.5 mmol), 0.2 mmol dNTPs, 2.5 U ampliTaq, and 0.3 µmol/mL of JV12Y (9). Samples were denatured for 3 min at 94°C and subjected to 40 cycles (94°C for 1 min, 37°C for 1 min 30 s, and 74°C for 1 min) before linearization at 74°C for 7 min. Amplified DNA was detected by electrophoresis in a 2% agarose gel and visualized under blue light after SYBR-Gold (nucleic acid gel stain) (Molecular Probes, Leiden, the Netherlands) staining. The specificity of the detected noroviruses was confirmed by Southern blot hybridization as described previously (5). RT-PCR products of appropriate size (327 bp) were gel purified (QIAquick PCR purification kit, Qiagen, Hilden, Germany) and cloned into a plasmid vector (pGEM-T Easy Vector, Invitrogen, Leek, the Netherlands). Plasmid DNA was purified and amplified by PCR using specific plasmid M13 forward and reverse primers according to manufacturer instructions. Amplified DNA was confirmed to be norovirus specific by Southern blot hybridization (using the same protocol as described above) before sequencing using the BigDye

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Terminator Cycle Sequencing Ready reaction Kit (PE Applied Biosystems, Foster City, CA, USA). Multiple sequence alignments were performed on the 145 sequenced bases with sequences of known genetic clusters available from GenBank, and phylogenetic trees were generated by using Bionumerics software (V2.0 Applied Maths, Kortrijk, Belgium).

Amplification of RNA detected from the stool sample and 3 of the 4 effluent water samples from 1987 yielded a norovirus-specific 327-bp band after gel electrophoresis of the RT-PCR product (data not shown). The presence of norovirus was confirmed by Southern blot hybridization of the amplified cDNA. RT-PCR products derived from the stool sample and 1 effluent water sample were successfully cloned and sequenced. The multiple sequence alignment and the resulting phylogenetic tree (Figure) showed high similarity between norovirus amplified from stool and the GGII.4 Hu/NLV/Grimsby/95/UK strain (GenBank accession no. AJ004864) (score: 143/145 nt). In the same way, high similarity was found between norovirus amplified from effluent and the GGII.6 Hu/NLV/Seacroft/1990/UK (GenBank accession no. AJ277620) (score: 144/145 nt). Results were not likely due to contamination, as the stool sample was positive for a norovirus strain different from the effluent sample, and the negative controls for RNA extraction and RT-PCR were negative (data not shown).

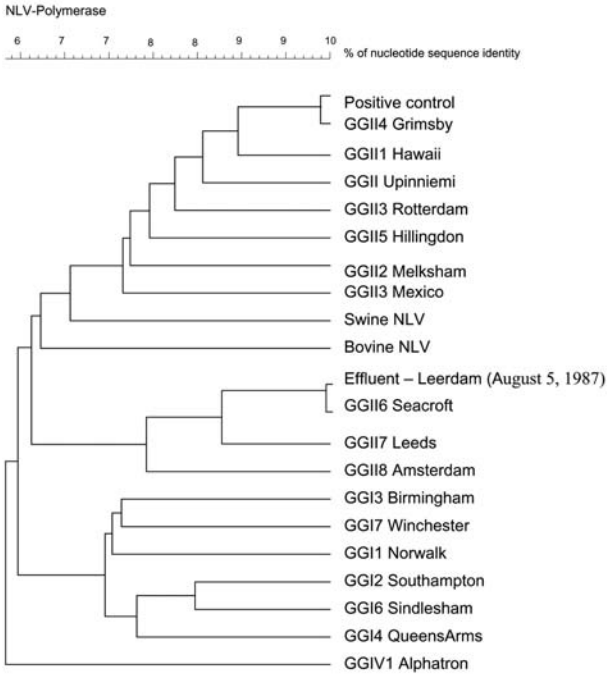

Figure. Phylogenetic analysis of the positive stool sample, the 1987 effluent sample, and referenced norovirus strains based on 145 nt of the RNA-dependent RNA polymerase sequence.

Conclusions

Historically, Seacroft strain was first detected and sequenced from a stool sample collected in 1990 in the United Kingdom (12). Noroviruses are generally more easily detected in clinical samples in which the virus concentrations are higher. Furthermore, norovirus strains present important genetic variations that can explain commonly reported, false-negative RT-PCR results (9,13). For those reasons, norovirus prevalence may be underestimated, especially in environmental samples in which virus concentrations are low and RT-PCR inhibition may occur. Their detection in stool samples enables optimization of primers that can subsequently be used to screen water samples. In that context, our observation confirms, retrospectively, the potential usefulness of environmental surveillance as a tool for monitoring virus infections in the population. Indeed, our results show that Seacroft strain had already spread in the environment at least 3 years before its reported characterization from a clinical sample. Moreover, this strain has been detected in the middle of summer (August 5, 1987), which confirms that norovirus infections do not exclusively occur during winter (4). Finally, our results show that environmental archival samples stored at low temperature with beef extract as cryoprotector may profit from current virologic detection methods. Thus, retrospective studies may provide information about geographic and seasonal distribution of emerging or previously undetectable viral strains. Forthcoming virus detection methods may provide useful information about current environmental samples. For example, no method is available to ascertain the presence of infectious norovirus and such methodology should be developed (2). We confirmed the presence of infectious F-specific phages and somatic coliphages in all 4 archival samples after 17 years of storage at –70°C, following ISO/FDIS 10705-1 and 10705-2 protocol, respectively (data not shown). We also cultured enteroviruses on buffalo green monkey cells (BGM) and detect plaques by monolayer BGM plaque assay (data not shown). Similar counts were established for the enteroviruses in 1987 (1–22 PFU/g of concentrate) and in 2004 (0.5–29 PFU/g of concentrate). Therefore, if frozen concentrates also conserve the integrity of norovirus, new detection protocols may help to identify infectious noroviruses in the environment. From a methodologic point of view, long-term retrospective virologic studies based on screening of archival samples have 2 advantages: 1) using the same methodology to generate results allows easier comparison, and 2) it can be applied to many samples already collected over a period of years. When this approach is used, important knowledge on pathogenesis and disease progression in clinical settings has already been acquired (15).

Moreover, environmental samples potentially differ from clinical samples in 2 important ways. First, environmental samples consist of pathogenic viruses derived from different persons that represent large populations, whereas clinical samples represent single persons. Therefore, environmental samples potentially contain more variant strains. Indeed, in environmental samples, both symptomatic and asymptomatic patients contribute to the dissemination of virus strains. These strains that can multiply in their host without causing disease are neglected when analyzing clinical samples, which are usually collected from patients with acute gastroenteritis symptoms. Second, viruses that are discharged in the environment through contaminated wastewater are subjected to diverse physical, chemical, and biologic inactivation or degradation factors (e.g., sunlight, wastewater treatment). These factors favor selection of the most persistent variant strains in the environment. Therefore, these strains have a higher probability of reaching and infecting persons through waterborne transmission. In that context, environmental samples may be considered a source of information about emerging waterborne viruses.

In conclusion, using long-term retrospective studies to analyze stored environmental and clinical samples may be a promising way of increasing our knowledge about the emergence of novel pathogens in waterborne disease transmission.

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Dr. Skraber is a postdoctoral fellow working currently with public health professionals at the National Institute for Public Health and the Environment, the Netherlands. His research interests include the detection of human viruses and bacteriophages by cell culture and molecular methods in different environmental samples such as water, sediment, and biofilms.

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Rats, Lice, and Zinsser

Gerald Weissmann*

Like many of my colleagues in academic medicine, I caught my first whiff of science from popular books about men and microbes. By the time we had finished high school, most of us had read and often reread Paul de Kruif's Microbe Hunters; Sinclair Lewis' Arrowsmith; and Rats, Lice and History by Hans Zinsser. It's hard nowadays to reread the work of de Kruif or Sinclair Lewis without a chuckle or two over their quaint locution, but Zinsser's *raffiné* account of lice and men remains a delight. Written in 1935 as a latter-day variation on Laurence Sterne's The Life and Opinions of Tristam Shandy, Zinsser's book gives a picaresque account of how the history of the world has been shaped by epidemics of louseborne typhus. He sounded a tocsin against microbes in the days before antibiotics, and his challenge remains meaningful today: "Infectious disease is one of the few genuine adventures left in the world. The dragons are all dead and the lance grows rusty in the chimney corner…. About the only sporting proposition that remains unimpaired by the relentless domestication of a once free-living human species is the war against those ferocious little fellow creatures, which lurk in dark corners and stalk us in the bodies of rats, mice and all kinds of domestic animals; which fly and crawl with the insects, and waylay us in our food and drink and even in our love" (1).

Figure. Hans Zinsser (1878–1940), author of Rats, Lice and History.

Despite the unwieldy subtitle "Being a study in biography, which, after twelve preliminary chapters indispensable for the preparation of the lay reader, deals with the life history of TYPHUS FEVER," Rats, Lice and History became an international critical and commercial success. Zinsser's romp through the ancient and modern worlds describes how epidemics devastated the Byzantines under Justinian, put Charles V atop the Holy Roman Empire, stopped the Turks at the Carpathians, and turned Napoleon's *Grand Armée* back from Moscow. He explains how the louse, the ubiquitous vector of typhus, was for most of human history an inevitable part of existence, "like baptism, or smallpox"; its habitat extended from hovel to throne. And after that Murder in the Cathedral, the vectors deserted Thomas à Becket: "The archbishop was murdered in Canterbury Cathedral on the evening of the twenty-ninth of December [1170]. The body lay in the Cathedral all night, and was prepared for burial on the following day…. He had on a large brown mantle; under it, a white surplice; below that, a lamb's-wool coat; then another woolen coat; and a third woolen coat below this; under this, there was the black, cowled robe of the Benedictine Order; under this, a shirt; and next to the body a curious hair-cloth, covered with linen. As the body grew cold, the vermin that were living in this multiple covering started to crawl out, and, as . . . the chronicler quoted, 'The vermin boiled over like water in a simmering cauldron, and the onlookers burst into alternate weeping and laughter...'"(1).

Zinsser's literary range and magpie intellect prompted reviewers of the day to compare him to an earlier Harvard Medical School author, Oliver Wendell Holmes. Indeed, the first few chapters of Rats, Lice and History sound a lot like Holmes' The Autocrat of the Breakfast Table. Both works offer clean drafts of political and poetic history "dipped from the running stream of consciousness"—to use Holmes' phrase, later made famous by William James. Rats, Lice and History is still in print, but it's playing to a different audience. A Yale academic worries that readers "may be puzzled by the acerbic references to the literary dandies of the interwar period" (2), while a scroll down Amazon.com yields another complaint: "Some of the writing assumes that all readers were educated under an aristocratic university system, so that there are bits thrown in Latin and Greek, not to mention French and other modern

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languages" (3). Be that as it may, Zinsser's assumption of an "aristocratic university system" did not prevent the book from becoming a best-seller in 1935 or from undergoing 75 subsequent printings. And as for those literary dandies: T.S Eliot? Gertrude Stein? Lewis Mumford? Edmund Wilson?

Zinsser followed up with As I Remember Him: The Biography of R.S., a third-person autobiography that survives as a distinguished work of literature. The R.S. in the title is an abbreviation of "Romantic Self," or the last letters, inverted, of Hans Zinsser's first and last names. In it, the author spells out his warm view of medicine as a learned profession: "There is in it a balanced education of the mind and of the spirit which, in those strong enough to take it, hardens the intellect and deepens the sympathy for human suffering and misfortune" (4).

As I Remember Him was written 2 years before Zinsser's death at age 61 of lymphatic leukemia in 1940. A selection of Book of the Month Club, it reached the best-seller list as its author lay dying. News of its warm reception by book reviewers filtered into the obituaries. The volume was widely popular among doctors of my father's generation; the copy I first read sat on his office shelves, wedged between Axel Munthe's The Story of San Michele and Romain Rolland's Jean-Christophe.

As I Remember Him tells the story of Zinsser and his cohort of American physicians, who "were more fortunate than they knew, because they were about to participate in a professional evolution with few parallels" (4). Zinsser lived to see American medicine develop from a "relatively primitive dependence upon European thought to its present magnificent vigor" (4). He describes how over the years the torch of medical science passed from England to France to Germany. The flow of American medical students followed its path, which had led in central Europe to "a powerful reaction of the basic sciences upon medical training and a true spirit of research [that] pervaded medical laboratories and clinics" (4). The Golden Age of German medicine ended abruptly in 1933 when, as Zinsser lamented, "common sense became counter-revolution."

Zinsser, a New Yorker, was of German liberal stock of the 1848 generation. "The spirit of this age of my German grandfathers was one of growing philosophical materialism and the *Freie Deutsche Gemeinde* (free German communities) or 'ethical culture societies' of the sort carried on for many years by Felix Adler" (4). Zinsser's father, a free-thinking industrial chemist, raised him in a cultivated Westchester home, with trips abroad, private tutors, music, and riding lessons. He prepped for Columbia at a small private school (now the Dwight School) on 59th Street, run on ethical culture lines by Dr. Julius Sachs. At Columbia College, he fell under the spell of George Woodberry, the Mark van Doren of his day, and became enchanted by the poet and essayist who had been hailed "the American Shelley" by James Russell Lowell. Woodberry introduced Zinsser to New England enlightenment and sparked his lifelong love affair with poetry.

Zinsser's Columbia years reinforced his distrust of credulity, later strengthened by the skepticism of German enlightenment as interpreted by H.L. Mencken. Heine, the skeptic, was the Zinsser family hero, and Zinsser often repeated the poet's final words. Asked to turn to God, that He might pardon him at death, Heine had replied, "God will forgive me. It's his business." It was in this spirit that Zinsser differed from William James. "I have been utterly incapable of that 'over-belief' which William James postulates as necessary to faith. Moreover, to give religious experience—as he does—a merely pragmatic value seems both to be begging the question and to be making light of a grave problem" (4).

At Columbia, Zinsser may have fallen under the spell of Woodberry in the humanities, but in science, his mentor was Edmund B. Wilson (no relation to the writer). Zinsser vastly admired Wilson, a founder of developmental genetics in the United States. Wilson and his assistants, Zinsser knew, were the direct spiritual offspring of the Darwinian period; they had known Haeckel and Huxley. Wilson himself had described how chromosomes divide before cell cleavage and are united in pairs in the new cells. Zinsser became a convert to experimental biology after many hours in Wilson's cytology laboratories and entered Columbia's College of Physicians and Surgeons in 1903. Two of his contemporaries in the class of 1904 were his future colleagues in bacteriology, Oswald Avery, who went on to prove that bare DNA was the stuff of genes, and Joseph Thomas, father of Lewis Thomas.

Zinsser cut his teeth on research while still in medical school, earning an M.A. in embryology and studying the effects of radium on bacterial survival. (I wonder about the relationship between radium research in those early days and Zinsser's leukemia). His record earned him a prized internship at Roosevelt Hospital, then the primary teaching hospital of Columbia. Zinsser and other house officers at Roosevelt lived in a small world of their own, sharing the hazing rituals of medical training and "walking out," as dating was then called, chiefly with Roosevelt nurses, among them Grace Peck: "An interne [sic] who doesn't sooner or later fall in love with a nurse is usually a depraved fellow" (4).

The Roosevelt house officers in those days worked the wards and clinics and rode ambulances. William Carlos Williams was also an intern at Roosevelt, and his accounts of Hell's Kitchen (5) jibe with those of Zinsser. Zinsser's tale of answering a 2 a.m. call to a tenement house could be set as the beginning of a Williams poem: I found a

man/Who had been shot in the chest./He was lying diagonally/across a small room/lighted by a single gas burner./He was bleeding heavily into his clothes/He also had a scalp wound/Which bled profusely./My business was to get him to the hospital …/I got him there alive … but he died" (4).

Zinsser picked up at Columbia what William Carlos Williams had acquired at Penn: the spirit of pragmatic humanism that flourished in the "aristocratic" American universities of John Dewey's era. Williams' "No ideas but in things" became an anthem of the generation. Its direct predecessor was James Russell Lowell's postbellum "Commemoration Ode." Zinsser would have seen a connection between William's plea for truth in things and Lowell's ode to veritas: "No lore of Greece or Rome/No science peddling with the names of things,/Or reading stars to find inglorious fates,/Can lift our life with wings … /But rather far that stern device,/The sponsors chose that round thy cradle stood/In the dim, unventured wood,/The Veritas that lurks beneath/The letters unprolific sheath…" (6).

Medical schools stopped peddling with the names of things and started looking for veritas in the laboratory soon after the Flexner report of 1910. "Oh, Abraham Flexner!" Zinsser intoned, "We hail you the father—or, better the uncle—of modern medical education" (4).[1] Flexner's exposé of the practitioner-dominated medical diploma mills that flourished at the turn of the century was the critical step in making American doctors members of a learned profession. Before then, it was all words, words, words and precious little experience; medical students sat on benches rather than working at them. After Flexner, American medical schools embarked on a century-long effort at empirical, laboratory-based medical instruction that became the envy of the world, "a phoenix rising." Zinsser would not be pleased that nowadays, as in 1933 Germany, common sense is again becoming a victim of counter-revolution. In our culture of HMOs, healthcare providers, insurance scams, and for-profit hospital chains, he'd worry that the phoenix of our youngest science might return to the "ill-smelling ashes of a big business" (4).

When their days at Roosevelt Hospital were over, William Carlos Williams became a pediatrician in Rutherford, New Jersey; Joseph Thomas entered medical practice in Flushing; and Zinsser became a microbe hunter in academia. In 1915, he accompanied the American Red Cross Sanitary Commission to investigate a devastating outbreak of typhus in Siberia. After much trial and error, he eventually succeeded in isolating the European form of the microbe that caused typhus and worked hard at developing a vaccine against it. He also moved rapidly into the new science of immunology (7).

Zinsser's scientific gifts were not limited to microbe hunting. He became a prolific medical writer and editor. His Textbook of Bacteriology went through many editions. From 1928 to 1940, he regularly published poems in the journal first edited by James Russel Lowell and named by Holmes, the Atlantic Monthly. Zinsser later explained that his cultural life bridged "the period between Emerson and Longfellow to T.S. Eliot and James Joyce" (7).

After faculty positions at Stanford and Columbia, Zinsser was appointed to the chair at Harvard in 1923. Harvard at the time was a phoenix rising and its dean, David Linn Edsall, could truly report to his trustees that "There can be little doubt that the school has acquired the standing of being the best place in the country and perhaps anywhere for advanced training in research and for advanced training of teaching and research personnel" (8).

Until the Flexnerian revolution came to Boston, indeed from the time of its founding in 1782, Harvard's Medical School had functioned chiefly as a cozy nursery for Yankee practitioners. In the days of Holmes' deanship (1847–1853), the school offered little encouragement for laboratory research to either faculty or students. In 1870, Holmes' clinical colleague James Clark White urged rigorous standards of laboratory and bedside teaching—matching those of Paris, Berlin, or Vienna—in Boston, at Harvard Medical School: "When I find the young men of Europe flocking to our shores and crowding our native students from their seats and from the bedside, when the fees of our best lecturers are mostly paid in foreign coin, and when thousands of wealthy invalids from across the sea fill the waiting-rooms of our physicians, then I will confess that I am wrong, and that of the two systems of education ours is the best. Until then I shall seek in the spirit and working of their schools the secret of their success, the cause of our failings" (9).

Its provincial air had prompted the taunt that Harvard was the best medical school in Boston. But by the 1920s, thanks to a new campus, a crop of young, full-time professors, and an ebullient Dean Edsall, innovation was in the air. Rote learning had largely yielded to learning by doing, electives had sprouted, experimental spirit had spread to the clinics, and Harvard was well on its way to becoming the best medical school in the country.

Edsall, a close friend and advisor to Flexner, assumed the deanship in 1918. He took medical education as a personal challenge and set about recasting Harvard into a world-renowned academic medical center. Frugal Edsall told of snaring Zinsser from New York, bragging that "we got this professor for $2,000 less than we could have

[1]For contributions of Greek culture to modern medicine, see the first Another Dimension published in Emerging Infectious Diseases (Myrianthopoulos NC. The philosophic origins of science and the evolution of the two cultures. Emerg Infect Dis. 2000; 6:77–82. Available from http://www.cdc.gov/ncidod/eid/vol6no1/myrianthopoulos.htm).

because of [his] personal income and because he wanted to come here" (8).

The "here" to which Edsall brought Zinsser in 1926 was the neoclassic campus north of the Charles. Its open piazza was framed by buildings well-endowed with laboratory space for students and faculty. Edsall's medical acropolis attracted Zinsser, Otto Folin, Edwin Cohn, and John Edsall (the dean's son) in biochemistry; Lawrence Henderson and Walter B. Cannon in physiology; and S. Burt Wolbach and Tracy Mallory in pathology. Among the clinicians were William B. Castle, Soma Weiss, Herman Blumgart, and Maxwell Finland. Other contemporaries included future Harvard Nobelists John F. Enders, Thomas Weller, George Minot, William P. Murphy, and George Hitchings. Zinsser was in tune with Edsall's social views; despite internal opposition, the dean had appointed Alice Hamilton (1869–1970), a pioneer of industrial medicine, first woman assistant professor, not only in the medical school but in all of Harvard University (10).

Zinsser's scientific career flourished at Harvard. His work on typhus carried him to Mexico and China. In Mexico, he went after the *Rickettsia prowasekii* that caused disease, and he worked out innumerable approaches to a vaccine. He also detailed the epidemiology of a recurrent variant of typhus in European immigrants (Brill-Zinsser disease). Zinsser's work on typhus in Serbia, Mexico, and China spelled it out: lice require dirty humans, bad weather, and crowding—as in tents and barracks. That's why typhus is the stuff of war and tragedies and has, as he predicted, outlived Hans Zinsser. During World War II, typhus spread through North Africa and the Pacific Islands and devastated central Europe, where it was the second leading cause of death in German concentration camps. On or about March 31, 1944, typhus killed Anne Frank in Bergen-Belsen, only 2 weeks before the British Army came in to stamp out the lice (11). American troops were protected by a vaccine based on the one developed by Zinsser and Castaneda and applied on a large scale. Although epidemic typhus declined at the end of World War II with the advent of DDT (12), *R. prowasekii* is making a comeback. The largest recent outbreak since World War II was in Burundi in the mid–90s, where modern molecular techniques were used to show that a single outbreak of "jail fever" sparked an extensive epidemic of louseborne typhus in the refugee camps of Rwanda, Burundi, and Zaire—countries racked by ongoing civil war and genocide (13). There was also a brisk outbreak in Russia in 1997. In Europe in the past and Africa today, persons who "recovered" from the epidemic typhus of their youth suffer relapses of Brill-Zinsser disease and have become a reservoir of new louse-borne epidemics (12).

Work on typhus was not Zinsser's only contribution. He was also a pioneer in the study of autoimmunity, our allergy to self induced by microbes. He was drawn to its study by the leading cause of cardiac disease in the interbellum years, rheumatic fever. Zinsser had been studying allergy to the streptococcus for several years, and in 1925 published a seminal hypothesis, now accepted wisdom. Entitled "Further Studies on Bacterial Allergy: Allergic Reactions to the Hemolytic Streptococcus," it argued that, "Failure to find the organisms themselves … suggested either a toxic or allergic pathogenesis. Such reasoning is especially applicable to the various forms of arthritis, in which it is at least logical to think of an allergic association" (14).

We now know that "autoimmune" reactions to the microbe are responsible, but we are still in the dark as to how the disease comes about. It remained for Zinsser's student, Albert Coons to put us on the right track. Zinsser would have been pleased. In brief, Coons found that if a fluorescent molecule were chemically hooked to purified antibody, this labeled antibody could be added to samples of tissue to find the suspected antigen (15). This powerful method, immunofluorescence, was discovered by Coons and Melvin Kaplan in the 1950s to test Zinsser's suggestion that rheumatic disease is due to what might be called friendly fire: our immune defenses against the microbe are launched against our own tissues because the strep and we share look-alike components. The immune system is therefore tricked into treating the host as if it were as invader that requires disposal. Zinsser laid the groundwork for much of what we know, or think we know, of rheumatic diseases today.

Zinsser was as fine a teacher as he was a scientist. His infectious enthusiasm for pure science brought him the best and brightest students, who eventually filled chairs of bacteriology, immunology, medicine, and public health the world over and transmitted Zinsser's broad, humanistic concerns to their students. Lewis Thomas was one of those vectors, and I confess that I get twinges of what might be called a Brill-Zinsser recurrence of sentiment when I'm asked about a lifetime of teaching in medical school: "… as we grow wiser we learn that the relatively small fractions of our time which we spend with well-trained, intelligent young men are more of a privilege than an obligation. For these groups are highly selected and they force a teacher continually to renew the fundamental principles of the sciences from which his specialty takes off. So while we are, technically speaking, professors, we are actually older colleagues of our students, from whom we often learn as much as we teach them" (4).

Zinsser died of a hematologic malignancy. The last chapter of As I Remember Him forecasts in stoic detail the events of Zinsser's terminal illness. He writes of himself in the third person: "As his disease caught up with him, R.S. felt increasingly grateful for the fact that death was com-

ing to him with due warning, and gradually. So many times in his active life he had been near sudden death by accident, violence, or acute disease . . . But now he was thankful that he had time to compose his spirit, and to spend a last year in affectionate and actually merry association with those dear to him" (4).

Zinsser's legacy is indelible in the two cultures of writing and science; his well-written books remain in print, and we live with his science. He taught us how we get typhus, created a successful vaccine against it, and told us how it can recur as Brill-Zinsser disease. He was the first to reckon that rheumatic diseases result from the friendly fire of our own armaments against microbes. But, his finest contribution was to warn us, from the field, from the podium, and in his writing, that "lice, ticks, mosquitoes and bedbugs will always lurk in the shadows when neglect, poverty, famine or war lets down the defenses" (1). The shadows remain.

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Emerging Infectious Diseases: 10 Years Running

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With this volume, Emerging Infectious Diseases (EID) turns 10—a time probably between childhood and adolescence in journal years. All of us associated with the journal are very proud of this milestone, but at the same time we are aware of the need to continually assess progress toward original goals and make changes when and where needed.

EID's genesis stems from advice to the Centers for Disease Control and Prevention (CDC) promulgated by the 1992 Institute of Medicine report, Emerging Infections: Microbial Threats to Health in the United States (1). EID was envisaged as a vital component of CDC's effort to communicate the threat of emerging infections worldwide. Fully and for the most part externally peer reviewed, the journal would have an international editorial board and would seek a global readership base. Its broad-based content would mirror the complex microbial, demographic, genetic, economic, technologic, behavioral, social, and other factors (including nefarious ones) that contribute to infectious disease emergence. The format would include views and summaries of major disease topics and trends, reports of new agents, reservoirs or foci of infection, and timely or preliminary results of relevant research. To make possible swift global dissemination of this compelling content, the journal would be fully electronic and be distributed free of charge, a concept being robustly discussed in 2005 by many editors and publishers under the topic of "open access." Special features were soon added to emphasize the links between science, public health, and the human condition.

If submissions and subscriptions are a measure of reader interest, Emerging Infectious Diseases has enjoyed some success during its first decade. Readership (infectious disease professionals in academia, clinical practice, industry, public health, and related disciplines) has grown from 3,000 subscribers in 1995 to >45,000 (print and online, >6,000 international). Submissions are increasing and number >120 per month. Although most submissions come from scientists in the United States and western Europe, manuscripts continue to arrive in substantial and increasing numbers from every part of the globe. The journal, which is widely indexed, has a consistently high impact factor (fourth most cited of 41 infectious disease journals, Institute of Scientific Information citation reports, 2003).

Electronic publishing, the dynamic concept driving Emerging Infectious Diseases, has made huge strides during the journal's first decade. Ahead-of-print publication, a founding feature of Emerging Infectious Diseases, is now common practice in the scientific journal community. Online-only publication of technical and lengthy but valuable parts of articles has also been adopted by many journals. Multiple links to references, databases, and other relevant information have become standard. Interactive features and instant access to authors and editors have revolutionized scientific dialog. EID's impulse to swiftly provide urgent information free of charge to a global audience has been reaffirmed by several recent movements toward free access to scientific data.

During its 10-year journey, Emerging Infectious Diseases has had some advantages. Providing unrestricted access to all content has allowed the journal to reach its intended audience of public health professionals around the globe. In-house production has made possible timely posting of articles on the Web as soon as they are cleared for publication. A small but uniquely competent and flexible production team, dedicated associate editors and editorial board, and thousands of volunteer expert reviewers have energized our journal's growing contribution to the public health community.

Like all anniversaries, our journal's 10th is a combination of reflecting on the past and looking into the future. From the public health perspective, the world's current situation is every bit as volatile as it was 10 years ago, perhaps even more so. New infectious diseases and etiologic agents (e.g., avian influenza, Nipah virus infection, SARS) continue to surface relentlessly. Other diseases are finding new niches. West Nile virus encephalitis, endemic to the Eastern Hemisphere, became established in the northeastern United

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States in 1999 and continues to spread each year. As predicted within the public health community, multidrug-resistant bacterial infections have become increasingly commonplace, yet development of new antimicrobial drugs has failed to keep pace. Against this backdrop of naturally occurring problems, intentional release of pathogenic organisms has surfaced as a threat to public health and global security. The Institute of Medicine revisited the issue they brought to the world's attention in 1992 with another report in 2003 (2). Their advice is not dissimilar to that of the Red Queen to Lewis Carroll's Alice in Wonderland: "…it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!" (3).

From the journal publishing perspective, continued emergence of new diseases and agents compels rapid dissemination of relevant scientific and public health information on the Web. As electronic access has become more commonplace, Web audiences have increased, and while in 1995 we had to educate readers on how to access our electronic journal, now we strive to keep up with their increased sophistication and need for additional research tools. In the ever-changing world of electronic publishing, speed is no longer the greatest challenge. Traditional publishing bottlenecks are diminishing. Web-based submission and peer review shrink the time from manuscript submission to publication. Online editing and production have advanced to the benefit of the final product. The question is no longer how fast information can be published but how content quality can keep up with technologic speed (4). When anthrax spores were disseminated through the U.S. postal system, Emerging Infectious Diseases published online a peer-reviewed article describing the first 10 cases. This definitive article (5), published online 5 days after submission, provided healthcare professionals the information needed to recognize potential new cases. When SARS burst onto the public health scene, Emerging Infectious Diseases published an entire issue with >40 peer-reviewed articles on a disease that 12 months earlier was not known to exist (see http://www.cdc.gov/ncidod/EID/vol10no2/contents_v10n2.htm). The technology that allowed these and other cases of expedited publication continues to advance. However, the ability to collect and interpret data on unknown diseases and uncharacterized agents follows a different timetable, in which quality must continue to take precedence over speed.

Electronic publishing provides solutions and poses challenges. Online-only publication for selected sections of journal materials may, in the short term, ease space constraints that threaten the journal's ability to remain inclusive. Expansive indexing will further refine electronic search capabilities, increase links to large electronic databases, and improve the pace and quality of emerging infections and other research. The breadth and complexity of online activities will continue to demand increased technical expertise and exceptional flexibility from all involved with the journal—authors, reviewers, editors, and production staff.

As Emerging Infectious Diseases begins its second decade, its primary goal remains to communicate the threat of emerging infections worldwide and reduce the global impact of these infections, particularly among the young, the old, and the immunocompromised. The principal difference between 1995 and now is the pace, both of current events and of expected publication. Our challenge for the coming decade is not only to keep up with an increasing pace, but also to set the pace whenever we can, or in the words of one track coach, "Start off as fast as you can, and then gradually pick up speed."

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Malaria and Travel to the Dominican Republic

To the Editor: The rise in international travel to malaria-endemic areas in recent years has been followed by an increase in the number of cases diagnosed in countries where malaria is not endemic (1). Tourist areas of the Dominican Republic have traditionally been considered to be low risk for malaria transmission. However, over the past few years, sporadic descriptions of imported falciparum malaria in travelers to these destinations have been described (2,3). In spite of these findings, neither the World Health Organization nor the Centers for Disease Control and Prevention recommend antimalarial chemoprophylaxis for trips to the Dominican Republic's main tourist resorts (4,5). We report a new case of imported malaria caused by mixed *Plasmodium vivax* and *P. malariae* infection, with unique clinical features, after a standard tourist trip to Puerto Plata (on the northern coast of the Dominican Republic).

A 31-year-old man with no relevant medical history was treated in the internal medicine department of our hospital. He reported a history of poorly defined malaise, night sweats, sleeplessness, tinnitus, and episodic diarrhea with no pathologic products during the previous 6 days. He did not report fever, chills, or headache. Two weeks earlier, he had spent 10 days in Puerto Plata in a tourist resort, without traveling to any other place. He had not received any antimalarial chemoprophylaxis. Physical examination showed no abnormalities. Laboratory values, including levels of sodium, potassium, liver enzymes, creatinine, and coagulation factors, as well as results of hemogram and chest radiograph, were within normal limits. A blood film showed trophozoytes of *P. vivax* and *P. malariae*. In a stool specimen, *Entamoeba histolytica*, *Trichiuris trichura*, *Endolimax nana*, and *Blastocystis hominis* were observed; stool cultures were negative.

Treatment was initiated with chloroquine (4 doses) and primaquine for a period of 14 days; metronidazole and paromomycin were administered for the intestinal infestations. Symptoms resolved in 48 hours, and control blood films showed clearance of the parasitemia. Two months after the end of treatment, the patient remained asymptomatic.

We describe a new and unusual case of imported vivax-malariae malaria. Two characteristics of our patient's case bear mention. First, the place of acquisition of the infection and the species of *Plasmodium* involved are notable. The Dominican Republic is considered a low-risk area for malaria, although some places in the west, on the Haitian border, are malaria-endemic. In addition, according to available information, autochthonous malaria cases increased after Hurricane George (3,003 cases in 1999, compared to 2,000 in 1998) (6). Previously described sporadic cases of imported malaria from the Dominican Republic included those in tourists who traveled to Punta Cana, in the eastern part of the country. All these cases were caused by *P. falciparum*. To our knowledge, no cases of *P. vivax* or mixed *P. vivax*/*P. malariae* infection have been described after travel to the Dominican Republic (2,3). From January 1999 to September 2003, TropNetEurop (a European surveillance network of tropical and imported diseases) noted 618 cases of *P. vivax* infection imported to Europe. The most common areas of acquisition of *P. vivax* infection were the Indian subcontinent (17%), Indonesia (12.1%), South America (11.4%), and West Africa (11.4%). Only 0.2% of the cases of *P. vivax* infection were acquired in the Caribbean, none of them in the Dominican Republic (7).

Second, the clinical features were atypical. Malaria usually starts as a febrile syndrome, accompanied by chills, headache, malaise, and arthromyalgia. However, sometimes symptoms are unspecific. In fact, ≤10% of patients do not exhibit fever or chills, and some report only poorly defined complaints or other atypical symptoms. Among these, gastrointestinal symptoms are the most frequently reported (8). In the present case, the syndrome could have been easily explained by the intestinal infestations detected in stool studies, and malaria would have been overlooked if the clinician had not taken into account this disease in the diagnostic workup.

In summary, clinicians should include malaria in the diagnostic workup of tourists who become ill after traveling to the Dominican Republic. Species other than *P. falciparum* may be the cause of the disease; these species likely induce more atypical forms of malaria.

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Buruli Ulcer Distribution in Benin

To the Editor: *Mycobacterium ulcerans* disease, commonly called Buruli ulcer, is an emerging infectious disease in West Africa (1,2). Several forms of Buruli ulcer exist; large, chronic ulcerations or indurated plaques of the skin are the most frequent manifestations of the disease (1), and bone is sometimes involved (3). Little is known about the focal epidemiology of Buruli ulcer; incidence, prevalence, and other data are usually reported at the national or district level (4). These data convey the importance of the disease but do not show the wide variations that occur at the village level within a given district. In 2002, we investigated the disease in an arrondissement (Gnizounmè) in an area in which Buruli ulcer is endemic, the commune of Lalo in Benin. Prevalence rates of Buruli ulcer varied from 0.58 to 32.62 per 1,000 inhabitants of villages in the same arrondissement. For Gnizounmè Arrondissement, the overall prevalence was 10.70 per 1,000 inhabitants. These results confirmed that distribution of Buruli ulcer must be determined at geopolitical divisions lower than district or national levels, as is frequently assumed to be the case.

An inverse relationship exists between the prevalence of Buruli ulcer and distance from the Couffo River, which drains the arrondissement of Gnizounmè. A comparison of the relevant data for Assogbahoué and Tandji villages shows that the number of patients per 1,000 inhabitants increases gradually from 0.58 to 32.62 as the distance from the river decreases from 10 to 1 km.

Recently, aquatic insects have been considered potential vectors of *M. ulcerans* (5,6). These aquatic insects can fly many kilometers from their source (7). This finding may partially explain how patients who live farther distances from their source of water become infected, but not as often as those who live closer. Some water bugs obtained from water collection points along the Couffo River in the village of Tandji were found to be positive for *M. ulcerans* by using PCR with specific insertion sequence *2404* as a target (8).

If we consider domestic water sources in the arrondissement of Gnizounmè, only Tandji (32.62 Buruli ulcer patients per 1,000 inhabitants) used water directly from the Couffo River. Other villages employed protected water sources for domestic purposes (boreholes, cisterns, or piped water from artesian wells). These results are similar to Barker's findings in Uganda, which showed that families who used unprotected sources of water for domestic purposes had higher prevalence rates of Buruli ulcer than those who used boreholes (9). Consequently, besides the possible influence of distance from the river on disease prevalence through potential vectors, such as insects or other factors, we hypothesize that the use of river water for domestic purposes may also play a role in the elevated prevalence of the disease in Tandji village. If this hypothesis is confirmed, preventive public health programs based on strategies that provide protected water supply systems to villages must be developed to reduce the frequency of the disease.

Determining the complex relationship between distance from the Couffo River and the numbers of cases and level of protection of water supply is difficult. Our findings argue for the need to perform additional epidemiologic studies to understand more completely the key factors that determine the distribution of the disease in the entire commune of Lalo.

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Mycobacterium tuberculosis Drug Resistance, Abkhazia

To the Editor: Drug-resistant tuberculosis (TB) has been identified as a major problem in the former Soviet Union, and was recently surveyed in the Aral Sea regions of Dashoguz (Turkmenistan) and Karakalpakstan (Uzbekistan) (1). However, few data are available for the Caucasian region and published reports have focused mainly on prisons (2,3).

We report a drug resistance survey for first- and second-line anti-TB drugs conducted in Abkhazia, a Caucasian region of 8,600 km^2 with approximately 250,000 inhabitants, at the western end of Georgia on the Black Sea. The collapse of the Soviet Union lead to disruption of TB control activities in all Eastern bloc regions (4). In Abkhazia, the shortage and poor quality of drugs, self-medication, and poor adherence to the therapy became even more evident during the war with Georgia in 1993 and the international embargo that followed. A TB program based on the World Health Organization/International Union against Tuberculosis and Lung Disease (WHO/IUATLD) recommendations was initiated in Abkhazia with the support of Médecins Sans Frontières (MSF) in 1999. In 2000, monitoring of drug resistance was started for new cases and previously treated case-patients. The study was performed in collaboration with the Guliripchi TB Hospital, MSF, and the Istituto Superiore di Sanità (ISS), a WHO/IUATLD Supranational Reference Laboratory for anti-TB drug resistance.

Sputa were collected from all case-patients attending Gulirip-chi TB Hospital in Sukhumi, the capital of Abkhazia, from September 2000 to April 2004. Patients were either referred by their practitioners or came spontaneously because TB was suspected. Diagnosis, treatment, and hospitalization were provided free. Samples were treated as previously described (5). Of 489 sputa collected from individual patients, 447 were culture positive (246 from new case-patients and 201 from previously treated case-patients) and 42 were culture negative; of these, >90% showed a negative, doubtful, or 1+ smear result. Susceptibility to first-line (streptomycin, isoniazid, rifampin, and ethambutol) and second-line (kana-mycin, ethionamide, capreomycin, cycloserine, *p*-aminosalicylic acid, and ofloxacin) drugs was determined by the proportion method on Middlebrook 7H10 agar. The critical concentrations used were streptomycin, 2 μg/mL; isoniazid, 0.2 μg/mL; rifampin, 1 μg/mL; ethambutol, 5 μg/mL; kanamycin, 5 μg/mL; ethionamide, 5 μg/mL; capreomycin, 10 μg/mL; *p*-aminosalicylic acid, 2 μg/mL; and ofloxacin, 2 μg/mL (6–8). Cycloserine was used at a concentration of 30 μg/mL (9). If a strain was resistant to ≥1 first-line drugs, the susceptibility to all second-line drugs was determined.

Data on resistance to the first- and second-line drugs are given in the Table. The strains isolated from 35.8% of the new case-patients and 57.2% of the previously treated case-patients were resistant to ≥1 first-line drugs. The highest monoresistance was seen for isoniazid and streptomycin in both new and previously treated case-patients while monoresistance to rifampin and ethambutol was low (<1%). Multidrug-resistant (MDR) strains (i.e., strains resistant to at least isoniazid and rifampin) were observed in 4.9% of the new cases and 25.4% of the previously treated case-patients. Strains resistant to isoniazid and streptomycin were isolated from 6.9% of the new cases and 8% of the previously treated case-patients. Resistance to second-line drugs was high (15.9% in new cases and 35.7% in previously treated case-patients), with the highest values being observed for kanamycin (4.5% in new cases and 21.7% in previously treated case-patients) and ethionamide (8% in new cases and 16.5% in previously treated case-patients). Twenty-five percent and 52.9% of the MDR strains isolated from new and previously treated case-patients, respectively, showed resistance to ≥1 second-line drugs.

Few data have been reported on drug resistance to first- and second-line drugs in the former Soviet Union and in the Caucasian region (1–4). Overall, in Abkhazia, monoresistance to isoniazid was higher than in Karakalpakstan and Dashoguz (1), while monoresistance to streptomycin was lower. MDR-TB in new and previously treated case-patients showed levels intermediate between these 2 regions. Resistance to kanamycin

and ethionamide was 14.3% and 12.8%, respectively, while resistance to ofloxacin was low (1.5%). Fluoroquinolones have not been commonly used in Abkhazia and former regions of the Soviet Union. Currently, regimens for the treatment of MDR-TB in Abkhazia combine an intensive phase for a minimum of 6 months with at least 4 drugs to which the MTB strain is susceptible, including 1 parenteral agent and 1 fluoroquinolone (ofloxacin), followed by a continuation phase of at least 15 months with ≥ 3 drugs.

This is the first survey reporting drug susceptibility data for MTB within the Caucasus. It indicates that the prevalence of MDR strains is similar to that in other central Asia regions (1). Our results are representative of the present situation in Abkhazia since sampling systematically covered all TB cases for the period examined. The Guliripchi TB Hospital of Sukhumi is the only TB treatment center in the region, and all cases were included in the study. Overall, our data show that second-line drug resistance is present in Abkhazia, particularly among cases with MDR, and suggest the adoption of strategies for access and correct use of second-line drugs (10).

Table. First-line and second line antituberculosis drug resistance in 447 *Mycobacterium tuberculosis* strains collected in Abkhazia from September 2000 to April 2004*

	No. new cases (%)	No. previously treated cases (%)	Total no. (%)
Total tested	246 (100)	201 (100)	447 (100)
Any first-line resistance	88 (35.8)	115 (57.2)	203 (45.4)
Monoresistance			
H only	31 (12.6)	28 (13.9)	59 (13.2)
S only	20 (8.1)	11 (5.5)	31 (6.9)
R only	1 (<1)	1 (<1)	2 (<1)
E only	2 (<1)	1 (<1)	3 (<1)
Any drug resistance			
Any H resistance	65 (26.4)	102 (50.7)	167 (37.4)
Any S resistance	51 (20.7)	80 (39.8)	131 (29.3)
Any R resistance	13 (5.3)	52 (25.9)	65 (14.5)
Any E resistance	14 (5.7)	35 (17.4)	49 (11)
H and R resistance			
MDR†	12 (4.9)	51 (25.4)	63 (14.1)
HRES only	7 (2.8)	24 (11.9)	31 (6.9)
HRS only	4 (1.6)	22 (10.9)	26 (5.8)
HRE only	0	3 (1.5)	3 (<1)
HR only	1 (<1)	2 (<1)	3 (<1)
H + other resistances			
HS only	17 (6.9)	16 (8)	33 (7.4)
HES only	3 (1.2)	7 (3.5)	10 (2.2)
HE only	2 (<1)	0	2 (<1)
R + other resistances (RE, RS, or RES only)	0	0	0
Total tested to second-line drugs	88 (100)	115 (100)	203 (100)
Any second-line resistance	14 (15.9)	41 (35.7)	55 (27.1)
KM	4 (4.5)	25 (21.7)	29 (14.3)
ETH	7 (8)	19 (16.5)	26 (12.8)
CM	3 (3.4)	6 (5.2)	9 (4.4)
PAS	2 (2.3)	4 (3.5)	6 (3)
OFL	0	3 (2.6)	3 (1.5)
CS	0	0	0
Total MDR strains resistant to second-line drugs‡	3 (25)	27 (52.9)	30 (47.6)
MDR + KM	2 (16.7)	10 (19.6)	12 (19)
MDR + ETH	0	5 (9.8)	5 (7.9)
MDR + KM + ETH	0	5 (9.8)	5 (7.9)
MDR + others§	1 (8.3)	7 (13.7)	8 (12.7)

*H, isoniazid; S, streptomycin; R, rifampin; E, ethambutol; KM, kanamycin; ETH, ethionamide; CM, capreomycin; PAS, *p*-aminosalicylic acid; OFL, ofloxacin; CS, cycloserine.
†MDR, multidrug resistant (resistant to at least H and R).
‡Values in parenthesis are the percentages of MDR strains.
§For new cases: MDR + KM + CM + PAS (1 strain); for previously treated cases: MDR + KM + CM (2 strains), MDR + KM + PAS (1 strain), MDR + KM + ETH + CM (1 strain), MDR + ETH + OFL (1 strain), MDR + PAS (1 strain), MDR + PAS + CM (1 strain).

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Endogeneity in Logistic Regression Models

To the Editor: Ethelberg et al. (1) report on a study of the determinants of hemolytic uremic syndrome resulting from Shiga toxin–producing *Escherichia coli*. The dataset is relatively small, and the authors use stepwise logistic regression models to detect small differences. This indicates that the authors were aware of the limitations of the statistical power of the study. Despite this, the study has an analytic flaw that seriously reduces the statistical power of the study.

An often overlooked problem in building statistical models is that of endogeneity, a term arising from econometric analysis, in which the value of one independent variable is dependent on the value of other predictor variables. Because of this endogeneity, significant correlation can exist between the unobserved factors contributing to both the endogenous independent variable and the dependent variable, which results in biased estimators (incorrect regression coefficients) (2). Additionally, the correlation between the dependent variables can create significant multicollinearity, which violates the assumptions of standard regression models and results in inefficient estimators. This problem is shown by model-generated coefficient standard errors that are larger than true standard errors, which biases the interpretation towards the null hypothesis and increases the likelihood of a type II error. As a result, the power of the test of significance for an independent variable X_1 is reduced by a factor of $(1-r^2_{(1|2,3,....)})$, where $r_{(1|2,3,....)}$ is defined as the multiple correlation coefficient for the model $X_1 = f(X_2,X_3,\ldots)$, and all X_i are independent variables in the larger model (3,4).

The results of this study clearly show that the presence of bloody diarrhea is an endogenous variable in the model showing predictors of hemolytic uremic syndrome, in that the diarrhea is shown to be predicted by, and therefore strongly correlated with, several other variables used to predict hemolytic uremic syndrome. Similarly, Shiga toxin 1 and 2 (*stx1*, *stx2)* genes are expected to be key predictors of the presence of bloody diarrhea, independent of strain, due to the known biochemical effects of that toxin (5,6). Because the strain is in part determined by the presence of these toxins, including both strain and genotype in the model means that the standard errors for variables for the Shiga-containing strains and bloody diarrhea symptom are likely to be too high, and hence the significance levels (p values) obtained from the regression models are higher than the true probability because of a type I error.

This flaw is a particular problem with studies that use a conditional stepwise technique for including or excluding variables. The authors note that they excluded variables from the final model if the significance in initial models for those variables was less than an α level (p value) of 0.05. Given the inefficiencies due to the endogeneity of bloody diarrhea, as well as those that may result from other collinearities significant predictors were likely excluded from the study, although this cannot be confirmed from the data presented.

The problems associated with the endogeneity of bloody diarrhea can be overcome by a number of approaches.

For example, the simultaneous equations approach, such as that outlined by Greene (7), would have used predicted values of bloody diarrhea from the first stage of the model as instrumental variables for the actual value in the model for hemolytic uremic syndrome. Structural equations approaches, such as those suggested by Greenland (8), would also be appropriate. However, bloody diarrhea is not the only endogenous variable in their models, and extensive modeling would be necessary to isolate the independent effects of the various predictor variables. Given the small sample size, this may not be possible.

The underlying problem in the study is the theoretical specifications for the model, in which genotypes, strains, and symptoms are mixed, despite reasonable expectations that differences in 1 level may predict differences in another. For example, the authors' data demonstrate that all O157 strains contain the *stx*$_2$ gene and have higher rates of causing hemolytic uremic syndrome and bloody diarrhea. This calls into question the decision to build an analytic model combining 3 distinct levels of analysis. Such a model depends on the independence of the variables to gain unbiased, efficient estimators. The model of the relationships one would develop from a theoretical perspective would predict the opposite (Figure). We expect that the genotypes (by definition) will predict the strain, and that strains have a differential effect on symptoms. The high level of inter-variable correlation due to these relationships, coupled with the decision to exclude variables based on likely inefficient p values, raises questions concerning the reliability of the results and conclusions. In particular, the conclusions that strains O157 and O111 are not predictors of hemolytic uremic syndrome deserve to be revisited; other excluded variables may also be significant predictors when considered under an appropriate model. These problems point to the need to ensure proper specification of analytic models and to demonstrate due regard for the underlying assumptions of statistical models used.

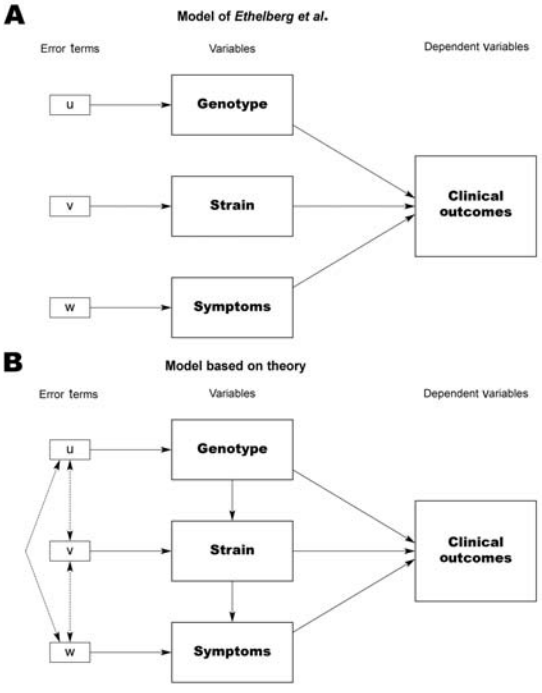

Figure. Model for determining virulence factors for hemolytic uremic syndrome

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In response: We appreciate Avery's interest (1) in our article (2), although we believe the critique of the methods is largely based on misunderstandings. We developed a model for the risk of progression to hemolytic uremic syndrome (HUS) containing 3 variables: whether the infecting Shiga toxin–producing *Escherichia coli* isolate had the *stx*$_2$ gene, age of the patient, and occurrence of bloody diarrhea. The critique relates to the fact that bloody diarrhea and *stx*$_2$ are not independent, since we showed that *stx*$_2$ was strongly associated with progression to HUS (odds ratio [OR] =

18.9) and also weakly associated with development of bloody diarrhea (OR = 2.5) (2). Avery uses the term endogeneity as it is used in econometric analyses; however, the term "intermediary variable," i.e., a factor in the causal pathway leading from exposure to disease, is more frequently used in epidemiology. In this context, we chose to consider bloody diarrhea as a potential confounder (3). A confounder is a risk factor but is also independently associated with the exposure variable of interest and is not regarded as part of the causal pathway (see online Figure at http://www.cdc.gov/ ncidod/EID/vol 11no03/05-0071-G.htm). Bloody diarrhea may act as a confounder if patients with bloody stools are treated differently by the examining physicians or if, for instance, unknown virulence factors contribute to the risk of having bloody stools.

A second line of critique of our methods apparently develops from the idea that virulence factors determine the serogroup. This idea, however, is a biological misconception. In fact, virulence genes and serogroup are independent at the genetic level, and an important point of our article is that HUS is determined by the virulence gene composition of the strain rather than the serogroup.

Regardless of the status of the bloody diarrhea variable, excluding it from the model doesn't change the conclusions of the article. A revised model contains only the significant variables age and stx_2 (Table). Serotype O157 is still not an independent predictor of HUS, and this result is robust.

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Table. Risk factors for HUS among 343 STEC patients, Denmark 1997–2003, comparison of models with and without bloody diarrhea as a variable*

Determinant	No. of patients	No. (%) with HUS	Original model, OR (95% CI)	New model, OR (95% CI)
eae				
Negative	111	0 (0.0)		
Positive	232	21 (9.1)	NI	NI
stx_2				
Negative	159	1 (0.6)	1	1
Positive	184	20 (10.9)	18.9 (2.4–146)	24.6 (3.2–187)
Age				
≥8 y	178	3 (1.7)	1	1
≤7 y	165	18 (10.9)	11.4 (3.2–41.3)	9.7 (2.7–34.1)
Bloody diarrhea				
No	218	6 (2.8)		
Yes	125	15 (12.0)	4.5 (1.6–12.7)	EX
O157				
No	262	10 (3.8)		
Yes	81	11 (13.6)	NS	NS

*HUS, hemolytic uremic syndrome; STEC, Shiga toxin–producing *Escherichia coli*; OR, odds ratio; CI, confidence interval; NI, not included (test not appropriate); NS, not significant; EX, excluded from model.

Rectal Lymphogranuloma Venereum, France

To the Editor: Lymphogranuloma venereum (LGV), a sexually transmitted disease (STD) caused by *Chlamydia trachomatis* serovars L1, L2, or L3, is prevalent in tropical areas but occurs sporadically in the western world, where most cases are imported (1). LVG commonly causes inflammation and swelling of the inguinal lymph nodes, but it can also involve the rectum and cause acute proctitis, particularly among men who have sex with men. However, LGV serovars of *C. trachomatis* remain a rare cause of acute proctitis, which is most frequently caused by *Neisseria gonorrhoeae* or by non-LGV *C. trachomatis* (2). In 1981, in a group of 96 men who have sex with men with symptoms suggestive of proctitis in the United States, Quinn et al. found that 3 of 14 *C. trachomatis* infections were caused by LGV serovar L2 (3). In France, 2 cases of rectal LGV were reported in an STD clinic in Paris from 1981 to 1986 (4). In 2003, an outbreak of 15 rectal LGV cases was reported among men who have sex with men in Rotterdam; 13 were HIV-infected, and all reported unprotected sex in neighboring countries, including Belgium, France, and the United Kingdom (5). At the same time, a rise in *C. trachomatis* proctitis (diagnosed by using polymerase chain reaction [PCR]; [Cobas Amplicor Roche Diagnostic System, Meylan, France]) was detected in 3 laboratories in Paris and in the *C. trachomatis* national reference center located in Bordeaux. To identify the serovars of these *C. trachomatis* spp., all stored rectal specimens were analyzed by using a nested *omp*1 PCR-restriction fragment length polymorphism assay. The amplified DNA product was digested by restriction enzymes. Analysis of digested DNA was performed by elec-

trophoresis. Patterns were compared visually with reference patterns (6).

From January 1, 2003, to March 31, 2004, a total of 44 of 124 male rectal swabs were positive for *C. trachomatis*. Of those, 38 were identified as belonging to the L2 serotype, which confirms the diagnosis of rectal LGV. Epidemiologic information was retrospectively obtained by clinicians through review of medical records, telephone interview, or both. A complete history was available for 14 of the 38 cases. All 14 men reported unprotected anal sex with anonymous male sex partners in France, and none reported a stay in an LGV-endemic area. Their mean age was 40 years (31–50); 8 were HIV-infected, and 9 had another concomitant STD. The mean duration of symptoms before LGV diagnosis was 50 days (range 11–120 days). All 14 patients had symptoms of acute proctitis, including rectal pain, discharge, and tenesmus, and 3 (all HIV-infected) had fever. Deep, extended rectal ulcerations were reported in 8 patients, 3 of whom were HIV-infected and had lesions suggestive of rectal carcinoma. In 1 patient in whom a late diagnosis was made 4 months after the onset of symptoms, a rectal tumorlike stricture was observed. All 14 patients were treated with tetracycline for a mean duration of 16 days (range 10–60 days).

An information campaign among microbiologists and clinicians and a sentinel LGV surveillance system were launched in April 2004. Subsequently, LGV was diagnosed in 65 additional male patients, some retrospectively. In total, rectal LGV was diagnosed in 103 patients from July 2002 to August 2004 (Figure).

Prompt diagnosis and treatment is indeed paramount to prevention and control. Diagnosis may be further hampered because rectal LGV may mimic other conditions such as rectal carcinoma or Crohn disease. Treatment duration should be no shorter than 21 days, and follow-up examinations should be conducted until all signs and symptoms have resolved (7,8). If left untreated, rectal LGV could lead to serious complications such as rectal stricture (1). If recently exposed to infection, sexual contacts should receive prophylactic treatment to prevent reinfection and to eliminate a potential reservoir. The emergence of rectal LGV, characterized by deep mucosal ulcerations and frequently occurring in HIV-infected men who have sex with men, is a serious concern for the gay community in Europe.

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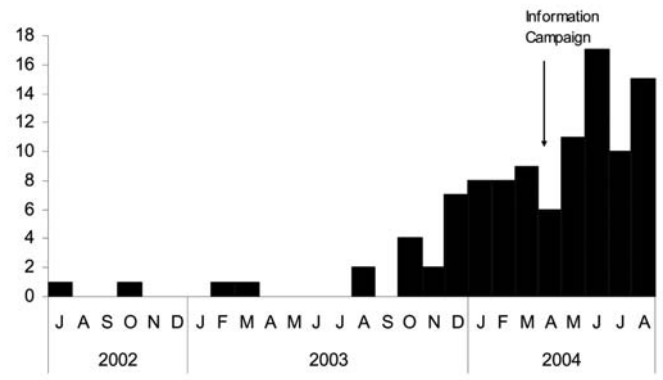

Figure. Number of rectal lymphogranuloma venereum cases diagnosed in men in France, July 2002–August 2004.

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Pertussis in Soldiers, Israel

To the Editor: The role of adults as reservoirs of pertussis was previously well established (1–7). Young army recruits undergoing basic train-

ing in the Israeli Defense Force constitute a unique adult population because of their special living and service conditions. This and the fact that they are not vaccinated with the diphtheria, tetanus, and pertussis (DTP) vaccine after the age of 1 year (unlike children in most of the Western countries) led us to hypothesize that this semiclosed population may have an exceptionally high risk for pertussis. These young soldiers are on leave on weekends, during which time they come in close contact with susceptible family members, including young infants, and may thus facilitate the "import" and "export" of pertussis between the military setting and the general population. An outbreak of pertussis that recently occurred among infantry soldiers (8) indicated the need to conduct the present study, in which we sought to evaluate the prevalence and incidence of pertussis among young soldiers in the Israeli Defense Force.

We conducted 2 concurrent studies. The first was a 15-month (November 2001–March 2003), laboratory-based surveillance study of pertussis, which included 110 trainees who complained of persistent coughing (case definition: cough lasting 10–90 days) upon admission to compound clinics. Samples obtained from these patients were tested by a commercial enzyme-linked immunosorbent assay (ELISA) (PanBio, East Brisbane, Queensland, Australia) for the presence of immunoglobulin (Ig) A against a *Bordetella pertussis* sonicate and by an in-house IgA-detection ELISA directed against pertussis toxin as previously described (9). Results for IgA to *B. pertussis* sonicate were calculated as arbitrary ELISA units obtained according to the manufacturer's instructions. A result of 9 U (a cutoff point that was previously shown to provide a 98.5% specificity for the diagnosis of recent pertussis infection ([8]) or higher was considered positive. Results for IgA to pertussis toxin were calculated as arbitrary ELISA units according to a calibration curve of a serially, double-diluted, positive standard. The cutoff point was calculated by adding 3 standard deviations to the mean value of a group of 40 healthy study participants. A positive result in either test was considered a confirmed case of pertussis.

We conducted another substudy to estimate incidence. This substudy included 278 trainees who were interviewed regarding the occurrence of persistent cough and seeking of medical care during the preceding 6 months. We multiplied the prevalence of laboratory-confirmed pertussis found in the first study by the incidence of study participants with persistent cough who sought medical care in this study. The result was multiplied by 200,000 to receive incidence estimation for 100,000 person-years.

The median duration of cough among the 110 case-patients was 14 days, their median age was 19 years, 94 (85.5%, 95% confidence interval [CI] 77.5%–91.5%) were males, 71 (64.5%, 95% CI 54.9%–73.4%) were born in Israel, and 85 (77.3%, 95% CI 68.3%–84.7%) were in basic training when they visited the clinic. Twenty (18.2%, 95% CI 11.5%–26.7%) and 14 (12.7%, 95% CI 7.1%–20.4%) of the patients were positive for IgA antibodies to *B. pertussis* and pertussis toxin, respectively. Twenty-five patients (22.7%, 95% CI 15.3%–39.7%) were positive by either test. Significant variations were recorded during the follow-up period. The first period (November 2001–May 2002) was characterized by a high prevalence of pertussis among the 72 patients enrolled, with 19 (26.4%, 95% CI 16.7%–38.1%) and 14 (19.4%, 95% CI 11.1%–30.5%) positive for IgA to *B. pertussis* and pertussis toxin, respectively. In the second period (August 2002–March 2003), although characterized by the same median duration of cough (14 days), a substantially lower prevalence of pertussis was observed among the 38 patients enrolled, with only 1 (2.6%) and 0 patients, respectively, showing positive results in either of the 2 tests ($p < 0.01$ for differences between the 2 periods for both diagnostic methods).

The frequency of clinical symptoms observed in patients positive for pertussis by at least 1 ELISA (n = 25) was similar to those observed in patients negative for pertussis by both ELISAs (n = 85), with the exception of post-tussive emesis (40% versus 25%, respectively) and fever (4% versus 21%). These differences were not significant.

Of the 278 basic training respondents, 17 (6.1%, 95% CI 3.6–9.6%) reported a persistent cough (>2 weeks) during the preceding 6-month period; 13 (4.7%, 95% CI 2.5%–7.9%) had sought medical care. When we extrapolated from this sample and from the laboratory-confirmed prevalence of 22.7% among patients with persistent coughing who sought medical care (and thus came to the clinics), the incidence rate was 2,132 cases per 100,000 person-years (95% CI 440–6,240).

The prevalence of pertussis found in this study is comparable with that previously reported among U.S. Marine corps trainees, university students, and other civilian adult populations (1–7). Complete case-capturing could not be performed. However, the high clinical similarity between pertussis-positive and other cases of prolonged cough renders selection bias unlikely. The prevalence of disease in this study showed significant changes in relation to time in contrast to previous studies (3). This difference may be because our present study was conducted in a semiclosed population characterized by epidemic occurrence of the disease.

The incidence of pertussis reported in this study (2,132 cases per 100,000

person-years) is substantially higher than the findings of Nennig and others (10) among an urban population in San Francisco (176 cases per 100,000 person-years). This finding may be due to the difference in immunization practices between Israel and the United States (5 doses of DTP vaccine in the United States with the last 1 administered between the ages of 4 and 6 years, compared with only 4 doses of the vaccine during the first year of life in Israel) or to crowded living conditions of the recruits. Our findings emphasize the need for revaccination against pertussis of young adults in Israel, primarily of those at high risk for pertussis, such as army recruits.

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Food Safety for First Responders

To the Editor: Relatively few published reports of intentional food contamination are available (1–5). However, after the anthrax attacks of 2001, biologic terrorism vulnerability assessments have determined that intentional food poisoning is a plausible means to widely disseminate pathogens, with potentially devastating effect (6). As a consequence, food security has emerged as one of the major priorities for bioterrorism preparedness (7–10). We describe a naturally occurring incident that demonstrates the potential for premeditated food contamination to target specific populations who are critical for protecting public safety, in this instance, a city police force. Measures to mitigate the risk of this scenario type are provided.

On the evening of December 19, 1998, local hospital emergency departments notified the Hawaii Department of Health that an unusually high number of police officers were coming to the hospitals with acute gastroenteritis. Earlier that day, several police-affiliated support associations had cosponsored a holiday event. The food was catered by a food service establishment that routinely provided meals at the police department headquarters in Honolulu. Active and retired police officers and their family members participated in the event. The food service at the event consisted of "bento lunch boxes" containing luncheon meat, a hot dog, teriyaki beef, fried chicken, and rice. Approximately 1,100 lunches were distributed at the event, some of which were taken offsite to be consumed by others, including on-duty officers at satellite police stations.

Interviews were conducted with a convenience sample of 394 persons who ate the bento lunch, of which 145 (37%) reported becoming ill with diarrhea (81%) or vomiting (80%). Illness onset occurred a mean of 4 hours after eating the lunch (1.5–8 hours); the mean duration of illness was 14 hours (2–96 hours). Incapacitation of police officers and their family members as a result of the illness was substantial with 25% absent from work for a half day, 42% for 1 full day, 18% for 2 days, and 15% for more than 2 days. *Staphylococcus aureus* was recovered from 7 of 8 stool specimens, of which 6 were positive on *S. aureus* toxin testing. Analyses of the lunch items found between 18 million to 3 billion *S. aureus* colonies per gram in the implicated foods. Luncheon meat, teriyaki beef, and hot dogs were positive on *S. aureus* toxin assays. *S. aureus* isolates obtained from food and clinical specimens were indistinguishable by pulsed field-gel electrophoresis. The quantity of lunch boxes produced for this holiday event exceeded that which the catering facility routinely produced while adhering to recommended food-han-

dling guidelines. A facility inspection and review of the caterer's procedures identified improper holding temperatures for potentially hazardous foods as the likely cause of the outbreak.

In this incident, prompt action by the police department, which employed an agency-wide radio communications system to warn officers not to eat lunches obtained for later consumption, reduced the number of persons who would have become ill from staphylococcal foodborne infection. In spite of this effort, the outbreak still had a considerable impact on the staff of the police department. The attack rate for those exposed was high, and three quarters of those who became ill missed ≥1 days of work. If an agent causing greater severity of illness, e.g., botulinum toxin, had been introduced into the bento lunches instead of *S. aureus*, the ensuing outbreak might have strongly compromised the department's ability to ensure public safety.

Despite modifications in food security industry regulations because of the Bioterrorism Act of 2002, intentional contamination of food items on a smaller scale remains a potential danger that needs to be addressed. As the incident we described demonstrates, under certain circumstances, terrorists may be able to substantially impair first response agencies, including police departments, through a limited but targeted foodborne attack. By incapacitating first responders, terrorists might maximize the impact of a larger, coordinated event. Just as maintaining the physical security of the Strategic National Stockpile is a priority in preventing a secondary attack, reasonable steps must be taken to ensure that our emergency workforce is protected from a targeted foodborne assault.

As a result of this outbreak, we have recommended that whenever entire units, departments, or shifts of first responders in our jurisdiction are involved in shared dining activities, efforts should be made to obtain food items from more than 1 caterer for each meal. If departmentwide events necessitate using a single caterer, efforts should be taken to identify and mitigate the threat of intentional food tampering and there should be rigorous adherence to standard safe food-handling procedures to minimize the potential for naturally occurring outbreaks (7). Our recommendations here are similar to those employed by airlines to protect pilots and copilots on long flights by serving separate meals prepared in different kitchens (11).

Our intention is to share with other preparedness agencies our observation that first response assets might be compromised by something as seemingly innocuous as a holiday party. Appropriate planning may reduce the risk of intentional food contamination that targets security forces or first responders, either as an isolated strike or as part of a larger, coordinated terrorist attack.

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Rickettsiae in Ixodid Ticks, Sicily

To the Editor: Members of the spotted fever group rickettsiae are intracellular bacteria usually associated with ixodid ticks, which are transferred to vertebrates by salivary secretions and within ticks transtadially and transovarially. Several tickborne rickettsiae cause human or animal diseases and, in the last 10 years, the increased use of molecular-based

identification methods has resulted in new spotted fever group rickettsiae being characterized in ixodid ticks throughout Europe (1). Until recently, no rickettsiae, other than *Rickettsia conorii*, were reported in Italy. Since 2002, *R. helvetica* and Israeli spotted fever *Rickettsia* (*R. conorii* complex) have been detected in *Ixodes ricinus* and *Rhipicephalus sanguineus*, respectively (2–4). In Italy, Mediterranean spotted fever is endemic. This disease appears to occur more commonly in some central and southern regions (5); in 2002, more than half (498 of 890) of the cases of Mediterranean spotted fever identified in Italy and reported to the Ministry of Health came from Sicily.

Because of the relatively high prevalence of rickettsial diseases in southern regions, we analyzed tick samples collected during 2001 and 2002 from herbivores (bovines, ovines, donkeys) from Sicilian farms in Corleone (Palermo Province) to determine the diversity of spotted fever group rickettsiae in various ixodid tick species. DNA from 238 tick samples from various genera (*Dermacentor*, *Rhipicephalus*, *Hyalomma*, *Haemaphysalis*, *Ixodes*) was extracted; in some cases, individual ticks of the same species collected from the same animal were pooled. Polymerase chain reaction screening and sequencing with primers for the gene encoding the cell surface antigen (*sca*4) (previously known as "gene D") and the 17-kDa antigen gene were performed as previously reported (6,7). A total of 7 positives were found, and the sequences obtained were compared to other bacterial sequences present in the GenBank database (Table). A 469-bp fragment with 100% identity to the *R. slovaca* sca4 sequence (AF155054) was obtained from 2 *Dermacentor marginatus* and 1 *Haemaphysalis punctata*. A 403-bp fragment with 99.75% identity (1-bp difference) to the sca4 sequence from *R. africae* (AF151724) was found from 1 *Hyalomma marginatum*, and a 423-bp fragment with 100% similarity to *R. conorii* sca4 sequence (AE008626) was found from *Rhipicephalus turanicus*. Finally, a 489-bp fragment with 99.79% identity (1-bp difference) to *R. aeschlimannii* sca4 sequence (AF163006) was obtained from 2 *H. marginatum* samples. The levels of identity between the 17-kDa antigen sequences (ranging in length from 351 to 419 bp) obtained during this study and those in GenBank were generally lower than those for sca4 because the 17-kDa antigen gene has not been sequenced for most of the *Rickettsia* spp. identified here on the basis of sca4 sequences. One exception was the fragment obtained from the *Rh. turanicus* sample, which had 100% identity with the *R. conorii* 17-kDa antigen sequence (AE008675). All sca4 and 17-kDa antigen gene sequences described in this study have been deposited in the EMBL database (accession no. AJ781411-AJ781420).

Table. Identification of *Rickettsia* spp. in tick samples collected from herbivores, Corleone (Palermo Province), Italy, 2001–2002

No. of ticks infected/total no. of ticks examined (tick species)	Minimum-maximum infection rate (%)	*Rickettsia* spp. identified (% identity with *sca*4 of spotted fever group rickettsiae)
Dermacentor marginatus (2/7)	28.5	*R. slovaca* (100)
Haemaphysalis punctata (1/15)	6.6	*R. slovaca* (100)
Hyalomma marginatum (2*/24)	8.3–20.8	*R. aeschlimannii* (99.79)
Hyalomma marginatum (1/24)	4.1	*R. africae* (99.75) *Rickettsia* sp. strain S (99.25) *R. honei* (99.0)
Rhipicephalus turanicus (1†/52)	1.9–7.6	*R. conorii* (100)

*Two pools of 2 and 3 ticks were positive.
†One pool of 4 ticks was positive.

Among the numerous rickettsia species recently described in Europe, *R. africae* and *R. slovaca* are known as human pathogens (8), and the first case of *R. aeschlimannii* infection in humans has recently been reported (9). African tick bite fever caused by *R. africae* is known as an imported disease in patients returning from sub-Saharan Africa or the West Indies (8), but our report raises the possibility that the rickettsial agent is actually present in European ticks from genera other than *Amblyomma*. *R. slovaca* has been shown to be responsible for a human disease known as tick-borne lymphadenopathy (10). Considering the problem of cross-reaction between different spotted fever group rickettsiae during serologic tests, our findings underscore the importance of using antigens from other spotted fever group rickettsiae, in addition to that of *R. conorii*, to obtain a more specific diagnosis of rickettsioses in Italy (10). Considering the large number of tick species present in Italy, and their infection with different spotted fever group rickettsiae, identifying the tick species responsible for a bite could be helpful for accurate diagnosis.

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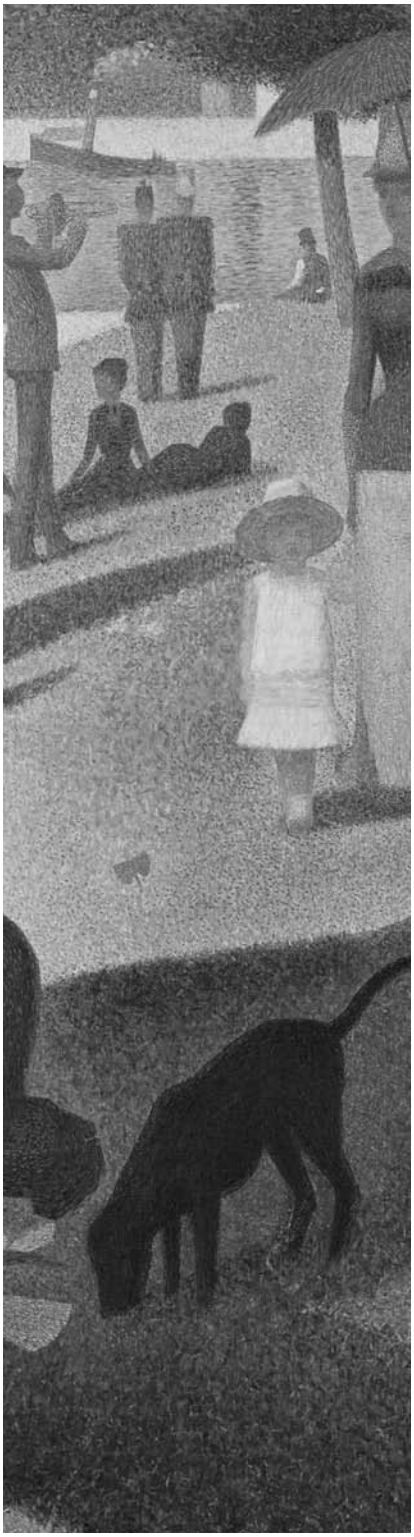

George Seurat (1859–1891)
Sunday Afternoon on the Island of
La Grande Jatte, detail (1884–86)
Oil on canvas (2.08 m x 3.08 m)
The Art Institute of Chicago,
Helen Birch Bartlett Memorial Collection

Optics and Biologic Connectedness

Polyxeni Potter*

"...It is this passion for beautiful colors that makes us paint as we do… and not the love of the 'dot,' as foolish people say," wrote painter Paul Signac in his journal (1). He was defending the art movement started by his good friend and fellow artist George Seurat and built upon by Signac himself, Camille Pissarro, and others. This movement, divisionism or pointillism, was Seurat's artistic contribution during a brief but extraordinary life.

Parisian from a middle-class family, tall, and handsome, Seurat enjoyed a comfortable life and proper education. He showed early talent for drawing, studied sculpture, and attended the prestigious École Des Beaux-Arts. A competent photographer, he became interested in the workings of light, particularly in black-and-white images. This interest grew as he studied optics and the processes at work on the silver particles of photographic film (2). During his art studies, particularly under the tutelage of a student of Ingres, he came to believe in a systematic approach to art.

Nicknamed "le notaire" (the notary) for his immaculate attention to his appearance, Seurat was temperamentally suited for a scientific approach to art (3). Idiosyncratically bent toward order and control and gifted with formidable observational skills, patience, concentration, and painstaking adherence to detail, he embarked on a style of painting based on color and structure that was cerebral and calculated.

Like the impressionists, Seurat was interested in the relationship between natural light and the application of paint, only he wanted to create an impression not on the canvas but in the mind of the viewer. Influenced by the work of French chemist Michel-Eugène Chevreul (1786–1889), he believed that next to each other, colors appear as dissimilar as possible, both in optical composition and tonal value (4). Seurat's color theory, in which the viewer plays a key role in perception, influenced the development of modern art.

His artistic goal, Seurat once said, was to show "modern people, in their essential traits, move about as if on friezes, and place them on canvases organized by harmonies of color, by directions of the tones in harmony with the lines, and by the directions of the lines" (5). In his best known work, images are tightly structured as if on a grid, the figures systematically placed in relation to each other in permanent, non-negotiable arrangements. Pure color is used directly from the tube, in static "points" clearly separate but intended to merge in the viewer's eye, producing a confluent image brighter than any achieved with brushstrokes. Like many scientific experiments, Seurat's daring process had unexpected results. The points remained visible, akin to tesserae in a mosaic, but produced a shimmering translucent effect (5).

A Sunday Afternoon on the Island of La Grande Jatte, on this month's cover of Emerging Infectious Diseases, is Seurat's masterpiece and one of the best-known works of the 19th century. The placid scene in an island park on the Seine shows a local crowd during a moment of leisure outdoors. Seurat's version of this commonplace event is revolutionary. As figures register in the viewer's eye, they seem suspended in mid-moment, levitating yet permanently fixed. Prototypes rather than likenesses, they represent workers in shirt sleeves, fashionable couples, children at play, soldiers in uniform. Seurat did not dwell

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on their faces, nor did he offer anything but their frontal or profile forms—classical, refined, distinct, balanced, and frozen in time. The iconic setup, like backdrop in a period drama, impassionedly places people, animals, and objects in a suddenly interrupted scene, creating a spellbinding visual effect.

As much interested in the science as in the art of painting, Seurat used figures as scene building blocks. Elegantly curved and grouped in harmonious ensembles, the figures are isolated from each other and detached from the beauty around them. And like separate dots of color, they do not fully blend, their shimmering presence only a means to a perfect artistic end.

Seurat's own life embodied the personal isolation seen in Sunday on La Grande Jatte. Even though surrounded by friends and supported by family, he was intensely private, even secretive, about his affairs. His parents did not know that he had a child until he was taken ill, possibly with diphtheria (6). He died precipitously at age 31, while hard at his innovative work. Signac encapsulated his friend's achievement, "He surveyed the scene and has made these very important contributions: his black and white, his harmony of lines, his composition, his contrast and harmony of color, even his frames. What more can you ask of a painter?" (1).

Seurat was not interested in the emotional or evolutionary connectedness of the crowd in La Grande Jatte. The nannies, belles and beaux, the playful pet monkey, even the stray dog foraging picnic crumbs in the foreground, are locked into themselves. Had Seurat been interested in biologic rather than optical accuracy, he might have ventured beyond visual perception of the crowd on the lawn. And between the dots, he might have found invisible connectedness, the glue that binds humans, monkeys, stray dogs, vegetation. Impervious to optics and inaccessible to the naked eye, biologic connectedness abounds.

Around the world, as in La Grande Jatte, scrounging animals share the landscape with humans. Along with scraps of food, they gather data that properly transcribed can be valuable. In African forest villages, loose dogs living near hunters and eating dead animals become exposed to Ebola and carry antibodies to the virus. Their destinies intertwined with ours in a way inaccessible to Seurat, the dogs may become predictors of human disease as their serologic status signals the presence of virus in the community (7).

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Upcoming Issue

Look in the April issue for the following topics:

Recurring Methicillin-resistant *Staphylococcus aureus* Infections in a Football Team

Antimicrobial-resistant Invasive *Escherichia coli*, Spain

Staphylococcus aureus Bacteremia, Australia

Echovirus 30, Jiangsu Province, China

Experimental Infection of Prairie Dogs with Monkeypox Virus

Canine *Echinococcus granulosus* Infection, Wales

Bed Bug Infestations in an Urban Environment

European Bat Lyssavirus in Scottish Bats

Influenza Outbreak Control in Confined Settings

Web-based Investigation of Multistate Salmonellosis Outbreak

Mycobacteria in Nail Salon Whirlpool Footbaths, California

Coxiella burnetii in Bulk Tank Milk Samples, United States

Complete list of articles in the April issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

March 16–18, 2005
Focus on Fungal Infections 15
Sheraton Bal Harbour
Miami, Florida, USA
Contact: 770-751-7332 or c.chase@imedex.com
http://www.imedex.com/calendars/infectiousdisease.htm

April 9–12, 2005
Society for Healthcare Epidemiology of America (SHEA) Annual Meeting
Los Angeles, California, USA
Contact: 703-684-1006
http://www.shea-online.org

May 1, 2005
International Society of Travel Medicine (ISTM) offers certificate of knowledge in travel medicine exam (Given prior to the opening of 9th Conference of the ISTM)
Contact: exam@istm.org
http://www.ISTM.org/

May 1–5, 2005
9th Conference of the International Society of Travel Medicine
Lisbon, Portugal
Contact: +49-89-2180-3830
http://www.ISTM.org/

May 3–5, 2005
Controlling Infectious Agents and Other Contaminants in Healthcare Facilities through Planning and Design
Madison, WI, USA
Contact: 800-462-0876
http://epd.engr.wisc.edu/emaG041

May 9–11, 2005
The Eighth Annual Conference on Vaccine Research
Baltimore Marriot Inner Harbor Hotel
Baltimore, MD, USA
Contact: 301-656-0003 ext. 12 or vaccine@nfid.org
http://www.nfid.org

EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.11, No.2, February 2005

Hazards of travel

EMERGING
INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje. org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word's table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/ EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only two. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be 1,000–1,500 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have one Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only (effective January 2005). Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.