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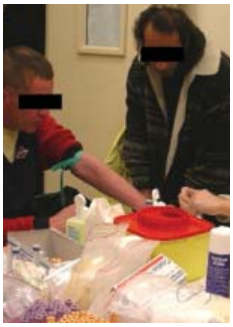

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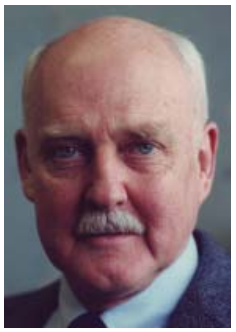

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Underreported Threat of Multidrug-Resistant Tuberculosis in Africa

Yanis Ben Amor, Bennett Nemser, Angad Singh, Alyssa Sankin, and Neil Schluger

Multidrug-resistant tuberculosis (MDR TB) in Africa may be more prevalent than previously appreciated. Factors leading to development of drug resistance need to be understood to develop appropriate control strategies for national programs. We gathered estimates of MDR TB rates for 39 of 46 countries in Africa. The relationship between MDR TB rates and independent factors was analyzed by using correlation and linear regression models. Our findings indicate that drug resistance surveys in Africa are critically needed. MDR TB rates must be assessed in countries without these surveys. In countries that have conducted a drug resistance survey, a new survey will determine evolution of drug resistance rates. We found no correlation between high MDR rates and TB incidence, HIV/TB co-infection rates, or year of introduction of rifampin. Results show that the retreatment failure rate was the most predictive indicator for MDR TB. Current category II drug regimens may increase MDR TB.

Global control of tuberculosis (TB) has been jeopardized by 2 major threats: HIV/AIDS and multidrug-resistant TB (MDR TB). MDR TB is defined as strains of *Mycobacterium tuberculosis* that are resistant to at least isoniazid and rifampin (*1*). Drug resistance has reached alarming levels with the emergence of strains that are virtually untreatable with existing drugs. The recent report of an outbreak of extensively drug-resistant TB (XDR TB) in South Africa (*2*), with its extremely high case-fatality rate, has drawn wide attention. However, the more general problem of MDR TB, with an estimated 450,000 cases worldwide annually, has been recognized since the first World Health Organization (WHO) global survey on drug resistance in the late 1990s (*3*).

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According to the WHO Global Report on Anti-tuberculosis Drug Resistance in the World (*1*), MDR TB strains have emerged in all regions of the world. However, despite the dramatic increase in TB rates in Africa, MDR TB appears nearly absent from this continent, which, until recently, reported the lowest median levels of drug resistance.

Two explanations have been most commonly put forward to explain these reported low levels of MDR TB in Africa. The first explanation is the presence of well-functioning control programs in Africa. Eighty-nine percent of the population in the WHO-defined region of Africa is covered by directly observed therapy, short course (DOTS), which is similar to the global average (*4*). However, there is discordance between purported DOTS coverage and national TB program (NTP) efficacy. Each year, countries with the lowest case detection and cure rates are clustered in the WHO-defined Regional Office for Africa (AFRO) region (*4*). This suggests that NTPs in Africa are not performing better than their Eastern European or South American counterparts, where MDR TB rates have already reached alarming levels. The functional status of many programs in Africa is difficult to assess with certainty, and the high incidence rates on that continent indicate that programs may not be functioning well. Low case-detection rates alone may not lead to development of MDR TB. Other factors that might favor development of MDR TB include the availability of drugs on the open market and a private sector that delivers drugs to the population in an unregulated fashion.

The second explanation is the recent introduction of rifampin in Africa. It is often stated that because rifampin was only recently introduced in Africa on a large scale, there has been relatively little time for resistance to develop. However, development of rifampin resistance may be spurred by HIV infection, and such resistance appears to develop rapidly (*5*–*10*).

Our research described in this report indicates the possibility of a third and straightforward explanation: field results for Africa are still incomplete, despite the recent publication of the WHO Fourth Global Report (*1*). This report is the result of independent drug resistance surveys (DRSs) conducted throughout the world following strict guidelines. The number of countries participating in the overall survey was low, especially in the Africa region. Furthermore, one might speculate that those countries capable of providing DRS data may have been the ones most likely to have a well-functioning NTP, laboratory structures, and transport networks, which would bias overall reporting. However, even if this is not the case, overall low levels of reporting make findings of the report questionable.

Consequently, the low level of reported drug resistance in Africa may not be an accurate reflection of reality, and the limited evaluation may be responsible for this skewed portrayal. Lack of comprehensive national DRS data from all countries in Africa is a barrier to understanding the magnitude of prevalence and incidence of MDR. In light of the added threat of XDR TB within the African context of high HIV prevalence, a thorough assessment of TB drug resistance and evaluation of data-specific deficiencies is urgently needed.

Our report aims to provide a more accurate assessment of MDR TB in Africa by incorporating all the latest available data and published estimates. In addition to suggesting that the effect of MDR TB in Africa is higher than previously thought, our report explores trends and relationships among case detection, treatment success, retreatment failure, and HIV rates relative to MDR TB. We attempted to identify population-based factors associated with MDR TB. Given the limited healthcare funding and substantial incidence of HIV in Africa, even a relatively low but increasing tide of MDR TB can lead to disastrous consequences for this continent.

Methods

Search Strategy

PubMed and Medline databases were searched for reviews and original reports based on primary studies by using the keywords tuberculosis, *Mycobacterium tuberculosis*, multidrug-resistant, and Africa. English and French language articles were reviewed.

Study Selection

Data were assembled into a comprehensive composite on the basis of several criteria. Published MDR TB rates from WHO were prioritized because of their reliability and because they are the result of DRSs conducted throughout the world following strict guidelines: new patients were clearly distinguished from those with previous TB treatment, and optimal laboratory performance was ensured and maintained through links with supranational reference laboratories.

Second, data on MDR TB were collected from peer-reviewed journals. Articles were considered only if specific criteria were met: 1) new patients needed to be distinguished from patients with prior TB treatment; 2) the number of confirmed TB cases needed to be statistically significant; and 3) the study needed to be nationwide or the population covered needed to be greater than one fourth of the total population.

For countries where data were unavailable through WHO reports or recent peer-reviewed journals, published formulaic estimates by Zignol et al. were used (*11*). The formula considers 9 independent variables considered likely to be associated with drug resistance and used in regression analyses. Resulting estimates compare favorably with known MDR TB values.

Data Extraction

The following data were tabulated for all forms of TB for all countries under scrutiny. Presurvey and postsurvey variables were annual country-specific indicators averaged from 1995 to the year of the drug resistance survey (presurvey) and from year after drug resistance survey to 2005 (postsurvey). These variables included TB incidence rate, case detection rate, treatment rates (category I: cured, completed, failure, default and success), and retreatment rates (category II: cured, completed, failure, default, and success). Year 2005 variables were published MDR TB estimates for new cases of pulmonary TB, HIV prevalence among adults 15–49 years of age, TB-related death rate, HIV/TB co-infection rate, male-to-female ratio of case notification rate, and health funding per capita (US dollars) in 2002 obtained from the United Nations Development Program (http://hdr.undp.org/statistics/data/indic/indic_52_1_1.html).

Combined MDR TB rates were compiled for 39 of the 46 countries in the AFRO region. For 21 of those 39 countries, MDR TB rates were gathered from either WHO reports or peer-reviewed articles. For the remaining 18 countries, MDR TB rates were reported by using a formula described by Zignol et al. (*11*). No DRS data or formulas were available for 6 countries, Cape Verde, Comoros, Liberia, Mauritania, Sao Tome and Principe, and the Seychelles, which account for only 20,475 of the 2,528,915 TB cases in Africa (*4*). Additionally, the accuracy of annual data from Mauritius was deemed questionable and thus excluded from further analysis.

Regression Analysis of Continuous Variables

The unadjusted associations between continuous independent variables and MDR TB rates were evaluated by us-

ing a linear correlation matrix. Independent variables highly ($p<0.05$) or marginally ($p<0.10$) predictive of the outcome in unadjusted models were included in further linear multivariable modeling. In addition, interactions between case detection, treatment failure, and retreatment failure rates were tested by using univariate linear regression analyses. All analyses were conducted in Stata Statistics version 9.2 (StataCorp, College Station, TX, USA).

Analysis of Presurvey and Postsurvey Continuous Variables

The change between presurvey and postsurvey averages (average value from 1995 to year of drug resistance survey, and year of drug resistance survey to 2005, respectively) for each continuous independent variable was evaluated by using the nonparametric Wilcoxon signed rank test. Given the small sample size, these variables were not assumed to be normal. A change between presurvey and postsurvey was deemed statistically significant if the p value was <0.05 and marginally significant if <0.10.

Categorical Trend Analysis

In addition to relationships between continuous MDR TB rates and independent factors, a categorical analysis of MDR TB was conducted. Countries were divided into 3 categories (tertiles) on the basis of MDR TB rates: (0.0–1.7, 1.8–2.1, and ≥2.2 defined as low, middle, and high MDR rates categories, respectively). For each TB-related indicator, a univariate trend across the 3 MDR TB categories (low, middle, and high) was assessed. Trend analysis was conducted by using the nptrend function in Stata Statistics version 9.2 (StataCorp), which performs a nonparametric trend test of rank sums across ordered groups. A trend was deemed statistically significant if the p value was <0.05 and marginally significant if <0.10.

Results

MDR TB Rates in Africa

Data on prevalence of MDR TB among all TB cases collected from various WHO publications and published peer-reviewed articles are shown in Table 1. The 39 countries considered in this study encompass ≈99% of total estimated incident or prevalent TB cases (all forms) in the AFRO region. The proportion of MDR TB among all TB cases varies from 5.8% in the Democratic Republic of Congo to virtually 0% in Kenya. The median MDR TB rate was ≈1.9% (Table 2).

The situation in Africa for MDR TB among all combined TB cases is depicted in a series of related maps (Figure). The first map is reproduced and translated from the WHO Third Global Report published in 2004 (*40*) (Figure, panel A). The coloring scheme was translated into the 3 categories used in this report, and the continent appears particularly devoid of MDR TB.

Panel B of the Figure includes data from various recent WHO publications, peer-reviewed journal articles, and the Fourth Global Report published in 2008 (*1*). Indications of geographic clustering are apparent; countries with a high prevalence of MDR TB are clustered in the southeastern and central regions of Africa. Panel B of the Figure also shows the 11 countries that have MDR TB rates >2.0 of all combined TB cases, including Democratic Republic of Congo, Rwanda, Equatorial Guinea, and Senegal, which were not included the WHO Third Global Report.

Panel C of the Figure includes formulaic estimates. On the basis of these estimates, high levels of MDR TB are not only located in the southeastern and central regions, but also in West Africa, which differs from results of the WHO Third Global Report.

Effect of Year of Introduction of Rifampin

Dates of rifampin introduction were available for 11 countries (Table 3). Dates of introduction are clustered around the mid-1980s. The earliest and latest known introductions of rifampin were 1979 in South Africa and 1989 in Zambia. Zambia has a middle-level rate of MDR TB, South Africa has a high rate, and other countries have low rates. No clear association between date of rifampin introduction and MDR rates could be discerned.

Indicators for Continuous MDR Rates

Presurvey independent variables were evaluated through 3 types of linear regression analysis: correlation (Table 4), univariate, and multivariate regression (data not shown). Regarding associations with MDR rates, correlation analysis showed a significant association with retreatment failure rate ($p = 0.043$, $r^2 = 0.119$). Several other univariate and multivariate linear models, such as treatment failure × retreatment failure and case detection × treatment failure × retreatment failure, were marginally significant. However, no other models that used presurvey data were more predictive than retreatment failure rate alone. For postsurvey variables, univariate analysis (Table 4) also showed a statistically significant association between MDR rates and retreatment failure rates.

Comparison of Presurvey and Postsurvey Indicator Averages

Using the Wilcoxon signed rank test, we determined that 5 of 14 indicators showed a significant ($p<0.05$) change between presurvey and postsurvey years. Treatment default showed a decrease; incidence rate, treatment cured, treatment success, and retreatment death rate increased between time intervals.

Table 1. Prevalence of MDR TB among combined TB cases by country, Africa*

Country	MDR resistance value, % (95% CI)	Nationwide study	Year	Reference	References for other convenience sample surveys
Algeria	1.4† (0.5–2.7)	NA	NA	(*11*)	
Angola	2.1† (0.6–8.9)	NA	NA	(*11*)	
Benin	1.0† (0.2–3.9)	NA	NA	(*11*)	
Botswana	1.6‡ (0.8–1.9)	Countrywide survey	2002	(*1*)	(*17*,*18*)
Burkina Faso	2.6† (8.8–11.0)	NA	NA	(*11*)	(*19*)
Burundi	1.4§	Bujumbura survey	2006	(*20*)	
Cameroon	2.0† (0.6–8.9)	NA	NA	(*11*)	(*12*,*13*)
Central African Republic	2.2¶ (1.0–3.4)	Countryside survey	1998	(*1*)	(*21*)
Chad	1.9† (0.5–9.0)	NA		(*11*)	(*22*)
Congo, Brazzaville	1.8† (0.5–8.2)	NA	NA	(*11*)	
Côte d'Ivoire	5.4† (3.2–8.4)	NA	NA	(*11*)	(*23*)
Democratic Republic of Congo	5.8¶ (0.6–10.3)	Kinshasa survey	1999	(*1*)	
Equatorial Guinea	3.4§	5 of 18 survey districts	2004	(*24*)	
Eritrea	1.9† (0.5–9.0)	NA	NA	(*11*)	
Ethiopia	2.5¶	Countrywide survey	2005	(*1*)	(*25*–*27*)
Gabon	1.8† (0.6–8.1)	NA	NA	(*11*)	
Gambia	0.4¶ (0.0–1.4)	Countrywide survey	2000	(*28*)	
Ghana	1.9† (0.5–8.7)	NA	NA	(*11*)	
Guinea	2.1¶ (1.0–3.0)	Sentinel sites survey	1998	(*1*)	
Guinea-Bissau	2.6† (0.8–1.4)	NA	NA	(*11*)	
Kenya	0¶ (0.0–1.1)	Nearly countrywide survey	1995	(*1*)	(*29*–*31*)
Lesotho	1.6¶ (0.4–2.6)	Countrywide survey	1995	(*1*)	
Madagascar	0.7¶ (0.7–10.3)	Countrywide survey	2007	(*1*)	(*14*)
Malawi	1.9† (0.5–9.3)	NA	NA	(*11*)	(*15*,*16*)
Mali	1.5† (0.3–7.9)	NA	NA	(*11*)	
Mauritius	1.4† (0.4–6.4)	NA	NA	(*11*)	
Mozambique	3.5¶ (2.5–4.6)	Countrywide survey	1999	(*1*)	(*32*,*33*)
Namibia	1.5† (0.4–7.1)	NA	NA	(*11*)	
Niger	2.7† (0.8–11.5)	NA	NA	(*11*)	
Nigeria	2.0† (0.6–9.3)	NA	NA	(*11*)	
Rwanda	4.6	Countrywide survey	2005	(*34*)	
Senegal	4.3¶ (0.8–10.6)	Countrywide survey	2006	(*1*)	
Sierra Leone	3.1¶ (0.3–4.0)	Nearly countrywide survey	1997	(*1*)	
South Africa	3.1¶ (2.2–3.0)	Countrywide survey	2002	(*1*)	(*2*,*35*–*39*)
Swaziland	1.9¶ (0.5–3.1)	Countrywide survey	1995	(*1*)	
Togo	2.1† (0.6–9.5)	NA	NA	(*11*)	
Uganda	1¶ (0.1–1.6)	3 district surveys	1997	(*1*)	
Tanzania	1¶ (0.6–9.8)	Countrywide survey	2007	(*1*)	
Zambia	1.8¶ (0.8–3.1)	Countrywide survey	2000	(*1*)	
Zimbabwe	2.2¶ (1.3–4.0)	Nearly countrywide survey	1995	(*1*)	

*TB, tuberculosis; MDR TB, multidrug-resistant TB; CI, confidence interval; NA, not available.
†Formulaic.
‡National Drug Resistance survey.
§Convenience sample survey.
¶World Health Organization.

Indicators for MDR TB Categories

When considered through nonparametric trend analysis, there was a marginally significant positive association between MDR TB categories and retreatment failure rates (data not shown). Median retreatment failure rates were higher within relatively higher categorized MDR TB countries.

Discussion

Our visual mapping indicates that MDR TB is likely to be more prevalent in Africa than previous reports indicated. The latest WHO Global Report on Anti-tuberculosis Drug Resistance in the World published in March 2008 (*1*) indicated rates of drug-resistant TB in Africa to be among the lowest worldwide. However, as of April 2008, 25 of the 46 countries in the AFRO region still had not completed a national study to investigate levels of MDR TB. By incorporating the latest national studies on MDR TB in Africa, and classifying countries on the basis of MDR TB rates in combined cases of TB, we found worrisome trends: 6 countries that were not included in the WHO Third Global

Table 2. Descriptive statistics for country-specific MDR rates and other TB-related factors, Africa*

Factor	Average, presurvey years (1995 to survey year)			Average, postsurvey years (year after survey to 2005)			Wilcoxon signed rank test†
	No., mean, median	Range	SD	No., mean, median	Range	SD	Z-score, p value‡
MDR rates	39, 2.21, 1.9	0.0–5.8	1.2				
Incidence rate/100,000/ y	39, 116, 109	22–228	40.6	33, 152, 150	25–308	70.2	–3.92, 0.0001§
Case detection rate (new ss+)	39, 47.2, 49.8	8.2–86.0	22.4	32, 55.5, 56.2	13.6–112.2	27.5	–1.52, 0.130
Treatment indicators							
Cured	36, 50.9, 52.3	17.1–74.6	14.2	32, 56.2, 59.0	16.6–78.4	14.5	–3.58, 0.0001§
Completed	39, 14.3, 12.4	3.1–47.4	9.8	32, 13.9, 11.1	1.3–38.5	8.9	1.27, 0.206
Died	36, 6.7, 6.1	2.6–19.5	3.3	32, 7.4, 7.2	0.7–17.1	3.5	–0.36, 0.721
Failed	36, 1.8, 1.6	0.0–7.8	1.5	32, 1.7, 1.3	0.2–5.3	1.2	–0.34, 0.738
Defaulted	36, 14.1, 13.2	4.4–41.4	6.8	32, 11.9, 10.9	2.6–39.7	7.4	2.48, 0.013§
Succeeded	36, 65.2, 67.0	33.0–83.5	10.3	32, 70.0, 70.5	37.1–90.3	10.3	–3.56, 0.0001§
Retreatment indicators							
Cured	35, 47.1, 48.0	11.1–71.3	16.2	29, 48.5, 52.0	2.9–72.4	17.3	–1.22, 0.221
Completed	35, 14.1, 12.7	0.0–46.6	8.7	29, 13.4, 9.8	0.5–40.8	10.0	1.49, 0.135
Died	35, 7.9, 7.7	0.0–22.7	4.1	29, 10.4, 10.1	1.6–21.4	4.5	–2.27, 0.023§
Failed	35, 3.6, 2.8	0.2–14.6	2.8	29, 3.2, 3.0	–8.9	2.2	0.18, 0.861
Defaulted	35, 14.6, 12.8	4.6–29.3	6.9	29, 11.9, 10.9	2.7–26.7	6.2	1.49, 0.135
Succeeded	35, 61.1, 62.6	30.3–81.3	13.1	29, 61.9, 64.6	23.1–81.8	14.2	0.18, 0.861
Year 2005 only variables							
Prevalence/100,000				38, 497, 513	55–936	178	
TB mortality rate/100,000/ y				39, 79, 73	2–304	48	
HIV/TB co-infection, %				39, 26.8, 19.0	0.5–75.0	20.5	
Male/female ratio: case notifications				36, 1.5, 1.5	0.7–2.6	0.4	
Health expenditures (US $ per capita)				39, 107, 51	15–689	131	

*MDR, multidrug resistance; TB, tuberculosis; ss+, sputum sample positive.
†Based on presurvey minus postsurvey values. A negative Z-score is indicative of an increase over time.
‡Marginally statistically significant trend (p<0.10).
§Statistically significant trend (p<0.05).

Report published in 2004 have MDR TB rates >2.0% of all combined TB cases. This finding suggests that completing DRSs for all or most countries in the AFRO region is urgently needed and that the MDR TB threat in Africa could be much higher than originally assessed by WHO in its previous report in 2004. Drug-resistant strains, along with HIV/AIDS, are causing the biggest challenge to efficient management and control of TB.

The lower rates of MDR TB in Africa, when compared with rates in Eastern Europe or South America, could be

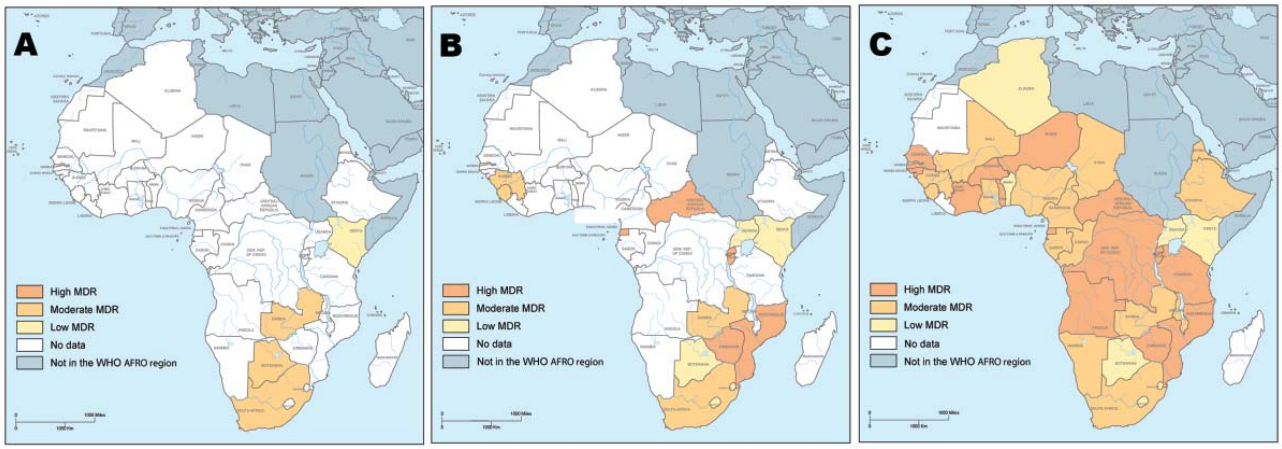

Figure. Prevalence of multidrug resistance (MDR) in Africa among combined tuberculosis cases. A) Data collected from the Third Global Report on Anti-tuberculosis Drug Resistance in the World of the World Health Organization (WHO) published in 2004 (*40*). B) Data from various recent WHO publications, peer-reviewed journal articles, and WHO's Fourth Global Report (*1*). C) Formulaic estimates of Zignol et al. (*11*). AFRO, WHO Regional Office for Africa.

Table 3. Years of introduction of rifampin in 11 African countries

Country	Date
Algeria	1980
Benin	1986
Botswana	1986
Côte d'Ivoire	1985
Gambia	1986
Malawi	1986
Mozambique	1986
Senegal	1986
South Africa	1979
Tanzania	1982
Zambia	1989

related to the fact that for many years Africa was neglected and TB was not treated. Alternatively, later introduction of rifampin in drug regimens is often cited as an explanation for these low rates. However, for the few countries for which data were available, we did not find a statistically significant relationship between year of rifampin introduction and level of MDR TB.

We subsequently set out to investigate linear associations between country-specific factors as well as categorical trends in TB management to discriminate levels of MDR TB in Africa. Retreatment failure rate was the most predictive indicator of MDR TB rates; other variables such as average case detection rate (1995–2005), average TB incidence rate (1995–2005), TB prevalence (2005), and HIV/TB co-infection (2005) did not show a linear relationship. Categorical analysis showed similar results.

One can speculate about the reasons that retreatment failure rates may be associated with higher rates of MDR TB. The relationship may simply be an association without causation, i.e., that retreatment fails because a given patient may already have MDR TB. In that sense, retreatment failure is simply a marker for preexisting MDR TB. However, it is also possible that retreatment may be a cause of MDR TB. Current WHO recommendations for retreatment regimens could lead to development of MDR TB or XDR TB in many instances because suggested retreatment regimens potentially amount to addition of 1 drug to a failing regimen, thereby intensifying the level of drug resistance.

We subsequently investigated whether combining the retreatment failure rate with other indicators in our model could generate a predictive combination for MDR TB rates. Whereas the univariate linear and the multivariate regression model showed a marginally significant association with either treatment failure, case detection rate, or both, retreatment failure as a sole indicator remained the most significant. This finding may indicate that, regardless of the successful record of a given NTP in detecting cases and subsequently successfully treating them with a category I regimen, the highest MDR TB rates were found in the countries with the highest retreatment failure rates (category II).

Table 4. Correlation analysis between country-level TB-related indicators and rates of MDR TB in African countries*

	Average, presurvey years		Average, postsurvey years	
Factor	Coefficient	p value†	Coefficient	p value†
Incidence rate/100,000/y	0.09	0.592	0.03	0.865
Case detection rate (new ss+)	–0.14	0.380	–0.10	0.585
Treatment indicators				
Cured	0.03	0.842	0.18	0.330
Completed	–0.13	0.446	–0.21	0.256
Died	–0.10	0.542	0.04	0.840
Failed	0.12	0.498	0.17	0.341
Defaulted	–0.07	0.664	–0.05	0.781
Succeeded	–0.08	0.653	0.08	0.676
Retreatment indicators				
Cured	–0.03	0.881	0.08	0.674
Completed	–0.20	0.258	–0.24	0.217
Died	–0.06	0.728	–0.11	0.588
Failed	0.34	0.043‡	0.41	0.029‡
Defaulted	0.04	0.798	0.12	0.540
Succeeded	–0.16	0.351	–0.07	0.728
Year 2005 only variables				
Prevalence/100,000			0.14	0.404
Mortality rate/100,000/y			0.02	0.894
HIV/TB co-infection (%)			–0.09	0.593
Male/female ratio: case notifications			–0.05	0.787
Health expenditures (US $ per capita)			–0.01	0.956

*TB, tuberculosis; MDR TB, multidrug-resistant TB; ss+, sputum sample positive.
†Marginally statistically significant trend (p<0.10).
‡Statistically significant trend (p<0.05).

We also investigated levels of variables reported in years after a DRS to determine whether the result of the survey affected these variables. Five of the 14 variables measured showed a statistically significant change over time: 4 variables (case detection rates, treatment cure rates, treatment success rates, and retreatment death rates) increased after the survey, and treatment defaulters decreased. Unfortunately, we cannot determine the effect of those 5 indicators on MDR TB rates because most countries surveyed reported only 1 DRS. Retreatment failure, which was shown to be the most predictive indicator of MDR TB rates, did not change over time. This finding suggests that MDR TB rates have increased in the years after the survey. To draw a parallel with physics, if the acceleration of an object remains constant over time, its speed increases. Therefore, it seems critical for countries who have already reported DRS results to undergo a second DRS to monitor the evolution of the MDR TB rate originally reported and help determine the effect of newly installed healthcare and TB treatment systems on drug resistance. For example, the only DRS reported from Kenya (conducted in 1995) reported an MDR TB rate of 0.0%. In 2008, the validity of this result is highly questionable given the recently published high MDR TB rates of neighboring countries (Figure).

This research was limited by the differing country-level data estimates and the ecologic study design. Twenty one of the 39 countries analyzed have TB estimates from WHO or peer-reviewed journals. However, the other 19 countries required formulaic estimates of MDR TB rates. The effect of these differing estimation methods depends on the congruity of future country-level estimates relative to the figures accepted in this study. Therefore, analyses should be repeated as more definitive estimates become available. Second, the ecologic study design, which analyzes these TB-related factors on a population level, limits the transference of any apparent relationships to the individual level. However, evidence shows that an effective DOTS program can limit the development of drug resistance on the individual TB patient level (*41*). Lastly, the small sample size of 39 countries limits the power to statistically determine differences. As a final note, this report hoped to realize 2 goals: to provide a composite of MDR TB in Africa and to identify relationships and trends.

Trends and relationships aside, MDR TB in Africa is a burgeoning obstacle that shows no signs of regression. The prevalence of MDR TB remains below the levels seen in Central Europe and parts of Latin America. However, in Africa, the tragically high HIV prevalence and limited funds and infrastructure dedicated to healthcare are serious factors. As the outbreak of MDR TB/HIV co-infection in New York City in the mid-1990s taught the medical community, this co-infection is pernicious, difficult, and overwhelmingly expensive to treat. This situation raises the question: if the continent is finding difficulty addressing TB, a well-defined disease caused by a well-defined agent, which is fully treatable with effective and affordable drugs through internationally recommended guidelines, then how will the continent fare against MDR TB and XDR TB?

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Preventing and Controlling Emerging and Reemerging Transmissible Diseases in the Homeless

Sékéné Badiaga, Didier Raoult, and Philippe Brouqui

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Describe practices to reduce the burden of HIV and hepatitis infection among the homeless.
- Identify how to screen for tuberculosis and treat tuberculosis in homeless settings.
- Describe the problem of scabies and body louse infections among the homeless.
- Specify the burden of illness associated with *Bartonella quintana* among the homeless and how to treat this infection.

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Homelessness is an increasing public health problem. Because of poor living conditions and limited access to healthcare systems, homeless persons are exposed to many communicable infections. We summarize the intervention measures reported to be efficient for the control and the prevention of common transmissible infections among homeless populations. Evidence suggests that appropriate street- or shelter-based interventions for targeted populations are the most efficient methods. Depending on the populations targeted, these interventions may include education, free condom distribution, syringe and needle prescription programs, chest radiography screening for tuberculosis, directly observed therapy for tuberculosis treatment, improvement of personal clothing and bedding hygiene, and widespread use of ivermectin for scabies and body louse infestation. Systematic vaccination against hepatitis B virus, hepatitis A virus, influenza, *Streptococcus pneumoniae*, and diphtheria is strongly recommended. National public health programs specific to homeless populations are required.

Homelessness is an increasing social and public health problem worldwide. According to the United Nations, "absolute homelessness" describes the conditions of persons without physical shelter. "Relative homelessness" describes the condition of those who have a physical shelter but one that does not meet basic standards of health and safety, such as and access to safe water and sanitation, personal safety, and protection from the elements (*1*). An estimated 100 million persons worldwide experience either absolute or relative homelessness (*2*). Homelessness is associated with numerous behavioral, social, and environmental risks that expose persons to many communicable infections, which may spread among the homeless and lead to outbreaks that can become serious public health concerns

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(*3*–*8*). Epidemiologic studies of homeless populations have reported the following prevalence rates for infectious diseases: 6.2%–35% for HIV infection (*6*,*9*–*13*), 17%–30% for hepatitis B virus (HBV) infection (*9*,*10*), 12%–30% for hepatitis C virus (HCV) infection (*9*,*10*), 1.2%–6.8% for active tuberculosis (TB) (*3*,*4*), 3.8%–56% for scabies (*11*,*12*), 7%–22% for body louse infestation (*5*,*11*,*13*,*14*), and 2%–30% for *Bartonella quintana* infection (*5*,*15*), which is the most common louse-borne disease in urban homeless.

The prevalence of these transmissible diseases among the homeless varies greatly according to living conditions. Homeless persons who sleep outdoors in vehicles, abandoned buildings, or other places not intended for human habitation are mainly street youth, female street sex workers, and persons with mental health problems (*1*). These persons are frequently injection drug users (IDUs), and they often engage in risky sexual behavior, which exposes them to both blood-borne and sexually transmitted infections such as HIV, HCV, and HBV (*6*,*9*,*10*). Homeless persons sleeping in shelters are mainly single men, but they also include single women, families with children, and mentally ill persons (*1*). The primary health concerns for this population are the overcrowded living conditions that expose them to airborne infections, especially TB (*7*), and the lack of personal hygiene and clothing changes that expose them to scabies, infestation with body lice, and louse-borne diseases (*5*). Homeless persons using single-room hotels or living with friends and family show a high prevalence of illicit drug use and risky sexual behavior that increases the risk for infections transmitted by blood and/or sex (*6*), and they also frequently live in overcrowded conditions that expose them to TB (*7*).

Homeless people face many barriers to accessing healthcare systems; these factors contribute to increasing the spread of infections (*1*). Implementing efficient strategies to survey and prevent the spread of communicable infections among the homeless is a public health priority. Strategies reported to be efficient for controlling or preventing communicable infections in the homeless are targeted interventions that focus on areas where homeless people are more likely to reside and are conducted with a mobile team that includes outreach workers (*8*,*16*–*19*). In this review, which concentrates on the primary communicable infections commonly associated with homelessness, we summarize the main intervention measures reported to be efficient in controlling and preventing these infections.

Interventions for Homeless at Risk for HIV and Hepatitis

The risk for HIV infection is higher in the following populations of homeless people: those engaged in sexual behavior such as sex work, receptive anal sex, and having multiple sexual partners; those who find it more difficult to use or obtain condoms (*6*,*10*); and those who use drugs in shooting galleries or who share syringes or other drug paraphernalia (*6*). Controlling the spread of HIV among the homeless requires interventions targeting high-risk groups such as youth, female street sex workers, and IDUs (*16*,*19*,*20*). For example, an intensive intervention program targeting homeless youth achieved a significant reduction of unprotected sex acts over 12 months ($p = 0.018$) and drug use over 12 months ($p = 0.019$) among female participants as well as a strong reduction in marijuana use over 12 months ($p = 0.082$) in male participants (*16*). The program involved training shelter staff and residents in small groups, providing access to health resources, and making condoms available easily and at no cost. An intervention program targeting homeless and crack-using African American women provided them with psychoeducational information and skills training on how to reduce HIV risk and drug use; the program significantly ($p = 0.03$) reduced the number of unprotected sex acts among participants, compared with control participants, at 6 months after the program was started (*19*). In Rhode Island, a prescription program to deliver syringes to high-risk underserved and diverse populations was conducted within the context of comprehensive drug treatment. The program recruited 327 persons and found that 86% saw a physician for syringe prescription at least 1 time, 46% at least twice, and 32% $\geq$3 times; this program demonstrated the feasibility and acceptability of such a program for and by its target population, and it reduced the number of injection drug–related risky behavior traits (*20*).

Factors predisposing to infections with HBV and HCV are much the same as those for HIV, with HBV a greater risk with of unprotected sex and HCV a greater risk with injection drug–related behavior (*9*,*10*). Therefore, intervention measures to prevent the spread of HCV among the homeless are the same as those noted above for HIV prevention (*16*,*19*,*20*). As for HBV infection, some evidence suggests that HBV immunizations for the homeless are feasible and effective. A study in New Haven, Connecticut, recruited 212 IDUs at syringe-exchange sites to undergo HBV vaccination. Most (63%) were vaccine eligible, including 23% of homeless persons; of the vaccine-eligible IDUs, 77% completed 2 vaccinations and 66% completed all 3 vaccinations (*21*). Homeless IDUs were more likely than other IDUs to complete the vaccination schedule, probably because the vaccination program and the syringe-exchange sites are areas where the homeless tend to congregate, providing more opportunities for them to access these services. An accelerated HBV vaccination schedule should be the regime of choice for homeless people, especially those with a past history of drug use. This recommendation is supported by a study in the United Kingdom that compared comple-

tion rates for a conventional HBV vaccine schedule (immunization at 0, 1, and 6 months) conducted among homeless in 1999 with the rates for an accelerated immunization schedule (immunization at 0, 7, and 21 days) in 2000. The completion rates for the accelerated vaccination regimen were almost 7 times higher than rates for the conventional one (*22*). A hepatitis A (HAV) outbreak has been reported among homeless persons and IDUs in Bristol (UK), possibly transmitted parenterally (*8*). The same city is the site of a successful HAV vaccination program for homeless persons and drug users to control HAV outbreaks and prevent transmission to the wider population (*8*). In June 2000, this program immunized 136 homeless persons and IDUs and 9 members of staff in shelters, hostels, drug services, and drop-in centers. The result was a significant ($p<0.001$) drop in HAV cases in the Bristol population (including homeless persons) from 90 cases (January–June) to 33 (July–December) (*8*).

Interventions for Homeless at Risk for TB and Airborne Diseases

TB incidence is higher in homeless populations than in the general population, as reported in San Francisco (270 cases/10^5 persons/year vs. 39.5 cases/10^5 persons/year) (*23*). Molecular epidemiology studies, using DNA fingerprinting, demonstrated that most TB cases occurring in the homeless are primary infections (*7*,*23*). The spread of TB among the homeless is related to recent person-to-person transmission, which produces outbreaks with large clusters in which >50% of persons are infected (7). Genotyping also identified homeless shelters as major sites of transmission (*7*,*23*). For example, in Los Angeles, California, from March 1994 through May 1999, 3 homeless shelters were sites of TB transmission for 55 (70%) of 79 homeless persons. Thirty-six of these 55 persons were infected in 1 large shelter in which 595 occupants shared 3 sleeping rooms. (*7*). Common individual risk factors for TB among homeless persons include alcohol abuse, poor nutrition, and HIV infection (*4*,*23*). Addiction to injection or inhaled drugs has also been reported as associated with TB in the homeless (*4*), but this association remains debated (*7*,*23*).

Interventions to control the spread of TB among homeless persons require early detection of cases and outbreaks in shelters, screening of those persons with whom the infectious person has had contact, and effective treatment of TB patients. According to a shelter-based screening program that used symptom evaluation, chest radiography, and in some cases sputum culture and tuberculin skin testing (TST), a TB infection rate of 1%–3% has been detected among sheltered homeless populations (*24*,*25*). Implementing mandatory shelter-based screening in several homeless shelters in the United States led to reduction of TB transmission among the homeless, as demonstrated by the reduction of genotype clustering in DNA fingerprinting analyses (*17*) (Table 1).

No consensus has been reached regarding the most effective diagnostic tools for screening for TB among the homeless. Logistically, TST is likely to be the simplest method to use because it requires only nurses and outreach workers (*17*). It was used successfully to identify TB infection in several intervention programs (*17*,*24*,*25*). However, TST lacks specificity, especially in areas where *Mycobacterium bovis* BCG vaccination is common. Spot sputum screening is also logistically easy, feasible, and efficient for identifying unsuspected TB cases in persons in shelters, and it can permit rapid detection of patients with smears that are acid-fast positive (*24*,*25*). However, 50% of TB patients' smears are negative, and the patients may be difficult to locate after culture results are known because the homeless tend to be very mobile. Screening by chest radiography either periodically in all residents or specifically in symptomatic persons (e.g., chronic coughers) is likely to be the most cost-effective approach, as was demonstrated in a jail setting (*26*). This strategy detected 42 cases of TB in 9,877 homeless persons in Los Angeles (*7*), and 2 cases of active pulmonary TB among 221 persons during a 1-night "snapshot" shelter-based survey (see "Snapshot Interventions," below) in Marseille, France (*27*).

Screening the contacts made by TB-infected homeless persons is more effective when it focuses on possible sites of transmission such as homeless shelters rather than when it investigates contacts of specific persons. By contrast, shelters and housing records are excellent sources of information for location-based investigation of contacts (*7*,*23*). The benefits for screening are clear: a 10% in increase in the number of chronically homeless persons with active TB who access treatment each year produced a 12.5% decline in future TB cases in this population after 10 years compared with the number expected without this intervention (*28*). The conditions for effectively treating TB in homeless persons include directly observed therapy (DOT) throughout the treatment course to ensure patient compliance and free medical care, including extended hospitalization and stays in convalescent-care institutions (*3*,*4*). Housing-based programs involving DOT have higher completion rates than programs in acute-care hospital settings.

In addition to TB, influenza, pneumococcal pneumonia, and diphtheria have been reported in the homeless (*29*,*30*). Although no reports on interventions to prevent these infections in the homeless have been published, it has been suggested that immunization against these diseases should be planned and delivered easily and at no cost to homeless people since this population is at high risk for outbreaks and severe illness (*29*,*30*).

Table 1. Communicable infections associated with homelessness*

Specific infections	Transmission route	Risk factors for infection spreading	References
HIV, hepatitis B, STIs	STIs	Sexual risk behavior traits: homosexuality/bisexuality, multiple sexual partners, crack and/or cocaine use, street sex work	(*6,9,10*)
HIV, hepatitis C, hepatitis B, hepatitis A	Blood-borne infections	Drug risk behavioral traits: sharing syringe, needle, and rinse water	(*6,8–10*)
Tuberculosis, influenza, diphtheria, pneumococcal pneumonia	Airborne infections	Overcrowding in shelters, alcohol abuse, drug addiction, malnutrition, HIV	(*3,7,17,23–25*)
Scabies, body louse infestation	Skin infections	Overcrowding in shelters, lack of personal hygiene, poor clothing and bedding hygiene.	(*5,11,13–15,18*)
Bartonella quintana infection, epidemic typhus	Louse-borne infections	High prevalence of body louse infestation	(*5,13–15*)

*STIs, sexually transmitted infections.

Interventions for Scabies, Body Louse Infestations, and Louse-borne Diseases

Scabies is transmitted by person-to-person contact or by contaminated fomites (e.g., clothes, bedding). It is more prevalent in the homeless than in the general population (*11*). The reported prevalence of scabies varies from 3.8% in shelter-based investigations (*11*) to 56.5% among hospitalized homeless persons (*12*). Human infestations with body lice occur when clothes are not changed or washed regularly, and close body-to-body contacts in crowded environments increase person-to-person transmission of body lice (*31*). In sheltered homeless populations, prevalence rates of body lice vary from 7% to 22% (*5,11,13,14*). In very poor hygienic conditions, an infection prevalence of 80% (*18*) and a single infected person carrying up to 600 lice have been reported (*14,18*). Scabies and body lice infestation generate severe pruritus, which leads to scratching, which may result in bacterial superinfections (*11*). In addition, the body louse is an efficient vector for *Bartonella quintana, Rickettsia prowazekii*, and *Borrelia recurrentis* (*3,5,15,32*). *Acinetobacter baumanii* has also been isolated from lice (*32*).

Controlling scabies, body louse infestation, and their effects on the homeless is a challenge. Classic therapeutic measures for scabies are based on bathing, followed by application over the entire skin of topical scabicides such as permethrin, lindane, benzyl benzoate, and crotamiton (*33*). Treatment with 200 μg/kg ivermectin, 2 doses administered 2 weeks apart, has been reported to be as effective as a single dose of a topical scabicide (*33*). Treatment of all close contacts and housemates is recommended, as well as careful washing of clothing and bedding (*33*). The therapeutic modality recommended for body lice is frequent changing and cleaning of clothing, including underwear and socks, as well as frequent treatment of bedding with insecticides or by boiling the sheets (*3,34*). In Marseilles, during a 4-year study of arthropod-borne infections among homeless people, we tried to treat, immediately and systematically, all persons in shelters who had scabies or body lice through complete clothing change, application of insecticide, administration of ivermectin as recommended, and education of shelter staff to change and treat the bedding frequently (*5*). Despite these efforts, no significant decrease in the prevalence of scabies and body louse infestation was observed during the study (*5*). In this population, a reduction in the prevalence of body lice infestation was seen after 3 doses of oral ivermectin were administered at 7-day intervals, but the effect was transient and disappeared by day 45 (*18*). In addition, a randomized, double-blind, placebo-controlled trial was conducted in Marseilles to evaluate the effect of a single dose of oral ivermectin on reducing the ectoparasite-based pruritus in the sheltered homeless population. This study showed that a single dose of oral ivermectin transiently reduces pruritus (S. Badiaga, unpub. data). These observations suggest that multiple repeated treatments of ectoparasite-based pruritus with ivermectin are an efficient and practical complement to classic therapeutic measures like frequent, complete changes of clothing and bedding to reduce scabies and body lice infestation in the homeless.

The most common louse-borne disease reported in the urban homeless is *Bartonella quintana* infection (*5*). *B. quintana* is a pathogen restricted to humans and was first described as the agent of trench fever during World War I (*34*). Emergence of *B. quintana* among the homeless was recognized in the early 1990s by simultaneous reports of *B. quintana* endocarditis in 3 homeless persons in France (*35*) and *B. quintana* bacteremia in 10 homeless persons in Seattle, Washington, USA (*36*). Subsequent epidemiologic studies showed *B. quintana* seroprevalence rates of 2%–11% among nonhospitalized homeless (*5,14,15*) and 30% in hospitalized homeless persons (*15*). *B. quintana* bacteremia rates of 5.4% in 930 nonhospitalized homeless persons (*5*) and 14% in 71 hospitalized homeless persons (*15*) have been reported from Marseilles. *B. quintana* DNA has been identified in 101 (14.9%) of 678 lice collected from the sheltered homeless population in Marseille (*5*), as well as in lice collected from homeless persons in Japan (*14*).

B. quintana causes trench fever, chronic bacteremia that may last up to 78 weeks, endocarditis in alcoholic per-

sons without previous valvulopathy, and bacillary angiomatosis in HIV-infected persons (*34*). Chronic bacteremia may be identified by blood cultures in homeless persons seen in emergency departments, as reported in a study from Marseilles (*15*). The phenomenon of chronic bacteremia suggests that humans are the natural reservoir of *B. quintana*, as demonstrated by identification of the bacterium in erythrocytes from homeless persons with *B. quintana* bacteremia (*37*). In addition to delousing, which is the best way to prevent louse-borne diseases, antimicrobial drug therapy against bacterial agents may be important for eradicating reservoirs and preventing complications such as endocarditis in cases of *B. quintana* infection. A randomized, open, placebo-controlled trial demonstrated significant efficacy of doxycycline (200 mg orally once a day for 28 days) in combination with gentamycin (3 mg/kg intravenously once a day for 14 days) in homeless persons with *B. quintana* chronic bacteremia (*38*). A regimen of gentamycin for 14 days and doxycycline for 28 days is recommended for patients with endocarditis (*34*).

Epidemic typhus caused by *R. prowazekii* and relapsing fever due to *Borrelia recurrentis* are 2 other louse-borne diseases that tend to affect the urban homeless. Outbreaks of epidemic typhus occur when body louse infestations are more prevalent in the population, as observed in Burundi (*31*). To date, no outbreak of epidemic typhus or relapsing fever has been observed in the urban homeless, nor has evidence of *R. prowazekii* or *B. recurrentis* been found in lice collected from this population. Nevertheless, during a 4-year study of louse-borne diseases among sheltered homeless persons in Marseille, we detected a sporadic acute autochthonous case of epidemic typhus in a sheltered homeless person (*39*) and significantly higher seroprevalences of *R. prowazekii* antibodies (0.75% vs. 0% in blood donors, p = 0.05) and of *B. recurrentis* (1.61% vs. 0% of blood donors, p = 0.005) (*5*). In a massive outbreak of epidemic typhus observed in Burundi, doxycycline was efficient in controlling the outbreak among jail inmates, causing a decrease in the death rate from 15% to 0.5% after administration of a single dose of 200 mg (Table 2).

Snapshot Interventions

Yearly snapshot interventions in shelters, performed by large multidisciplinary teams, have been reported to be efficient for controlling or preventing infections among the homeless (*5*,*11*). These investigations can reach a category of homeless who do not usually seek healthcare. In Marseilles, since 2000, a large mobile team is sent once a year to perform these snapshot interventions in order to survey louse-borne disease in the 2 shelters designated for accommodating the homeless (*5*). This team comprises 30–40 persons, including physicians, residents, or fellows; nurses; and outreach workers. During interventions, homeless persons who choose to participate are interviewed and physically examined. Clothes are carefully screened for body lice, and specific treatment is given when appropriate. Nurses also collect blood and other microbiologic samples for serologic tests for louse-, flea-, and tick-borne diseases, as well as hepatitis, HIV; and syphilis. Arthropods are collected and PCR-screened for pathogens such as *Bartonella*, the epidemic typhus rickettsia, and *Borrelia recurrentis*. Depending on the epidemiologic situation, other surveys such as for the prevalence of TB and other respiratory diseases can be organized (*27*). In such cases, a pneumologist and radiography technologists using a mobile x-ray machine are added to the intervention team. These snapshot interventions led

Table 2. Interventions to control and prevent the spread of infections in the homeless*

Infections and specific interventions	References
HIV, HCV, HBV infections	
Tailored education of targeted population on reducing infection risk with provision of free condoms	(*16*,*19*)
Syringe prescription program and needle exchange programs	(*20*)
HBV, HAV infections	
HBV accelerated immunization	(*21*,*22*)
HAV immunization	*(8)*
Tuberculosis	
Shelter based-intervention with chest radiograph screening, sputum culture, tuberculin skin testing	(*17*,*24*,*25*,*27*)
Genotyping	(*7*,*23*)
Contact investigation through homeless shelters	(*7*,*23*)
Influenza, diphtheria, *Streptococcus pneumoniae* infections	
Systematic vaccination	
Scabies, body louse infestation	
Providing facilities for bathing and laundry; insecticide application to bedding in shelters	(*5*,*34*,*35*)
Ivermectin for scabies, body louse, and ectoparasite-based pruritus	(*18*,*34*)
Louse-borne diseases	
Doxycycline and gentamicin for persons with chronic *Bartonella quintana* bacteremia	(*39*)
Doxycycline for persons with epidemic typhus	(*32*)

*HCV, hepatitis C virus; HBV, hepatitis B virus; HAV, hepatitis A virus.

to successively identifying a high prevalence of louse infestation, louse-borne diseases such as *B. quintana* infection, and skin infections among this homeless population (*5*,*11*). Snapshot interventions have also identified the risk for the homeless of acquiring other louse-borne diseases such as epidemic typhus and relapsing fever, and enabled the first isolation of *A. baumanii* from lice (*5*,*32*,*39*). This strategy of wide systematic testing of infectious diseases in this population also led to the unexpected discovery of an outbreak of acute Q fever in a homeless shelter in Marseille (*40*). Epidemiologic investigations of this outbreak showed that exposure to wind from an abandoned slaughterhouse, used for an annual Muslim sheep feast, was the main risk factor for developing *Coxiella burnetii* infection (Figure).

Conclusions

Evidence suggests that appropriate public health interventions can be effective in preventing and controlling the spread of numerous transmitted diseases among homeless persons, which is a public health concern both for the homeless and the larger population. These interventions should be tailored to the targeted populations and focused on areas where the homeless are more likely to reside. The strategies reported to be efficient include tailored education; distribution of free condoms; implementation of a syringe and needles prescription program for HIV and HCV; systematic chest radiography for TB screening in shelters and DOT for TB; improvement of personal, clothing, and bedding hygiene; use of ivermectin to treat pruritus most often caused by scabies or body louse infestation; and immunizations against HBV, HAV, influenza, *Streptococcus pneumoniae,* and diphtheria. Implementation of systematic vaccination schedules to prevent communicable diseases in the homeless is a major public health priority. The success of these interventions requires the implementation of a national public health prevention program for the homeless. A yearly snapshot intervention is 1 means to achieve these objectives.

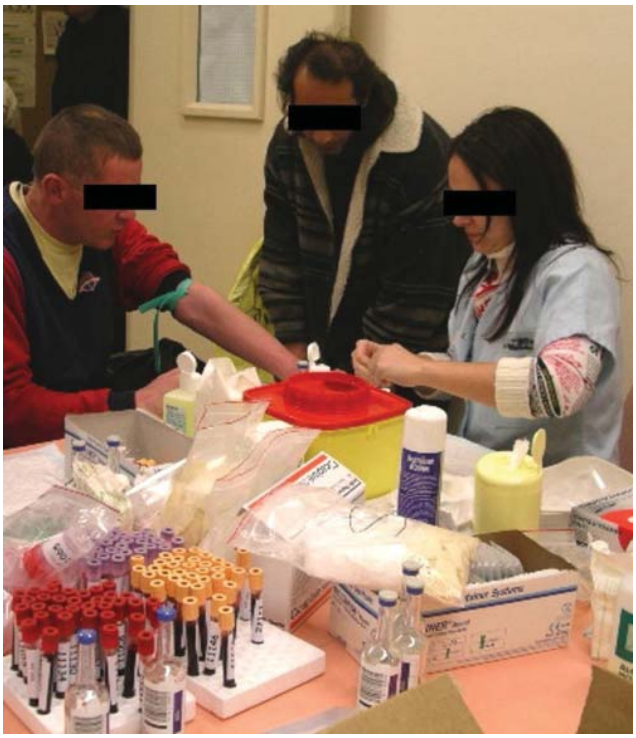

Figure. An intervention in a homeless shelter in Marseilles for infectious diseases survey.

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Questions on Mediterranean Spotted Fever a Century after Its Discovery

Clarisse Rovery, Philippe Brouqui, and Didier Raoult

Mediterranean spotted fever (MSF) was first described in 1910. Twenty years later, it was recognized as a rickettsial disease transmitted by the brown dog tick. In contrast to Rocky Mountain spotted fever (RMSF), MSF was thought to be a benign disease; however, the first severe case that resulted in death was reported in France in the 1980s. We have noted important changes in the epidemiology of MSF in the last 10 years, with emergence and reemergence of MSF in several countries. Advanced molecular tools have allowed *Rickettsia conorii conorii* to be classified as a subspecies of *R. conorii*. New clinical features, such as multiple eschars, have been recently reported. Moreover, MSF has become more severe than RMSF; the mortality rate was as high as 32% in Portugal in 1997. Whether *Rhipicephalus sanguineus* is the only vector and reservoir for *R. conorii conorii* is a question not yet answered.

Mediterranean spotted fever (MSF) is a tick-borne disease caused by *Rickettsia conorii*. It was first described a century ago as a disease that caused high fever and spots (*1*). Our knowledge about MSF has evolved since its first description. First, we thought that MSF was only limited to some regions of the world, i.e., southern Europe, North Africa, and India. In fact, an increasing number of regions have been reporting MSF cases, such as central Europe and central and southern Africa. Serologic techniques cannot distinguish among different rickettsiae species of the spotted group. Consequently, all rickettsioses with spotted fever group (SFG) antibodies were considered to have MSF in countries where this disease was endemic. Early clinical descriptions that relied only on serologic test results were likely to include infections related to multiple rickettsial species and were probably not describing a unique entity. For example, in France, emerging rickettsioses caused by bacteria, including *R. sibirica mongolitimonae, R. slovaca, R. felis, R. helvetica,* and *R. massiliae,* have been recently described (*1*). The first description of patients with MSF in southern France may have included patients with these emerging rickettsioses. With new molecular tools such as PCR and sequencing, we can now identify much more precisely the rickettsial agent responsible for the disease.

MSF is an emerging or a reemerging disease in some countries. For example, in Oran, Algeria, the first case of MSF was clinically diagnosed in 1993. Since that time, the number of cases has steadily increased (*2*). In some other countries of the Mediterranean basin, such as Italy and Portugal, incidence of MSF has substantially increased in the past 10 years.

Another point is that MSF was considered for 70 years a benign disease when compared with Rocky Mountain spotted fever (RMSF). In fact, because of the lack of medical interest in MSF, its real severity was long ignored. Although the mortality rate was evaluated to be from 1% to 3% in the early reports in the literature, the first description of a highly severe form of MSF was published in the early 1980s (*3*). At present, we know that MSF is at least as severe as RMSF and has a mortality rate as high as 32.3%, which occurred in Portugal in 1997 (*4*).

Although many hypotheses have been suggested, the nature and distribution of the reservoir of the rickettsiae in nature are still not answered. The aim of this review is to show the evolution in our knowledge of MSF in the past 10 years with an emphasis on epidemiology, clinical features, and severe forms.

Historical Background

The historical background of MSF is summarized in Table 1. MSF was described in Tunisia by Conor and Bruch (*1*) and was soon reported in other regions around the Mediterranean basin. The disease was thereafter also

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Table 1. Historical reports of MSF*

Year	Discovery	Authors†
1910	Description of the "fièvre boutonneuse de Tunisie" (7 cases)	Conor and Bruch
1925	Description of a cluster of MSF (8 cases) in Marseille, France, during the summer	Olmer
1927	Description of the inoculation eschar, the *tache noire* (black spot). Description of the disease associating fever, spots, and eschar as "Marseille fever"	Boinet and Pieri
1930	Experimental transmission of the disease by the brown dog tick	Durand and Conseil
1932	Demonstration of the transstadial and transovarian transmission of the agent of MSF in ticks. Demonstration of *Rickettsia* in infected ticks	Blanc and Caminopetros
1932	Isolation of the *Rickettsia* causing MSF in the vagina of infected guinea pigs and in infected ticks; named *R. conorii*	Brumpt
1982	First description of cases of malignant MSF	Raoult

*MSF, Mediterranean spotted fever.
†First or senior authors.

known as boutonneuse fever (spotted fever) because of the manifestation of a papular rather than a macular rash. The typical inoculation eschar, the *tâche noire* (black spot), was described in 1925 in Marseille by Boinet and Pieri (*5*). In the early 1930s, Durand and Conseil (*6*) proposed that the brown dog tick, *Rhipicephalus sanguineus,* was the vector in Europe after they inoculated humans with crushed ticks. Blanc and Caminopetros successfully repeated these experiments on humans and spermophiles (*7*). Brumpt showed that the SFG rickettsia was the causative agent, and in honor of Conor, this organism was named *R. conorii.* Blanc and Caminopetros showed that *R. conorii* could be transmitted through transovarial passage (*7*) in ticks and hypothesized that ticks could be the reservoir of *R. conorii* (Table 1).

Knowledge Gained about MSF in the Past 10 Years

New Information about the Agent

R. conorii is an obligate, intracellular, gram-negative bacterium (Figures 1, 2). In recent years, the rickettsial field has undergone a substantial evolution, particularly because of the technologic advances in molecular genetics. In the past decade, several rickettsial genomes, including that of *R. conorii* (*8*), have been sequenced. Availability of these genomic data have allowed, in turn, the development of global approaches, including proteomics and transcriptomics, powerful tools to gain a better knowledge of cell biology and interaction of rickettsiae with their host cells. Due to genome sequencing, the taxonomy of rickettsiae has undergone extensive reorganization. Until 2005, opinions were divided as to whether rickettsial strains related to *R. conorii* belonged to the same species or were distinct species. This included Israeli spotted fever rickettsia, Indian *R. conorii* strain (Indian tick typhus rickettsia [ITTR]), and Astrakhan spotted fever rickettsia (AFR) (Table 2). In fact, phylogenetically, these rickettsiae and *R. conorii* strain Malish (the agent of MSF) constitute a homogeneous cluster supported by significant bootstrap values and distinct from other *Rickettsia* spp. By estimating the degrees of genotypic variation among isolates of the *R. conorii* strains Malish, ISFR, ITTR, and AFR, Zhu et al. proposed that

Table 2. Distribution, vector, and main clinical features of the different subspecies of *Rickettsia conorii* complex

Rickettsia	Vector tick	Geographic repartition	Human disease name	Symptoms present, % patients			Fatal forms? (% patients)
				Fever	Inoculation eschar	Rash	
R. conorii conorii, isolates Malish, Moroccan Kenyan	*Rhipicephalus* sp., *Haemaphysalis leachii*	Mediterranean area (southern Europe, northern Africa), Croatia, Slovenia, Kenya, Somalia, South Africa, and surrounding the Black Sea (Turkey, Bulgaria, Ukraine, Romania)	Mediterranean spotted fever	91–100	20–87	93–100	Yes (0–18.1)
R. conorii israelensis	*Rh. sanguineus*	Israel, Portugal, Sicily	Israeli spotted fever	100	0–46	98–100	Yes (0–3.5)
R. conorii caspia	*Rh. sanguineus, R. pumilio*	Astrakhan region, Chad, Kosovo	Astrakhan spotted fever	100	23	94	No
R. conorii indica	*Rh. sanguineus*, *Boophilus microplus*, *H. leachii*	India, Pakistan	Indian tick typhus	100	Rare	100 (frequently purpuric)	No

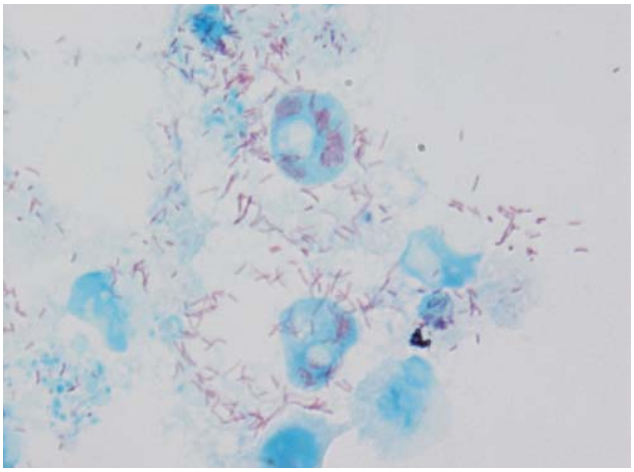

Figure 1. *Rickettsia conorii conorii* in Vero cells (red rods; magnification ×1,000).

R. conorii species nomenclature should be modified through the creation of the following subspecies: *R. conorii conorii*, *R. conorii caspia*, *R. conorii israelensis*, and *R. conorii indica* (*9*). These rickettsiae have discernable serotypes and cause diseases with distinct clinical features in defined geographic locations, but they are not genetically different enough to be considered as new species.

New Information about Epidemiologic Features

MSF is endemic to the Mediterranean area, including northern Africa and southern Europe. Cases are still identified in new locations within this region. Thus, some cases were recently described in Algeria, Malta, Cyprus, Slovenia, Croatia, Kenya, Somalia, South Africa, and in areas surrounding the Black Sea (Turkey, Bulgaria, and Ukraine). Spotted fever cases have been confirmed as MSF by the use of molecular tools in Portugal, Italy, Malta, Greece, Croatia, Spain, France, Turkey, Algeria, Tunisia, Morocco, Zimbabwe, Kenya, and South Africa. MSF is suspected to be endemic in Slovenia, Albania, Ukraine, Georgia, and Zimbabwe, but *R. conorii conorii* has not been isolated in human clinical samples in these countries.

MSF appears to be waxing and waning, as indicated by peaks in the number of MSF cases (Figure 3). Incidence of the disease sharply increased in the 1980s in Italy (*10*), Spain, and southern France (*11*). In some countries, MSF is reemerging. During the past decade in Portugal, the number of hospitalizations has increased from <200 to >400 cases per year (*4*). In Italy, a notable increase in case numbers was reported during the 1990s; cases peaked in 1999 at 699. In Bulgaria, MSF cases started to sharply decrease at the beginning of the 1960s and completely disappeared in the 1970s. However, in 1995, a peak of MSF disease with 236 cases was noticed in this country and reached 716 cases in 1997 (*12*). In Oran, Algeria, the first case of MSF was diagnosed in 1993; since that time, the number of cases has steadily increased to reach 134 in 2004 (*2*). Figure 4 illustrates the distribution and incidence of *R. conorii conorii* infection in countries where MSF is endemic.

Such variations have also been noted for RMSF (*13*). In the United States, the disease is in the midst of its third emergence since 1920, after peaking from 1939 to 1949, and again from 1974 to 1984 (*13*,*14*) (Figure 3). The causes of these variations in the incidence of MSF and other SFG rickettsioses are unknown. In most countries, no national epidemiologic surveillance of MSF cases is conducted. Only Italy and Portugal have a formal surveillance program. However, in these countries, surveillance is passive and not mandatory. Thus, in many countries, reported cases depend on the observers and can be affected by such variables as the need for international publications by physicians. For example, the dramatic increase in MSF cases in Oran, Algeria, is mainly due to the renewed interest in the disease by 1 physician (*2*). However, in countries that have a surveillance program, incidence of MSF cases actually varies in time. Another factor that limits the study of incidence of MSF in European countries is that nonspecific serologic tests are used for the diagnosis of MSF and could include other SFG rickettsioses. However, in Oran, when specific tests are used such as Western blot and molecular tools, *R. conorii conorii* appears to be the main etiologic agent of SFG rickettsioses in this area (D. Raoult, unpub. data). An increased number of ticks and increased human contact with the habitat of infected ticks are possible factors that would explain variations of incidence. In addition, the ecologic changes in the outskirts of large cities during the 1980s may have played an important role by moving rural sources to suburban zones. Climatic factors could also intervene, such as the increase of temperature, the lack of rainfall (for example, in Spain [*15*]), or the reduced number

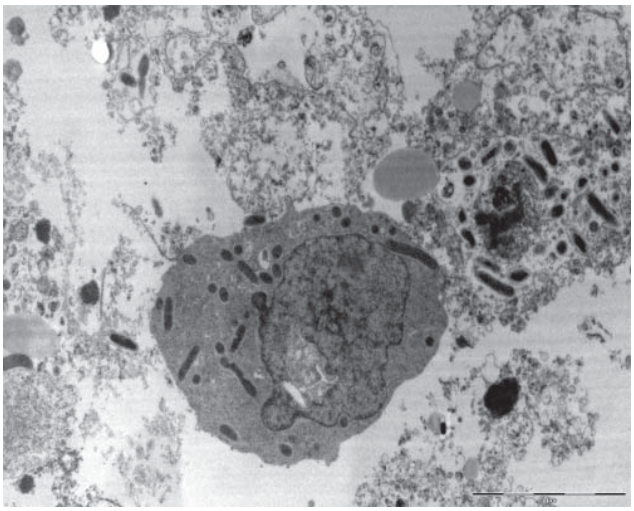

Figure 2. *Rickettsia conorii conorii* localized in cytoplasm of host cells as seen by electron microscopy (magnification ×100,000).

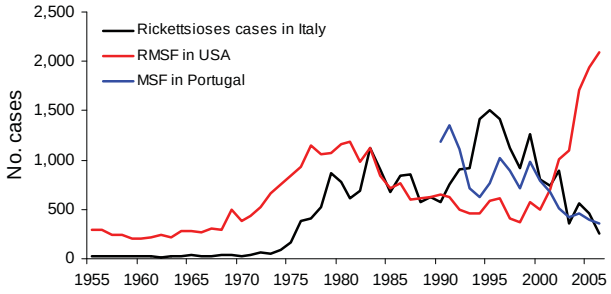

Figure 3. Fluctuation of incidence of Mediterranean spotted fever (MSF) in Italy and Portugal and of Rocky Mounted spotted fever (RMSF) in the United States, by year.

of days of frost during the past year in France (*16*). Climatic variations are suspected to play an important role in tick activity and, consequently, on rickettsial prevalence (*15*). It is also conceivable that undetected reservoir–vector systems have emerged or that the size of the reservoir has increased. This increase in MSF cases in the 1970s could also have been caused by a shift in effectiveness of prescribed antimicrobial drugs. In fact, before the 1970s, doxycline was used as an empiric therapy for patients with a fever of unknown origin. Finally, dramatic increases in MSF cases during the 1970s may be related to the advent of new diagnostic methods, such as microimmunofluorescence and the increased interest in traveling to several countries such as France, Italy, and Spain. Sporadic cases in non–disease-endemic countries are also observed as a consequence of tourism (*17*).

New Information about Clinical Features

The clinical description of MSF has not really changed since it was first described. MSF is characterized, just as the other rickettsioses, by fever, headaches, and maculopapular rash. Most of the studies reporting series of patients with MSF could have been affected by many factors. Descriptions of the first clinical cases, which were diagnosed on the basis of serologic test results alone, surely included infections related to other species of rickettsiae. Clinical descriptions with a series of patients are also subject to biased observations. For example, the eschar (Figure 5) can be difficult to retrieve and can sometimes be atypical, for example, having the aspect of a furuncle, which is difficult to recognize. This could explain wide variations in the reported presence of an eschar (20%–86%) (*4*,*11*).

Eschars are rarely multiple. This observation was, however, reported in the early description of the disease by Olmer (*5*). More recently, 2 studies in Sicily found multiple eschars in 7 (1.08%) of 645 children (*18*) and in 6 (1.4%) of 415 children (*19*), respectively. In studies in Spain (*20*,*21*), multiple eschars were found in 3%–11.5% of patients and involved more children. These findings should, however, be interpreted with care. The role of *R. aeschlimannii* circulating in *Hyalomma* spp. in Spain has to be considered in the cases of multiple eschars. Indeed, *Rh. sanguineus* has a low probability of biting humans, and the infection rate by rickettsiae is low (<10%). Accordingly, the probability of being bitten simultaneously by several infected *Rh. sanguineus* is low. Conversely, *H. marginatum* ticks readily bite humans, and persons may receive multiple simultaneous tick bites (*1*). Moreover, in Spain, Fernandez-Soto et al. reported *R. aeschlimannii* in 6 species that frequently feed on humans; a total of 4,049 ticks were removed from 3,685 asymptomatic patients. In this study, *R. conorii conorii* was isolated from only 1 *Rh. sanguineus* (*22*). In this context, we can hypothesize that cases of spotted fever acquired in southern Europe and associated with the presence of several eschars can be caused by *R. aeschlimannii*. Nevertheless, multiple eschars also exist in MSF. In 2004, our laboratory confirmed a diagnosis of MSF in 9 patients by using PCR. Among them, 3 had multiple eschars, and 2 of the 3 had a severe form of MSF (D. Raoult, unpub. data). All of these

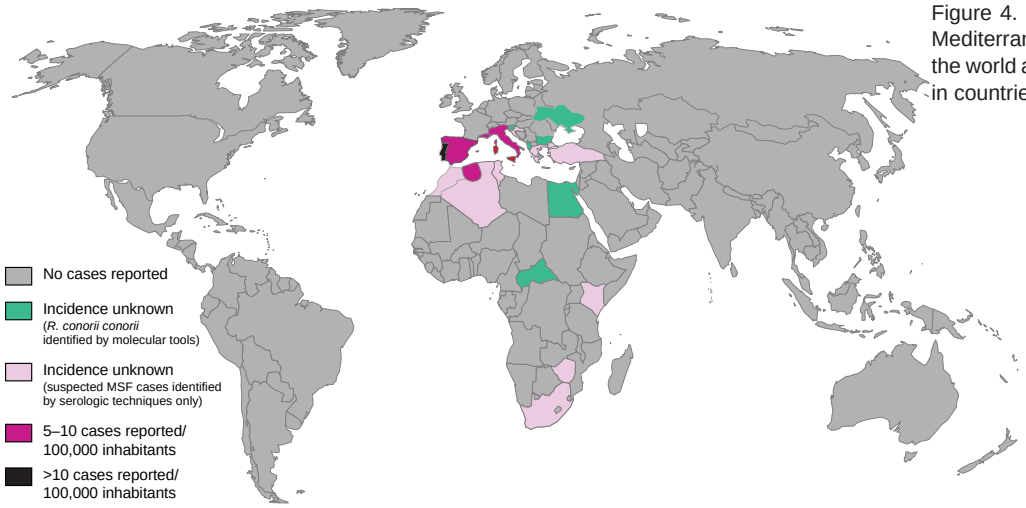

Figure 4. Distribution of the cases of Mediterranean spotted fever (MSF) in the world and incidence of the disease in countries where MSF is endemic.

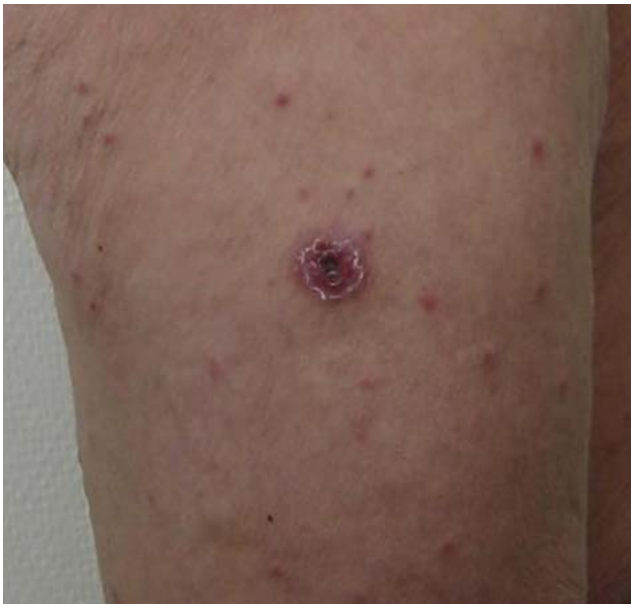

Figure 5. Typical eschar and spots on the leg of a patient with Mediterranean spotted fever.

patients were bitten in the southern of France. In Algeria, Mouffok et al. reported in a prospective study of 20 of 270 patients with multiple eschars (D. Raoult, unpub. data).

New Information about the Severity of MSF

Historical Background

Although mortality rates were determined to be 1%–3% in the early description, before the antimicrobial drug era, MSF was thought to be a benign illness with the proportion of deaths <1%. MSF was even named benign summer typhus. In comparison, RMSF was described as a very severe disease with a mortality rate of 65%–90% in western Montana (*23*). However, a few complications have been reported, including renal, neurologic, cardiac, phlebitis, and retinal complications. Severe forms of MSF were reported in 1981 (*3*); 4 of 6 patients with confirmed diagnoses died. Active surveillance of MSF in Marseille and the surrounding area in 1983–1984 found 7 (5%) of 142 patients with a severe form of MSF. At the same time, severe cases of MSF were described in Spain (*24*). Severe cases of MSF were similar to severe cases of RMSF with purpuric rash associated with neurologic manifestations and multiorgan dysfunction syndrome. At this time, *R. conorii conorii* was the sole agent in cases of spotted fever known in these countries. However, we could not exclude the possibility that some of these severe cases could be caused by rickettsiae discovered since this time. In fact, in 1997, in Portugal, some of the severe cases were caused by *R. conorii israelensis* (*4*). Recently, a severe spotted fever case caused by *R. australis* has been reported (*25*); the organism was previously considered to be responsible for a benign disease.

Temporal and Geographic Distribution of Severe Cases

Severity of MSF varies according to the time. For example, in 1983, in Salamanca, Spain, MSF was reported as a severe disease with complications occurring in 19% of the cases (*24*). During the 2 preceding years, in the same area, the annual incidence of complications was 3.7% (1981) and 4.34% (1982) (*24*). In 1997 in Beja, a southern Portuguese district, the mortality rate in hospitalized patients with MSF was 32.3%, the highest obtained there since 1994 (*4*). The mortality rate for the previous years was <15% in this region. This example illustrates not only a temporal, but also a geographic variation in the severity of MSF. Geographic variation in severity has also been reported for RMSF. In fact, in the United States, the overall case-fatality rate was 1.4% during 1997–2002 (*14*); however, in Brazil, the average case-fatality rate during 1995–2004 was 29.1% (*13*).

Recent MSF appears to be a more severe disease than it was in the past. Mortality rates were 3.2% in Oran, Algeria, in 2004 (*2*); 5.6% in Marseille, France, in 2003 (D. Raoult, unpub. data); and 32.3% in hospitalized patients in Beja, Portugal, in 1997 (*4*). This increase in severity has not been explained. Indeed, MSF is more quickly recognized and treated with more effective antimicrobial drugs than in the past. Thus, we would expect that MSF would be less severe. This was the case for RMSF, which had mortality rates in the United States of 2.4% during 1993–1996 (*26*) and 1.4% during 1997–2002 (*14*), in contrast to a mortality rate of 65%–90% at the beginning of the century. The first hypothesis could be that severe forms of MSF were not well recognized in the past. In fact, MSF was considered to be a benign disease before the 1980s (*27*). MSF was not a diagnosis that was evoked when patients were hospitalized in intensive care with a febrile rash. Another hypothesis is that more virulent strains of *R. conorii conorii* appeared. At present, however, the use of highly variable intergenic spacer sequences for multispacer typing of *R. conorii conorii* strains has not led to the identification of a more virulent strain (*28*). Remarkably, MSF is not a severe disease in children. No deaths or severe cases were noticed in 60 children in Barcelona during 1979–1980 (*29*). In Sicily, no severe forms were reported in 645 children during 1984–1996 (*18*) or in 415 children during 1997–2004 (*19*). Only 1 report of death (in a 16-year-old patient) was found in the literature (*30*). However, these studies could have been affected by recruitment bias. Other risk factors for severe MSF, other than advanced age, include the following: immunocompromized situations, chronic alcoholism, glucose-6-phosphate dehydrogenase deficiency, prior pre-

scription of an inappropriate antimicrobial drug, delay in treatment (*1*), and diabetes (*4*).

Unanswered Questions

Does *R. conorii conorii* Have Vectors Other than *Rh. sanguineus*?

Rh. sanguineus (Figure 6) is generally accepted as the main vector for *R. conorii conorii* in Europe and North Africa. No other species of ticks have been retrieved from the skin of humans infected with *R. conorii conorii*. *Rh. sanguineus* has a weak affinity for humans, as evidenced by the fact that only 3.5% of larvae, 2% of nymphs, and 5% of adults settle on humans when placed in direct contact with them (*31*). However, according to a 2003 report, 22 *Rh. sanguineus* (1 adult and 21 nymphs) were found attached to a homeless man with alcoholism, who was living with his dog near Marseille (*32*). Because this infestation was associated with the highest summer temperature noted in France in the past 50 years, the host-seeking and feeding behavior of *Rh. sanguineus* ticks may have been altered by the unusual climatic circumstances. Multiple eschars indicate that the same tick has bitten patients several times or that multiple ticks have bitten the patient. In southern France, we never noticed multiple eschars until 2004, when 2 patients had 3 eschars and 1 patient had 2 eschars (D. Raoult, unpub. data). Patients with multiple eschars were not observed in 2005. Multiple eschars could indicate recent modification of tick behavior related to unusual climatic circumstances of the previous year. Likewise, laboratory evidence has shown an association between changing temperature and changing behavior of *Rh. sanguineus* (D. Raoult, unpub. data).

In Africa, vectors other than *Rh. sanguineus* could intervene. PCR, followed by restriction fragment length polymorphism, on samples of hemolymph-positive ticks in Zimbabwe showed *R. conorii conorii* to be present in *Rh. simus* and *Haemaphysalis leachi* (*33*). In our laboratory, we recently isolated *R. conorii conorii* from *H. punctaleachi* collected in Uganda (D. Raoult, unpub. data).

What Is the Real Reservoir of *R. conorii conorii?*

Because of transovarial transmission, *Rh. sanguineus* was thought to be the reservoir for *R. conorii conorii* (*7*). However, this commonly accepted idea is now being challenged. In fact, only a small proportion of *Rh. sanguineus* ticks are infected with *R. conorii conorii*; infection rates are generally <15% (*34*). Moreover, most *Rh. sanguineus* ticks experimentally infected with *R. conorii conorii* die (*35*). This increased proportion of deaths in *Rh. sanguineus* has also been shown for naturally infected ticks after 1 generation (P. Parola, unpub. data). Curiously, *Rh. sanguineus* is found throughout the world, but *R. conorii conorii* is found only in some regions of the world. Dogs, the usual hosts of *Rh. sanguineus,* are also found everywhere. Even within endemic zones, microfoci exist. Early rickettsiologists such as Olmer in southern France and Blanc and Caminopetros in Greece have shown that foci of MSF are usually small with a low propensity for diffusion (*7*). Clusters in very limited geographic zones have also been observed for *Rh. sanguineus* that transmit *R. rickettsii* infection in Arizona (*36*). One explanation might be that transovarial transmission may occur for a limited number of passages and that *Rh. sanguineus* may only be the vector of the disease.

Figure 6. *Rhipicephalus sanguineus* adult tick, the suspected vector for *Rickettsia conorii conorii.*

Currently, we do not know the real reservoir for *R. conorii conorii*. Dogs serve as common transport hosts by bringing infected ticks closer to their owners. In certain zones of southern Europe, a correlation between the percentage of the canine population with antibodies to *R. conorii conorii* and the incidence of MSF in humans has been found (*37*). Seropositivity was even higher in dogs belonging to MSF patients (*37*). Dogs are transient reservoirs because of transient rickettsemia after infection; therefore, dogs do not seem to be an efficient reservoir for *R. conorii conorii*. Evidence has recently been shown that dogs can exhibit febrile illness related to infection with this bacterium (*38*). In the early description of MSF, Pieri showed that rabbits could be bacteremic without severe disease developing, which suggests that these animals could be a reservoir for *R. conorii conorii*. Le Gac et al. suggested that wild rabbits (*Oryctolagus cuniculus*) could play a role in the transmission of *R. conorii conorii* on the French Mediterranean

coast because a large drop in MSF cases occurred in 1952 during an outbreak of myxomatosis, which killed all the wild rabbits on the French Mediterranean coast. MSF reappeared in 1967 with the reappearance of wild rabbits (*39*). Ruiz Beltran et al. (*40*) found that 76.5% of wild rabbits and 25% of hares had antibodies to *R. conorii conorii* in Salamanca, Spain. Hedgehogs and other small rodents are also candidates for the reservoir because antibodies against rickettsiae have been detected in serum of these animals (*39*). Because *R. conorii conorii* has never been isolated in the Americas, its reservoir is most likely a mammal present only in the Old World that has yet to be determined.

Conclusion

Our knowledge regarding MSF has undergone notable changes within the past 10 years. Molecular tools have allowed us to better discriminate rickettsial species and subspecies of the SFG. We now know that >1 rickettsiosis can be present in the same country. Patients who have been included in series of MSF cases may have had other rickettsioses. Moreover, MSF has a wider distribution than previously described. The disease has emerged and reemerged in several countries in the Mediterranean basin. New clinical features, such as multiple eschars, previously suggested in the early description, have now been confirmed in MSF. MSF is becoming an increasingly severe disease with death rates ranging from 3.2% to 32%. However, questions persist regarding the vector and reservoir for this disease, which need to be addressed.

Dr Rovery is a specialist in infectious diseases in Marseille, France. His research interests are focused on rickettsial diseases, particularly *R. conorii* infection.

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Regulatory Systems for Prevention and Control of Rabies, Japan

Hiromi Takahashi-Omoe, Katsuhiko Omoe, and Nobuhiko Okabe

Japan is one of the few rabies-free countries. Although 3 imported cases of human rabies were seen in 1970 and 2006, no other cases have been reported for ≈50 years. The elimination of rabies in Japan is attributed to not only its geographic isolation but also to effective prevention and control measures, such as registration and vaccination of domestic dogs, required quarantine of susceptible imported animals, and national plans of action based on scientific research. Countermeasures against rabies have been upgraded; an improved management system for domestic dogs under the amended Enforcement Regulations of the Rabies Prevention Law has been in effect since April 2007. The latest regulatory systems for preventing and controlling rabies provide an effective model for elimination of the disease worldwide.

Rabies is a severe zoonotic viral disease that kills ≈55,000 persons annually in many countries of Africa and Asia (*1*). Because of the lack of specific and effective medical care for persons with clinical rabies (*2–4*), many countries have taken various measures to prevent and control rabies in animals. Rabies-free countries and territories are limited to islands such as Japan and New Zealand and to parts of northern continental Europe (*5*). Japan has been free of rabies for ≈50 years; the last cases of human and animal rabies were reported in 1954 and 1957, except for 3 imported cases of human rabies in 1970 and 2006 (Table 1) (*6–9*).

In Africa and Asia, human rabies is contracted primarily from rabid dogs. However, several wild animal species, including bats and foxes, are carriers and vectors for rabies and related viruses in the genus *Lyssavirus* (*10*,*11*). Although lyssaviruses have been isolated from wild animals in many countries, in Japan such viruses have not been reported in any animals during the past decade (*12*).

Japan has long been free of rabies because it is separated by water from countries in which the disease is endemic and because it has successfully managed rabies prevention and control. Management techniques include registration and vaccination of domestic dogs, legal regulations to quarantine susceptible imported animals, and national plans of action based on scientific research. Nevertheless, outbreaks of animal or human rabies, such as the cases in 2006, and recent increases in the international movement of people and animals have raised concerns.

A further cause for concern is the decreasing percentage of vaccinated domestic dogs among all registered dogs in Japan. According to data reported in 2006 (*13*), 4,910,047 6,635,807 of registered domestic dogs were vaccinated. However, because the percentage of registered dogs is assumed to be ≈50% of the total number of dogs in Japan, immunization coverage may actually be <40% (*14*).

Because of the increasing risk for domestic and international rabies outbreaks, Japanese central and local governments, in conjunction with coalitions of public health specialists such as veterinarians, physicians, and researchers, have developed several preventive measures. We present the country-level management systems in Japan, focusing on the latest legal regulations and plans of action. We believe that Japan's approach to preventing and controlling rabies is an effective model for the elimination of rabies throughout the world.

Legal Framework

Preventive measures against human and animal rabies in Japan are stipulated under 3 laws: the Rabies Prevention Law (no. 247, August 1950, and amended law no.

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Table 1. Annual transition of rabies outbreaks in Japan*

Year	No. cases in dogs (cats)	No. cases in humans	No. cases in livestock	Remarks
1945	94 (2)	1	19	
1946	24 (1)	1	5	
1947	37	17	1	
1948	141 (1)	45	2	
1949	614 (10)	76	2	
1950	867 (29)	54	12	Enforcement of Rabies Prevention Law
1951	319 (3)	12	18	Enforcement of Domestic Animal Infectious Diseases Control Law
1952	232	4	1	
1953	176	3	4	
1954	98	1	No data	
1955	23	0	No data	
1956	6	0	No data	
1957	0 (1)	0	No data	
1970	0	1	0	Imported case (returning traveler from Nepal)
2006	0	2	0	Imported cases (returning travelers from the Philippines)

*Data from the aggregate calculation by the Ministry of Health, Labour and Welfare (*6*).

160, December 1999); the Domestic Animal Infectious Diseases Control Law (no. 166, May 1951, and amended law no. 102, October 2005); and the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients with Infectious Diseases (Infectious Diseases Control Law; no. 114, October 1998, and amended law no. 30, May 2008) (Figure 1) (*15–18*). Under these laws, substantive efforts to prevent and control rabies have been adopted by central and local governments, relevant ministries, various concerned bodies, veterinarians, physicians, and researchers.

The measures enforced under these laws are divided into 2 categories: 1) day-to-day measures, such as the registration and vaccination of domestic dogs, and 2) export and import quarantine of animals that are susceptible to rabies. The former is managed primarily by the Ministry of Health, Labour and Welfare of Japan (MHLW) and the public health departments of local governments, and the latter is managed by the Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF), and quarantine stations. The usual preventive measures, emergency measures in case of an outbreak of human and/or animal rabies and related plans of actions, are promulgated in these laws. The essential features of these regulatory systems are described below.

Animal Rabies Control under the Rabies Prevention Law

The regulatory system to control rabies in pets and wild animals is based on the Rabies Prevention Law (*15*). The objectives of the law are to improve public health and contribute to public welfare by preventing outbreaks of rabies, controlling its spread in the event of an outbreak, and therefore eliminating the disease. The animals targeted under this law are dogs, cats, and other animals (e.g., raccoons, foxes, skunks) that have a high potential to infect humans.

This law focuses particularly on the development of daily administrative systems for domestic dogs (*19*). Under these systems, all dog owners are required to register their dogs and have them vaccinated against rabies. Owners must register their dog with the head of the nearest local government once during the animal's lifetime; after registration, the dog must wear a license tag. Regarding vaccination, dog owners must have their dog vaccinated against rabies once a year. After vaccination, the owner must take the vaccination certificate from the veterinarian who administered the vaccine to the head of the nearest local government, where

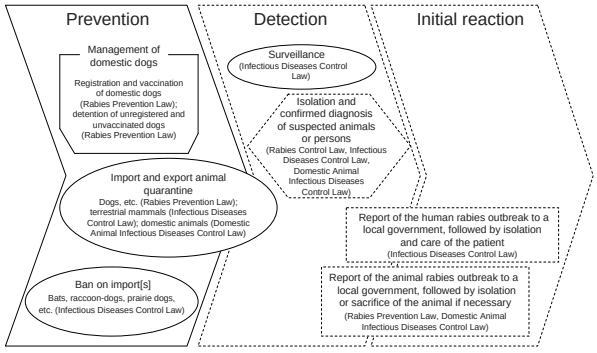

Figure 1. Regulatory framework for preventing and controlling rabies in Japan. Under 3 laws, countermeasures against rabies are divided into prevention, detection, and initial reaction. Infectious Diseases Control Law means Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients with Infectious Disease. Solid and dashed lines show ordinary and emergency countermeasures, respectively.

they will receive a certification tag that the dog must wear. Local governments are responsible for managing registration and vaccination of dogs and for assigning veterinarians who capture and detain unregistered or unvaccinated dogs.

To strengthen this management structure, 2 measures have been taken. One is the amended Enforcement Regulations of the Rabies Prevention Law, enacted in April 2007 (MHLW ordinance no. 52, September 1950, and amended ordinance no. 17, March 2007), which provides improved standards for licensing and certification of vaccinated dogs. The amendment offers 2 improvements: 1) the miniaturization of the license and vaccination certification tags so that they can be attached to smaller dogs and 2) the ability of local governments to choose the shape of the license and certification tags (*20*). The other measure is the approach by the Japan Veterinary Medical Association (JVMA) to strengthen rabies control (*14*). JVMA encourages dog owners to keep their animals vaccinated against rabies because actual immunization coverage is assumed to be <40% in Japan (*14*); nevertheless, the World Health Organization (WHO) recommends immunization coverage of at least 70% to control canine rabies in areas where the disease is endemic (*21*). Additionally, JVMA asks for public understanding and cooperation regarding rabies vaccination.

In addition to the management of domestic dogs described above, the law stipulates import and export quarantine for animals that are susceptible to rabies. The quarantine system, which is based on the latest scientific diagnostic knowledge and which makes use of examples from the UK quarantine system entitled the Pet Travel Scheme (*22*), has been enforced since November 2004 under the Regulations for Import and Export Quarantine of Dogs and Other Designated Animals (MAFF ordinance no. 68, November 1999, and amended ordinance no.75, November 2004) (Quarantine Regulation by MAFF). Under this system, dogs, cats, raccoons, foxes, and skunks are identified as animals subject to quarantine in a MAFF Animal Quarantine Service facility. The quarantine detention period is from 12 hours to 180 days, depending on the status of rabies outbreaks in the animal's region of origin and preparation of the required certification (Table 2). Detention for 12 hours is applicable for dogs, cats, raccoons, foxes, and skunks imported directly from rabies-free regions (designated regions) and dogs and cats vaccinated and inspected in regions other than designated regions. Detention for 180 days is required for all raccoons, foxes, and skunks imported from regions other than designated regions. Further details concerning the quarantine system, such as forms for notification and

Table 2. Detention period for quarantining imported animals under the Rabies Prevention Law

	Imported from designated regions (rabies-free regions)*		Imported from other regions	
Animals	Detention within 12 h	Detention for >12 h	Detention within 12 h	Detention for >12 h
Dogs and cats	Necessary procedures before import: prior notification concerning import† attached by a health certificate type A. Contents of type A certificate: 1. Individual identification by microchip‡; 2. residency in the exporting country for at least 180 d immediately before shipment to Japan, or since birth, or continuous residency in the exporting country since being directly imported to Japan; 3. no case of rabies in the exporting country for at least 2 y before exporting the animal; 4. clinical examination showing rabies-free (dog and cat) and leptospirosis-free (dog) proof	Extended quarantine period up to 180 d in the case of omissions in prior notification† attached by a certificate type A	Necessary procedures: prior notification concerning the import† attached by a health certificate type B. Contents of type B certificate: individual identification by microchip,‡ rabies vaccination using inactivated vaccines at least twice, rabies serologic test,§ a wait of at least 180 d between the date of blood sampling (day 0) and the date of arrival of an animal in Japan	Extended quarantine period up to 180 d in the case of omissions in prior notification† attached by a certificate type B
Raccoons, foxes, skunks	Necessary procedures before import: prior notification concerning the import† attached by a health certificate type C. Contents of type C certificate: individual identification by microchip,‡ rabies vaccination using inactivated vaccines at least twice, rabies serologic test,§ clinical examination showing rabies-free proof	Extended quarantine period up to 180 days in the case of omissions in prior notification† attached by a certificate type C		Necessary procedures: prior notification†, individual identification by microchip‡, clinical examination. Fixed quarantine period (180 d)

*Designated regions are Taiwan, Iceland, Sweden, Norway, United Kingdom (only Great Britain and Northern Ireland), Australia, New Zealand, Fiji Islands, Hawaii, and Guam. All regions were designated on June 7, 2005.
†When trying to import dogs or cats, the person must submit the advance notification described below to the Animal Quarantine Service, which has jurisdiction over the person's intended port of arrival, at least 40 d before arrival in Japan. Notification items include name, address, and contact number of the person submitting the notification; breed of dog/cat; number of animals; intended use; country of export; date and place of import; name and address of consignee/consigner; export location/destination; and individual identification data.
‡An International Organization for Standardization–compliant microchip (ISO11784 and ISO11785) should be used. If another type of microchip is used, a special reader for the microchip is needed.
§For the rabies serologic test, the neutralizing antibody titration test against rabies is necessary after the second vaccination. The test must be carried out by a laboratory designated by the Minister of Agriculture, Forestry and Fisheries of Japan. Test results must be $\geq$0.5 IU/mL.

certification, can be found in the practical guide by MAFF (*23*,*24*).

Animal Rabies Control under the Domestic Animal Infectious Diseases Control Law

The regulatory system to control rabies in livestock is based on the Domestic Animal Infectious Diseases Control Law (*18*). The law has been implemented to domestically and internationally promote the livestock industry by preventing the outbreak and spread of infectious diseases in domestic animals. Under this law, rabies in cattle, horses, sheep, goats, swine, buffalo, deer, and wild boars is designated as a "domestic animal infectious disease (infectious disease obligated to report)." Livestock intended for import and export are quarantined to prevent outbreaks and spread of rabies not only in Japan but also in other countries. As shown in Figure 2 (*25*), the quarantine detention period for the above animals differs according to species (cloven-hoofed animals or horses) and whether the animals are being imported or exported. For imported animals, double inspections have been implemented to detect 100% of infected animals before they are transferred to a farm. One inspection is a microbiologic test conducted at the MAFF Animal Quarantine Service facility; the other is the monitoring of physical condition of animals at the municipal livestock hygiene service center. Details on the quarantine system can also be found in the information manual of the animal quarantine system by the Japan External Trade Organization (*26*).

When rabies is suspected or confirmed in livestock, the diagnosing veterinarian or animal owner is required to report the case immediately to a prefectural or city governor through the director of the nearest animal public health center. It is also a legal requirement that the animal be isolated, and euthanized if necessary.

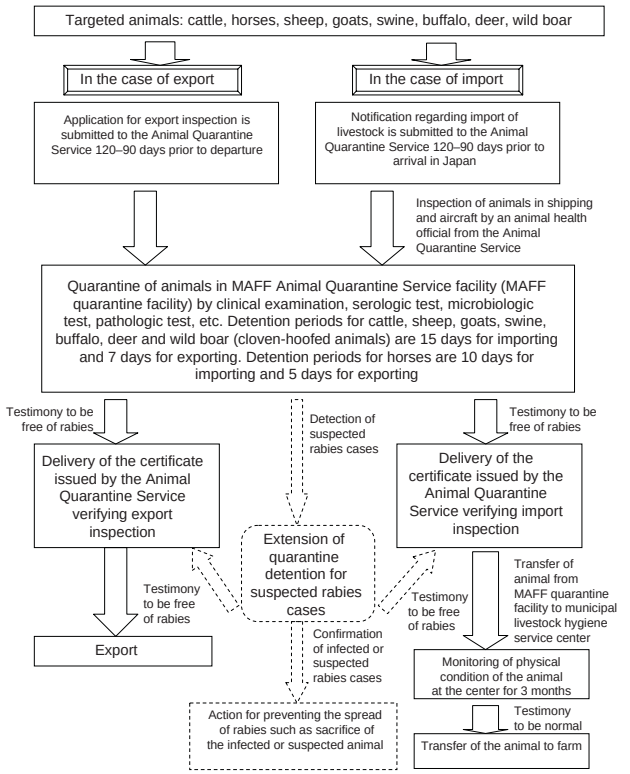

Figure 2. Flowchart of the inspection for rabies infection for importing and exporting animals under the Domestic Animal Infectious Diseases Control Law. The figure is based on our interpretation of data from reference (*25*). Dashed lines show emergency countermeasures. MAFF, Ministry of Agriculture, Forestry and Fisheries.

Animal Rabies Control under the Notification System for the Importation of Animals

To prevent the invasion of infectious diseases, including rabies, through animals imported into Japan, the Notification System for the Importation of Animals, authorized by the Infectious Diseases Control Law, has been in force since September 2005 (*27*). With respect to the use of quarantine to control rabies, the system requires terrestrial mammals being exported to be accompanied by health certificates declaring the animals to be free of the disease; the certificates are issued by government authorities of the exporting country. Target mammals include not only animals for distribution and exhibition in Japan but also animals for personal possession as pets; they do not include animals that have already been quarantined under the Rabies Prevention Law or the Domestic Animal Infectious Diseases Control Law or animals whose importation is banned by the Infectious Diseases Control Law (Chinese ferret badgers; bats; raccoon dogs; masked palm civets; prairie dogs; *Mastomys natalensis;* and all monkeys except those used for experimentation, research, and exhibition in Japan). Therefore, the notification system plays a complementary role in the quarantine specified under the Rabies Prevention Law and the Domestic Animal Infectious Diseases Control Law. Animal species that are quarantined under the above 3 laws are shown in Table 3.

Human Rabies Control under the Infectious Diseases Control Law

Japan's regulatory system for human rabies control is based on the Infectious Diseases Control Law (*16*,*17*). The objective of the law is to control outbreaks of infectious diseases, including zoonoses, and to prevent the spread of these diseases in humans. The law targets ≈100 kinds of infectious diseases (*28*) and stipulates the medical care for patients affected by the diseases to promote, improve, and upgrade public health in Japan. Regarding human rabies, the law requires reporting of disease cases

Table 3. Animals subject to quarantine and/or examination for rabies before importation into Japan

Law or regulation	Animals subject to quarantine	Animals requiring a health certificate	Animals banned from importation
Rabies Prevention Law (Regulations for Import and Export Quarantine of Dogs and Other Designated Animals*)	Dogs, cats, raccoons, foxes, skunks		
Domestic Animal Infectious Diseases Control Law	Cattle, horses, sheep, goats, swine		
Law concerning the Prevention of Infectious Diseases and Medical Care for Patients with Infectious Diseases	Monkeys used for research and exhibition, under specified conditions only		Chinese ferret badgers, bats, raccoon dogs, masked palm civets, prairie dogs, *Mastomys natalensis* rats, monkeys except those to be used for research or exhibition
Notification System for the Importation of Animals†		Terrestrial mammals except for Artiodactyla (e.g., cattle, sheep, goats); Perissodactyla (e.g., horses); Lagomorpha (e.g., rabbits); dogs, cats, raccoons, foxes, skunks, monkeys	

*This regulation is authorized by the Rabies Prevention Law.
†This regulation is authorized by the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients with Infectious Diseases.

promptly after diagnosis. In the instance of well-defined or suspected human rabies, the diagnosing physician must report the case immediately to the director of the nearest public health center, who will then forward the report to the local government.

National Standards for Rabies Control

The 2001 Guidelines on Rabies Countermeasures (MHLW Notification, November 2001, and supplement, January 2003) have been put into practice as the standard for preventing and controlling rabies according to the above laws (*29–31*). The guidelines are described in a comprehensive handbook for addressing an outbreak or suspected outbreak of rabies in Japan; they establish measures to guide government, medical, and other related institutions in taking suitable initial actions. These measures are based on a number of documents: Laboratory Techniques in Rabies, published by WHO (*32*); Laboratory Methods for Detecting Rabies, by the US Centers for Disease Control and Prevention (*33*); Rabies Contingency Plan in Hawaii (*34*); and Memorandum of Rabies, Prevention and Control, by the UK Department of Health (*35*). The latest guidelines include a supplement concerning the response to the increasing risk for rabies infection through rabid animals and the status of rabies outbreaks in the world.

The 2001 Guidelines on Rabies Countermeasures base specific countermeasures against suspected cases of animal and human rabies on the location of cases. These countermeasures are divided into 7 patterns to facilitate a quick response, depending on the situation (*29–31*). Each pattern involves role sharing between the Japanese central and local governments; networking among affected organizations such as veterinary hospitals, animal control facilities, and medical institutions; measures for dealing with people and animals that might come into contact with rabid animals; and specific examination procedures.

The 2 cases of human rabies in 2006 (*7–9*) were stringently controlled according to the 2001 Guidelines on Rabies Countermeasures, in terms of the initial response to a rabies outbreak and medical practice; the patients, however, died of the disease. It was possible to make a rapid, definitive diagnosis by detecting the rabies virus gene on days 2–3 (first case) (*7*,*8*) and on day 2 (second case) (*8*,*9*). For the first case, the health professional who treated the patients in the hospital worked smoothly with local governments, the National Institute of Infectious Diseases, and MHLW to enable urgent health advice to be given quickly to the related organizations such as quarantine stations and local governments on day 4. Concerning the second case, effective countermeasures published in an overseas case report and manual were also applied. The patient was isolated strictly, following the recommendations of the Centers for Disease Control and Prevention manual (*36*); in addition to isolation, the patient received the same medical care as that given to a patient who had survived (*2*,*3*).

Conclusion

Japan has successfully eliminated rabies because of its geographic isolation and because of the systematic management of susceptible animals and humans under the relevant laws and regulations. These effective preventive measures enforced under the regulatory systems serve as a model for elimination of the disease worldwide.

As a remaining task for controlling rabies in Japan, internal and international rabies surveillance should be maintained or increased in the years ahead. Previous reports suggest that no rabies or other lyssaviruses have been detected in animals during the past decade in Japan (Table 1)

(*6*,*12*); however, surveillance of domestic and wild animals that are possible hosts for infection in Japan should be followed up continuously because of the <40% immunization coverage of dogs (*14*).

In addition to domestic countermeasures against rabies, border control measures to eliminate possible importation of animal or human rabies cases should be strengthened. Regardless of quarantine system, which theoretically makes it possible to eliminate the entry into Japan of an animal infected with rabies or other lyssavirus, the risk for rabies in Japan is believed to be rising (*14*). This belief is because the international movement of people and animals is increasing and the illegal importation of rabid animals remains a possibility, as does the immigration of people who are unaware that they have been infected with rabies or other lyssaviruses. To eliminate these possibilities, it is necessary to control such animals thoroughly by stringent import quarantine and to highlight the risk for rabies infection to Japanese nationals, who tend to consider the disease to have been eradicated in Japan and therefore may be less vigilant than necessary.

Moreover, surveillance of rabies and lyssavirus infections in wild animals is needed for further rabies control internationally because several wild animal species are recognized as wildlife carriers of rabies and lyssaviruses worldwide. In recent years, our understanding of the epidemiology of rabies and lyssaviruses has changed substantially as a result of improved molecular approaches to virus variant identification and improved epidemiologic analysis techniques for rabies and lyssavirus infections. However, epidemiologic data from Asian countries have not been sufficiently collected and analyzed (*37*). Japan must survey the distribution of rabies and lyssavirus infections in nearby Asian countries from the standpoint of international cooperation in terms of control of rabies and improvement of the import quarantine system. Thus, Japan needs to promote surveillance of rabies and lyssavirus infections internationally, focusing on not only dogs but also other animals, especially wild animals. As a surveillance attempt, scientists in Japan and other Asian countries have epidemiologically and phylogenetically examined domestic and wild animals living in Asian countries (online Appendix, available from www.cdc.gov/EID/content/14/9/1368-app.htm) (*38*,*39*) and discussed a measure for developing a new type of rabies vaccine based on the surveillance data (*39*). Because more surveillance and analysis data regarding rabies and lyssaviruses diseases in Asian countries will be published, a network responsible for amassing and systematizing the data provided by scientists should be established by a coalition of not only scientists but also of governments and healthcare professionals, such as veterinarians and physicians, in Asian countries. Creating a new network for the control of rabies and lyssavirus diseases is timely, is of global interest, and represents a further contribution to the successful elimination of the diseases around the world.

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Forest Fragmentation as Cause of Bacterial Transmission among Nonhuman Primates, Humans, and Livestock, Uganda

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We conducted a prospective study of bacterial transmission among humans, nonhuman primates (primates hereafter), and livestock in western Uganda. Humans living near forest fragments harbored *Escherichia coli* bacteria that were ≈75% more similar to bacteria from primates in those fragments than to bacteria from primates in nearby undisturbed forests. Genetic similarity between human/livestock and primate bacteria increased ≈3-fold as anthropogenic disturbance within forest fragments increased from moderate to high. Bacteria harbored by humans and livestock were approximately twice as similar to those of red-tailed guenons, which habitually enter human settlements to raid crops, than to bacteria of other primate species. Tending livestock, experiencing gastrointestinal symptoms, and residing near a disturbed forest fragment increased genetic similarity between a participant's bacteria and those of nearby primates. Forest fragmentation, anthropogenic disturbance within fragments, primate ecology, and human behavior all influence bidirectional, interspecific bacterial transmission. Targeted interventions on any of these levels should reduce disease transmission and emergence.

Infectious diseases transmitted among wild nonhuman primates, humans, and domestic animals pose a serious threat to wildlife conservation, human health, and animal health (*1*,*2*). For example, outbreaks of Ebola hemorrhagic fever and anthrax have caused epidemic deaths in apes and local humans in West Africa (*3*,*4*), and human paramyxoviruses have caused repeated deaths in chimpanzees in Côte d'Ivoire (*5*). Emerging pathogens such as these are now regarded as important drivers of primate population declines (*1*,*6*).

Although people and domestic animals have shared habitats with nonhuman primates (primates hereafter) for centuries, the dynamics of these interactions have changed dramatically over the last several decades. The destruction of tropical forests worldwide has imperiled many primates (*7*). Today, most primates live in remnant forest fragments and isolated protected areas within habitat mosaics of farmland, pastures, and human settlements (*8*,*9*).

Several studies have demonstrated that fragmentation of tropical forests reduces primate biodiversity and alters primate demographics and behavior (*10*,*11*). Fragmentation also alters patterns of gastrointestinal helminthic and protozoan infection in certain species (*12–14*). Whether host susceptibility, transmission dynamics, or a combination of these factors drive such trends remains unclear (*15*). The effects of fragmentation on the dynamics of pathogen transmission between primates and other species, including humans, are largely unexplored.

The goal of this study was to assess the effects of forest fragmentation on rates and patterns of bacterial transmission among wild primates, humans, and livestock, and to examine how anthropogenic and behavioral factors affect these rates and patterns across a fragmented forest landscape. We targeted *Escherichia coli,* a common, genetically

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diverse gastrointestinal bacterium transmitted by a variety of modes, including directly and through the environment (*16*,*17*). Virulent forms of *E. coli* are of considerable concern as emerging zoonoses (*17*,*18*), and benign forms of the bacterium provide a useful system for understanding the transmission dynamics of a range of microbes with similar biologic and epidemiologic characteristics (*19*,*20*).

By examining genetic relationships among *E. coli* isolates from humans, livestock, and 3 species of primates, we inferred rates of bacterial transmission among populations of these species living in or near 3 fragments that differed in their degrees of anthropogenic disturbance. Combining bacterial genetic data with surveys of local residents allowed us to identify behavioral and demographic risk factors affecting bacterial transmission between humans and primates.

Materials and Methods

Study Site

The study took place in and near Kibale National Park, Uganda (Figure 1). Kibale is a 795-km^2 park located in western Uganda near the foothills of the Rwenzori Mountains (0°13′–0°41′N, 30°19′–30°32′E), consisting primarily of moist semideciduous and evergreen forest, which is transitional between lowland rainforest and montane forest (elevation range ≈1,100–1,600 m) and interspersed with grassland, woodland, wetlands, and colonizing forest (*21*,*22*). Kibale is notable for its high species diversity and density of primates and is considered a premier primate research site in sub-Saharan Africa (*23*). Outside of the protected areas of Kibale exist a series of forest fragments that sustain small populations of primates (*11*). These fragments typically occupy nonarable wet lowlands. For this study, we focused on 3 fragments, Bugembe, Kiko 1, and Rurama, which have been studied intermittently since ≈1994 (Table 1).

Study Species

We studied the 3 species of primates typically found in fragments near Kibale: red colobus (*Procolobus rufomitratus*), black-and-white colobus (*Colobus guereza*), and red-tailed guenons (*Cercopithecus ascanius*). The first 2 species are folivorous and can survive even on the depauperate forest vegetation of the fragments in which they reside (*11*). Red-tailed guenons are omnivorous primates that favor fruit and insects in undisturbed sections of Kibale (*24*) but survive in nearby fragments by habitually raiding crops from adjacent farmlands (*25*).

Small agricultural settlements surround each fragment (Table 1, Figure 1). Persons living in these settlements are primarily subsistence farmers. Their contact with primates occurs during excursions into fragments to extract forest resources (e.g., firewood, timber) or when primates leave fragments to raid crops. Primates must also cross pastures to move among disconnected habitats within fragments, thereby coming into close contact with livestock and their feces. Livestock included in this study were cattle (*Bos indicus* and *B. indicus* x *B. taurus* crosses) and goats (*Caprus hircus*), which are ubiquitous in the region. Humans and livestock in the region use common open water sources, such as open wells and streams, which tend to be located inside or near the edges of fragments, within primate home ranges.

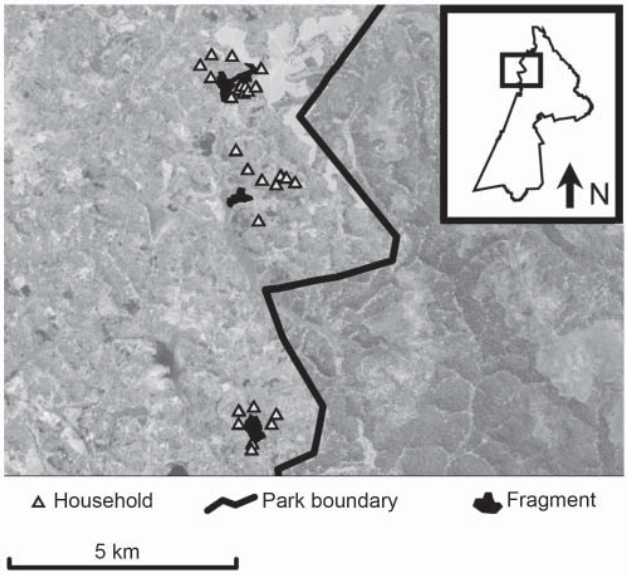

Figure 1. Map of study area within Kibale National Park, western Uganda (box), and forest fragments and households included in the study. Fragments are (from north to south) Kiko 1, Bugembe, Rurama (see Table 1 for details). Households, park boundary, and fragments are superimposed on a Landsat satellite image (30-m resolution).

Sample Collection and Human Surveys

We collected fecal samples from primates (n = 93) during behavioral observations in June and July 2005 (dry season). We sampled all primate social groups from the 3 fragments as well as primates of the same species living in nearby undisturbed areas of Kibale National Park. Once a primate was observed to defecate, we recorded its species, age, sex, and individual identity (if known). We took care to sample only those portions of the fecal material that had not contacted the ground, to avoid environmental contamination. Environmental contamination from other sources (e.g., canopy vegetation) would have been unlikely, since we have been consistently unable to recover *E. coli* from such sources despite repeated attempts (T.L. Goldberg, unpub. data). Samples were placed in sterile tubes and transported within 6 hours to our field laboratory.

Table 1. Characteristics of locations included in the study and sample sizes of households, persons, and *Escherichia coli* bacterial isolates*

	Characteristics					Sample sizes†					
Location	Area‡	Perimeter‡	Distance to park‡	Primate species present§	Disturbance¶	Household	Human	Livestock	BWC	RC	RT
Kibale National Park	792.73	197.20	0	BWC, RC, RT, + 9 others‡	Low	NA	NA	NA	10, 35	12, 46	7, 26
Bugembe fragment	0.66	1.48	1.69	BWC (11), RC (60), RT (10)	Medium	8	25, 52	27, 92	11, 42	9, 33	1, 4
Kiko 1 fragment	1.48	3.52	1.11	RC (4), RT (7)#	Very high	13	48, 139	16, 57	NA#	4, 13	4, 8
Rurama fragment	1.13	1.42	0.66	BWC (15), RC (15), RT (12)	High	7	26, 61	17, 53	13, 48	12, 46	10, 36

*BWC, black-and-white colobus (*Colobus guereza*); RC, red colobus (*Procolobus rufomitratus*); RT, red-tailed guenon (*Cercopithecus ascanius*); NA, not available.
†Pairs of values indicate sample sizes of individuals and bacterial isolates, respectively. Household numbers indicate sample sizes of households enrolled in the study; approximately twice the indicated number of households are associated with each fragment. Although many households surround Kibale National Park, nonhuman primates were sampled only from core undisturbed forest sites where home ranges do not overlap with human settlements. Livestock included both cattle (*Bos indicus* and *B. taurus* x *B. indicus* crosses) and goats (*Caprus hircus*), which are combined here and in subsequent analyses because results were essentially identical when the species were analyzed separately.
‡Area (km^2) and perimeter (km) were calculated by using the computer program ArcMap, version 9.1 (ESRI, Redlands, CA, USA), from point data gathered by walking the boundary of each fragment with a hand-held global positioning system unit. Distance to the park (km) was calculated as the shortest straight-line distance between the fragment and the park boundary.
§Numbers in parentheses indicate population sizes of each species in each fragment in July 2005. See Struhsaker (*21*) for a description of the primate fauna and ecology of Kibale National Park.
¶Disturbance rankings are based on quantitative measures of encroachment from Onderdonk and Chapman (*11*) and from Gillespie and Chapman (*12*), as well as on qualitative assessments of forest clearing rates and intensities of human activity gathered from ground surveys in 2005–2006.
#BWC had been extirpated from Kiko 1 fragment shortly before this study began, and RC were extirpated from the fragment shortly after sampling was completed, between January and July 2006. A combination of habitat destruction and hunting by domestic dogs led to the local extinctions of these 2 primate species.

Maps and ground surveys were used to identify households within 0.5 km of each fragment; members of all of these households were invited to participate in the study in 2004. In June and July 2005, concurrent with primate sampling, members of each participating household (n = 99 persons) were given self-contained, sterile bacterial transport systems containing Cary-Blair agar (BD CultureSwab, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and were instructed in the proper method for self-administering a rectal swab. Inoculated swabs were collected and transported to our field laboratory within 24 hours of distribution. At the same time, fresh fecal samples from livestock (n = 60) owned by participating households were collected in sterile tubes and transported within 6 hours to our field laboratory.

At the time of human sample collection, a survey was administered to each participant. The survey focused on demographic information, personal health, patterns of forest use, and interactions with primates during the month before sample collection. The survey was administered in the local language by trained field assistants who were also members of the local communities; researchers were never present during survey administration to avoid response biases associated with the presence of foreigners. This study was reviewed and approved by the institutional review board and institutional animal care and use committee of the University of Illinois before data collection.

Bacterial Isolation and Characterization

Swabs and fecal samples were streaked for isolation of *E. coli* onto individual MacConkey agar plates and incubated at 37°C for 24 hours in our field laboratory. Up to 6 putative *E. coli* colonies from each sample were transferred into tubes containing 0.1 mL tryptic soy agar and stored at room temperature for up to 4 weeks. Isolates were then shipped to the University of Illinois in the United States, re-isolated, subjected to standard biochemical tests for positive identification (*26*), and stored in 20% glycerol at –80°C for further analysis.

Confirmed *E. coli* isolates were genotyped by using Rep-PCR, which targets repetitive sequences dispersed throughout bacterial chromosomes (*27*). This method has high power for discriminating among *E. coli* isolates (*28*,*29*), and it can generate accurate phylogenetic information (*30*). DNA extraction, PCR, and electrophoresis protocols are described in detail elsewhere (*30*).

Analyses

Rep-PCR genotypes were stored in the computer program BioNumerics, version 4.0 (Applied Maths, Austin, TX, USA). Relationships among isolates were inferred from Rep-PCR genotypes by using published methods that maximize the correspondence of such inferences to the standard of multilocus sequence typing (*30*). Population genetic analyses available in the computer program

Arlequin, version 3.0 (*31*), were used to measure genetic differences among bacterial subpopulations. Specifically, analysis of molecular variance (AMOVA; *32*) was used to apportion genetic variation among different ecologically defined bacterial subpopulations, and the common genetic distance measure, F_{ST} (*33*), was used to quantify short-term genetic distances among populations of bacteria from different host species and locations. Complementary phylogenetic analyses were conducted to examine relationships among individual bacterial isolates and to infer directional interspecific transmission; these were performed with BioNumerics and the computer programs PHYLIP, version 3.57c (*34*) and MacClade, version 4 (*35*), following a previously published analytical framework (*36*). Regression analyses were used to investigate the effects of putative demographic and behavioral risk factors on genetic similarity between bacteria from individual humans and bacteria from the primates inhabiting that person's associated fragment (online Appendix Table, available from www.cdc.gov/EID/content/14/9/1375-appT.htm).

Results

A total of 791 *E. coli* isolates from 252 individual persons, livestock, and primates were analyzed, representing (in the case of humans and livestock) 29 households (Table 1). Humans ranged in age from ≈2 months to 77 years and consisted of 48% male and 52% female participants. Sample sizes of primates were low in some locations, but this was inevitable, given small primate population sizes (Table 1). Human and livestock samples represented ≈50% of households surrounding each fragment.

Phylogenetic analysis of bacterial genotypes identified 23 major clades (Figure 2), each containing between 2 and 142 unique genotypes. Some clades contained genotypes specific to particular species or locations; others contained genotypes from multiple species and multiple locations. Of the latter type, those containing isolates from both humans and primates tended to be phylogenetically clustered (Figure 2). Phylogenetic analyses of directional interspecific transmission (*36*) indicated no biases in transmission for different classes of directional transmission events (e.g., human to primate, primate to human). Analyses of molecular variance (Table 2) indicated that differences among species and locations accounted for only a small proportion of total bacterial genetic diversity (7.8% and 6.8%, respectively), and that individual fragments contained most bacterial genetic diversity (85.4%).

Pairwise bacterial genetic distances between metapopulations of primates, persons, and livestock are shown in Table 3. Both humans and livestock harbored bacteria significantly more similar genetically to those of primates in fragments than to those of primates in undisturbed locations within the national park. Humans and their livestock shared very similar bacteria, as indicated by an F_{ST} of only 0.03; this genetic distance was smaller even than that between bacteria from primates in fragments and bacteria from primates in undisturbed forest (F_S = 0.046), although this difference was not statistically significant.

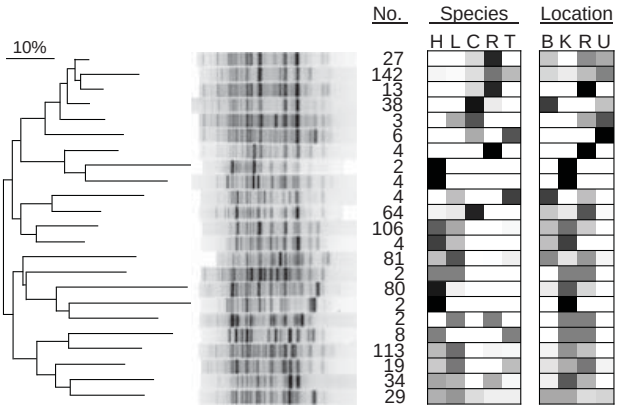

Figure 2. Dendrogram of genetic relatedness among 23 major clades of *Escherichia coli* from humans, domestic animals, and primates in 4 locations in and near Kibale National Park, western Uganda, derived from Rep-PCR genotypes. Major clades were identified from the full tree of 791 isolates by using the "cluster cutoff method" available in the computer program BioNumerics, version 4.0 (Applied Maths, Inc., Austin, TX, USA), which optimizes point-bisectional correlation across a range of cutoff similarity values to identify the most relevant clusters. A single representative bacterial genotype from each major clade is shown, and numbers of isolates falling within that clade are given (no.). Boxes indicate the host species and locations from which isolates in each clade were recovered and are shaded in proportion to the percentage of isolates in the clade from that species or location (0%, white; 100%, black). Species: H, human; L, livestock (cattle or goats); C, black-and-white colobus; R, red colobus; T, red-tailed guenon. Location: B, Bugembe fragment; K, Kiko 1 fragment; R, Rurama fragment; U, undisturbed locations within Kibale National Park. The dendrogram was drawn by using the neighbor-joining method (*37*) from a distance matrix generated from electrophoretic data that used optimized analytical parameters (*30*).

Figure 3 shows the results of interspecies bacterial genetic distance analyses conducted separately for each fragment. Across fragments, bacteria from humans were uniformly genetically similar to bacteria from their livestock. However, genetic similarity between human and primate bacteria varied among fragments. Human–primate bacterial genetic similarity was highest in the Kiko 1 fragment, followed by Rurama, and then by Bugembe. This pattern parallels the relative degrees of anthropogenic disturbance of the fragments themselves (Kiko 1 >Rurama >Bugembe; Table 1). Species-specific analyses (Figure 4) indicated that bacteria from both humans and livestock were more similar to bacteria from red-tailed guenons than to bacteria from black-and-white colobus or red colobus.

Table 2. Hierarchical analysis of molecular variance for *Escherichia coli* isolates collected from humans, nonhuman primates, and livestock associated with 3 forest fragments near Kibale National Park, western Uganda*

Variance component	Observed partition		ϕ statistic	p value†
	Variance	% Total		
Among species	0.672	7.76	ϕ_{CT} = 0.078	<0.001
Among locations within species	0.592	6.84	ϕ_{SC} = 0.074	<0.001
Within locations	7.395	85.41	ϕ_{ST} = 0.146	<0.001

*Bacterial isolates were collected from 5 species in 3 locations. Locations were defined as Bugembe, Kiko 1, and Rurama fragments, and species were defined as humans, livestock (cattle and goats), black-and-white colobus, red colobus, and red-tailed guenons. Data consisted of bacterial genotypes represented as series of binary loci scored for the presence/absence of bands at each of 97 electrophoretic positions, by using the "bandmatch" procedure of the computer program BioNumerics, version 4.0 (Applied Maths, Inc., Austin, TX, USA) and optimized analytical parameters (*30*). Analysis of molecular variance was performed with the computer program Arlequin, version 3.0 (*31*).
†Probability of having a more extreme variance component and ϕ statistic than the observed value by chance alone; probabilities were calculated from 16,000 random permutations of the data by using Arlequin, version 3.0 (*31*).

Four variables were retained in the final regression model that examined associations between human behavioral predictors and human–primate bacterial genetic similarity (online Appendix Table). Residence near a more disturbed fragment was the variable most strongly associated with increased genetic similarity between human and primate bacteria. Tending livestock, experiencing gastrointestinal symptoms, and fetching water from an open water source within the month before sampling were also associated with increased human–primate bacterial genetic similarity.

Discussion

This study provides evidence that forest fragmentation increases bacterial transmission between primates and humans and their livestock. Bacteria from humans and livestock near 3 fragments were more similar genetically to bacteria from primates in those fragments than to bacteria of primates from nearby undisturbed forest locations. Moreover, the degree of disturbance of the fragments themselves paralleled the degree of genetic similarity between human and primate bacteria. Phylogenetic analyses and analyses of molecular variance further indicated that bacterial gene flow among species was high and that no directional biases in bacterial transmission were evident, findings that indicate that transmission of *E. coli* from primates to humans and livestock was as likely as transmission in the other direction.

Chapman et al. (*15*) recently showed that red colobus in forest fragments near Kibale suffer increased gastrointestinal parasitism with helminths as a result of nutritional stress and that this effect has led to a decline in population. Gillespie and Chapman (*12*) documented that the degree of disturbance of a fragment (measured as the density of tree stumps) was an accurate predictor of prevalence of infection of red colobus with parasitic nematodes. However, neither of these studies sampled humans or domestic animals, and neither examined transmission explicitly. The results of our study suggest that fragmentation may exert a heretofore-undocumented negative influence on the risk of primate infection by increasing pathogen transmission rates between primates and other species. Our results also show that the degree of anthropogenic disturbance within a fragment affects the rate at which bacteria are transmitted among species. Fragmentation likely leads to elevated interspecific transmission rates by increasing ecologic overlap among species.

The especially close genetic relationship between bacteria from humans and bacteria from red-tailed guenons (paralleled in livestock) probably reflects the propensity of red-tailed guenons to enter human habitats to raid crops (*25*). Unlike colobines, which can subsist on leaves even in highly degraded fragments, red-tailed guenons, which eat a more varied diet consisting of a high proportion of fruits, are likely obligate crop raiders in fragments. The importance of this species as a crop raider is evident from the fact that persons in our study area engage in a variety of culturally unique practices specifically designed to deter crop raiding, especially of maize, by red-tailed guenons (*38*).

Table 3. Matrix of pairwise interpopulation F_{ST} values for *Escherichia coli* from humans, livestock, and nonhuman primates in Kibale National Park, western Uganda, and 3 nearby forest fragments

Bacterial population	F_{ST} (SEM)*		
	Livestock	Nonhuman primates in forest fragments	Nonhuman primates in undisturbed forest
Humans			
Livestock	0.030 (0.007)[1]		
Nonhuman primates in forest fragments	0.102 (0.024)[2]	0.090 (0.021)[2]	
Nonhuman primates in undisturbed forest	0.180 (0.052)[3]	0.151 (0.051)[3]	0.046 (0.013)[1]

*F_{ST} values (which can vary between 0 and 1) represent short-term genetic distances between bacterial populations and were calculated from Rep-PCR data by using optimal analytical parameters (*30*). Standard errors were estimated from bootstrap analyses with 1,000 replicates. Each of the 6 F_{ST} values shown is statistically significantly different from the null expectation of no genetic difference between populations, based on the bootstrap analysis (all p<0.01). Different superscript numbers indicate significantly different F_{ST} values (exact probabilities <0.05).

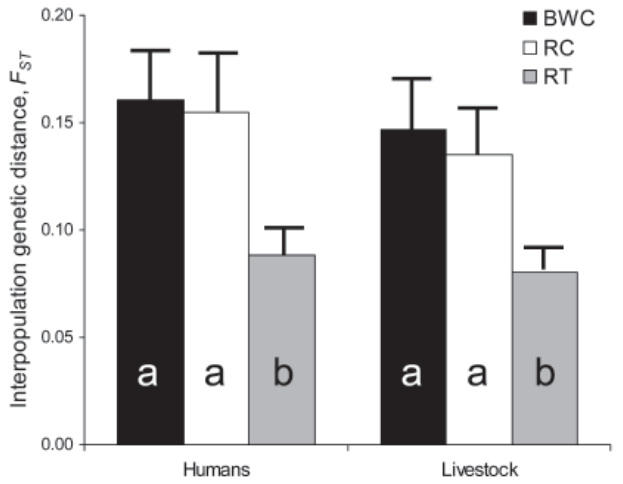

Figure 3. Interpopulation F_{ST} values between *Escherica coli* from humans in villages associated with 3 forest fragments near Kibale National Park, Uganda, and *E. coli* from livestock and primates in the same village or fragment, respectively. F_{ST} values between humans in each village and primates in undisturbed locations within Kibale National Park are shown for comparison. Error bars represent standard errors of the mean, estimated from bootstrap analyses with 1,000 replicates. Different letters within bars indicate statistically significantly different F_{ST} values (exact probabilities <0.05).

We initially suspected that diet and digestive physiology might influence the genetic similarity of *E. coli* among different host species (*39*), but our results do not support this hypothesis. Humans in our study, who as omnivores have single stomachs, harbored *E. coli* virtually indistinguishable genetically from the *E. coli* of their cattle and goats, which are herbivores with chambered stomachs. By extension, similarities in digestive physiology between humans and red-tailed guenons would not be sufficient to account for the close genetic relationship between *E. coli* from these species. We infer that spatial and ecologic overlap is the primary determinant of bacterial genetic similarity among populations of hosts in our system.

Persons who tended livestock and experienced gastrointestinal symptoms during the month before sampling harbored bacteria genetically similar to those of the primates in their associated fragment, whereas persons who did not engage in these activities or experience these symptoms harbored bacteria more distantly related to those of the same primates. Tending livestock, which are often grazed in or near fragments, may bring humans into close contact with primates. Fetching water from an open water source ($p = 0.07$ in our regression analysis; see online Appendix Table) may expose humans to water contaminated with bacteria of primate origin. We note that these variables accounted together for only 28% of variation in human–primate bacterial genetic similarity, indicating that most variation in this parameter remains unexplained.

We emphasize that the results of our risk analysis represent statistical associations and that they do not indicate direction of causality. For example, persons who experience gastrointestinal symptoms such as diarrhea may shed bacteria at high rates and thus be at increased risk of transmitting bacteria to primates; alternatively, persons who ingest microbes from primates might tend to experience gastrointestinal symptoms as a result. We also caution that our results might differ for pathogens more virulent than *E. coli*. For example, gastrointestinal disease would increase shedding of pathogens into the environment and affect host behavior. Our results are best interpreted as reflecting background patterns of bacterial transmission in the absence of confounders such as high virulence. Finally, we caution that assumptions inherent in our statistical analyses could affect the strength of the trends we have documented. For example, our analyses of genetic correspondence assume that parameters derived from maximum likelihood estimation are globally optimal, and our analyses of interpopulation genetic distances assume neutral molecular evolution (*33*).

We suspect that the patterns of bacterial genetic similarity we have documented reflect indirect transmission of microbes through the physical environment, such as through contaminated soil or water, rather than transmission by direct contact. For example, primates in the forest fragments near Kibale must come to the ground to cross open fields (often pastures) between habitat patches or to raid crops; this would increase their probability of encoun-

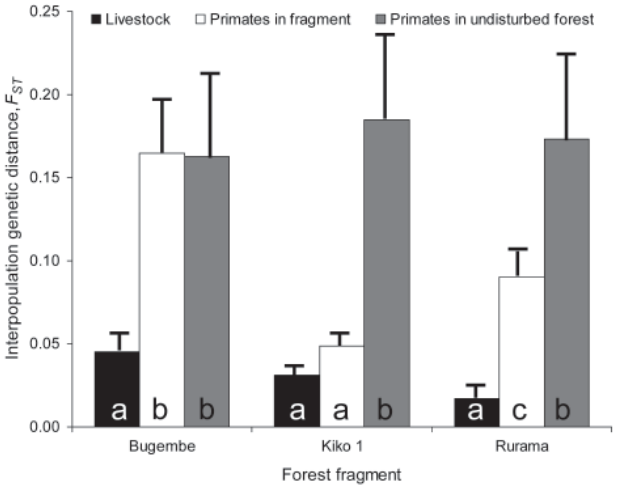

Figure 4. Interpopulation F_{ST} values between *Escherichia coli* from 3 species of primates in 3 forest fragments near Kibale National Park, Uganda, and *E. coli* from both humans and livestock living in villages associated with the fragments. BWC, black-and-white colobus; RC, red colobus; RT, red-tailed guenon. Error bars represent standard errors of the mean, estimated from bootstrap analyses with 1,000 replicates. Different letters within bars indicate statistically significant differences in F_{ST} values (exact probabilities <0.05).

tering bacteria of human or livestock origin. Similarly, the location of fragments in nonarable, wet lowlands creates ideal conditions for contamination of surface water with primate feces. Unfortunately, our varied attempts to recover *E. coli* from water, soil, and vegetation were largely unsuccessful, perhaps because of the heat and aridity of the western Ugandan dry season (*40*).

Zoonotic diseases with primate origins have had global effects on human health (*1*). In Uganda, a high prevalence of HIV renders a significant proportion of the population immunocompromised and thus susceptible to opportunistic infections. Countries like Uganda are also undergoing rapid demographic changes and correspondingly rapid changes in land use. Our finding that a land-use change (forest fragmentation) enhances bacterial transmission between primates and an immunocompromised human population raises concerns about the potential for epidemics of zoonotic disease to originate from disturbed ecosystems such as this. Forest fragmentation may, in other words, negatively affect human public health by increasing the risks for zoonotic disease transmission from animals in forest fragments.

Forests and the primates living in them are disappearing rapidly from this region of Africa, which unfortunately typifies locations throughout the Tropics. We have already documented the extinction of 2 primate species from 1 fragment, and we predict that, without intervention, all unprotected fragments and their primates will disappear from our study area within the next 2 decades. Our results indicate that extinction of local primates may be accompanied by "spikes" in anthroponotic and zoonotic disease transmission risk, which could threaten not only the health of other primates and conservation but also human health. Mitigating these risks could entail such interventions as building closed wells, managing the grazing patterns of livestock, and encouraging the persistence of primate food trees within fragments. Understanding in greater detail how forest fragmentation and associated land-use changes affect pathogen transmission among primates, humans, and domestic animals would be critical for designing rational intervention strategies to conserve wild primates, as well as to safeguard human and animal health.

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Pigs as Source of Methicillin-Resistant *Staphylococcus aureus* CC398 Infections in Humans, Denmark

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An emerging subtype of methicillin-resistant *Staphylococcus aureus* (MRSA), clonal complex (CC) 398, is associated with animals, particularly pigs. We conducted a matched case–control and a case–case study comparing 21 CC398 case-patients with 2 controls randomly selected from the Danish Civil Registry and 2 case-patients infected with MRSA other than CC398. On farms of case-patients, animals were examined for MRSA. Thirteen case-patients reported pig exposure. Living or working on farms with animals was an independent risk factor for CC398 in the case–control (matched odds ratio [MOR] 35.4, 95% confidence interval [CI] 2.7–469.8) and the case–case study (MOR 14.5, 95%CI 2.7–76.7). History of hospitalization was associated with an increased risk only in the case–control study (MOR 11.4, 95% CI 1.4–94.8). A total of 23 of 50 pigs on 4 of 5 farms were positive for CC398. Our results, corroborated by microbiologic testing, demonstrate that pigs are a source of CC398 in Denmark.

Methicillin-resistant *Stapylococcus aureus* (MRSA) is becoming increasingly recognized among persons in the community without established risk factors (*1*,*2*). MRSA primarily causes human disease and animals have not, until now, been considered a source of infection.

It has recently become apparent that animals, particularly pigs, can constitute a separate MRSA reservoir and be a source of a novel and rapidly emerging type of MRSA in humans; namely MRSA clonal complex (CC)398 by multilocus sequence typing (MLST) (*3*). MRSA CC398 consists of 8 MLST types (www.saureus.mlst.net), the predominant type being sequence type (ST)398, and a range of closely related protein A (*spa*) gene types (i.e., t011, t034, t108, and t1793) (*4*,*5*) .

Although transmission appears to be primarily between animals, indistinguishable isolates have been found in their human contacts, particularly those with occupational exposure (*3*–*7*). MRSA CC398 (ST398) was first detected in 4 pigs and 1 healthy pig farmer in France (*3*,*8*). Clinical infection was described in the daughter of a pig farmer in the Netherlands in 2004 (*7*). That study showed that 23% of pig farmers in a small survey in the same region were seropositive for MRSA CC398.

Denmark has a low incidence of MRSA. In 2006, only 706 new MRSA patients (colonization only or infection) were reported, which corresponds to 13/100,000 population (*9*). To maintain this status, Denmark has adopted a strict "Search and Destroy" policy, which includes active screening of at-risk persons at admission to a hospital (*10*). Prompted by reports from the Netherlands, we identified a small (<1% of all MRSA patients) but increasing number of MRSA CC398 human patients after 2003. Furthermore, CC398 was detected in a pig in Denmark in 2006 (*11*). With an annual production of ≈25 million slaughter pigs (www.dst.dk), Denmark has a large potential reservoir for MRSA CC398.

We report results of an analytical study of MRSA CC398, in parallel with systematic farm and microbiologic investigations, to identify risk factors for MRSA CC398 ac-

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quisition in persons in Denmark. Although farm and pig exposure have been postulated as risk factors after interviews with MRSA CC398 patients in other studies (*4*,*6*,*7*,*12*), no previous study has included interviews with control populations to determine if these exposures were higher than would be expected in the general population.

Methods

Surveillance

In Denmark, MRSA isolates from all human patients have been referred to Statens Serum Institut since 1988 for characterization and national surveillance. Epidemiologic and clinical information has been obtained prospectively since 1999 on all patients.

Case-Patients and Controls

We conducted a matched case–control study comparing human case-patients with MRSA CC398 during 2004–2007 with 2 population controls. In parallel, we conducted a case–case study comparing the same MRSA CC398 case-patients to 2 case-patients of community-detected MRSA of a type other than CC398 (non-CC398 case-patients). Eligible case-patients were persons with a confirmed diagnosis of MRSA with *spa* types related to CC398 (carriage or infection) during the study period: October 29, 2003 (first human diagnosed) to May 31, 2007. Where household clusters were identified, secondary case-patients were excluded from the study. Population controls were selected randomly from the Danish Civil Registry System and matched by sex, date of birth, and residence in the same municipality. Non-CC398 case-patients were selected from the national MRSA database and matched by sex, residence in the same region (Zealand, Jutland, or Funen), age group (± 10 years for adults and ± 3 years for persons <18 years of age) according to whether infected or a carrier, and similar time of diagnosis (90% ± 4 months) to limit differential recall bias. When >2 non-CC398 MRSA case-patients were identified within ± 4 months of diagnosis of the case-patient, 2 were randomly selected from the list generated.

Data Collection

After written informed consent was obtained, case-patients, controls, and non-CC398 case-patients were interviewed by using a structured telephone-administered questionnaire. Questions captured demographic and clinical data as well as information on known/identified risk factors for MRSA (including hospitalization and other medical exposures, contact sports, and travel) and hypothesized farm and animal exposures for MRSA CC398 (including living or working on a farm, exposure to farm animals, contact with farm workers, preference for eating, and contact with pets) in the year before case-patient diagnosis. Data were double-entered into Epidata version 3.1 (Statens Serum Institut, Copenhagen, Denmark) and collected and handled according to the requirements of the Danish Data Protection Agency. The study did not require ethical approval.

Statistical Analysis

Univariable and multivariable conditional logistic regression analyses were conducted to estimate matched odds ratios (MORs). Unmatched logistic regression was used for variables for which MORs could not be calculated because not enough controls were exposed to MRSA. Multivariable conditional logistic regression analysis included significant variables (based on a p value of 0.05) from univariable analysis. Stepwise exclusion was used, and variables were tested for significance by using the likelihood ratio test. Stata version 9.2 software (StataCorp, College Station, TX, USA) was used for all analyses.

Farm Investigations

For case-patients, controls, and non-CC398 case-patients reporting contact with production animals, the farm owner was contacted. If the owner consented, nasal swabs were taken from 10 randomly selected animals (from 10 different pens where possible) and tested for MRSA.

Microbiologic Analysis

Human isolates were tested by using PCR to identify the *mecA* gene (*13*), pulsed-field gel electrophoresis (PFGE) with the Harmony protocol (*14*), *spa* typing (*15*), and staphylococcal cassette chromosome mec (SCC*mec*) typing (*16*). Isolates were also tested for *lukF/lukS* genes encoding Panton-Valentine leukocidin (PVL) (*17*). Results of PFGE and *spa* typing were interpreted by using BioNumerics version 4.6 software (Applied Maths, Sint-Martens-Latem, Belgium). Because *spa* typing is acknowledged as being a proxy for MLST, the MLST clonal complex annotation was inferred on the basis of *spa* types. One of the human isolates was typed by MLST for confirmation. Furthermore, a random selection of 7 isolates were tested by PCR for the exotoxin genes *tst*, *eta*, and *etb* encoding staphylococcal toxic shock syndrome toxin 1, exfoliative toxin A, and exfoliative toxin B, respectively (*18*).

Animal swabs were plated directly on CHROM-MRSA agar (Becton Dickinson, Heidelberg, Germany) and blood agar (containing 5% bovine blood) and subsequently placed in selective broth (tryptic soy broth, 2.5% salt, aztreonam [20 mg/L], and cefoxitin [3.5 mg/L]; SSI Diagnostika, Hillerød, Denmark). After incubation for 24 hours, the CHROM-MRSA agar was inspected for putative MRSA. Subcultivation on CHROM-MRSA was conducted with samples from the enrichment broth. Possible MRSA

colonies were subcultivated on blood agar plates, identified by PCR for the *mecA gene* (*13*), and subjected to *spa* typing and SCC*mec* typing.

Human and animal isolates underwent antimicrobial drug susceptibility testing by using disc diffusion (D) with Neosensitabs (Rosco, Taastrup, Denmark) on Danish blood agar (SSI Diagnostika) or microbroth dilution (M) (*19*). Susceptibility tests were performed for tetracycline (D and M), erythromycin (D and M), streptomycin (D and M), kanamycin (D), norfloxacin (D), pencillin (D), clindamycin (D), fusidic acid (D), rifampicin (D), cefoxitin (D), ceftiofur (M), chloramphenicol (M), ciprofloxacin (M), florfenicol (M), spectinomycin (M), sulfamethoxazole (M), tiamulin (M), and trimethoprim (M).

Results

Descriptive Epidemiology

Thirty-one case-patients with MRSA with *spa* types related to MRSA CC398 were detected from October 29, 2003, through February 16, 2007. Of these, 6 were excluded from the study because they were secondary case-patients (3 family clusters). Of the remaining eligible case-patients, we were unable to interview 4 because of a death (n = 1) and refusal to participate (n = 3). The questionnaire was therefore administered to 21 of 25 primary case-patients. Median age of the case-patients was 29 years (age range 8 months to 80 years), and 13 (62%) were female. Three case-patients reported having Dutch relatives, and 2 case-patients had a connection to the People's Republic of China; 1 case-patient was an adopted child from China and another case-patient had adopted a child from China.

Ten case-patients (48%) reported having had an infection, of which all were skin and soft tissue infections. Moreover, sinusitis developed in 1 case-patient, and a severe invasive infection with multiorgan failure after knee surgery developed in another case-patient.

Univariable Analysis

Several exposure variables related to farms and animals were associated with CC398 in the case–control and case–case studies (Table 1). Case–control analysis also identified 4 medical-related risk factors (Table 1). No association was found in the case–control and case–case studies for the following exposures: travel 12 months before diagnosis, working in the healthcare sector, contact with primary healthcare sector (doctor, specialist), visiting an emergency department, presence of a person in the household with a skin condition, presence of a person in the household with staphylococcal infection, smoking daily, contact sports,

Table 1. Statistically significant associations by univariable analysis for infection with MRSA CC398, Denmark*

Exposure variable	No. (%) case-patients exposed, n = 21	Odds ratio (95% confidence interval) for MRSA infection	
		Case–control study, population controls, n = 42	Case–case study, non-CC398 case-patients, n = 39
Animal and farm-related exposures			
Lived or worked on farm with animals	14 (67)	22.1 (2.9–170.3)	11.6 (2.6–51.7)
Worked with animals or meat	11 (50)	16.2 (2.0–127.8)	∞†
Worked on farm with animals	10 (48)	∞†	∞†
Lived on farm with animals	9 (43)	6.9 (1.5–32.8)	7.9 (1.7–36.7)
Exposure to pigs	13 (62)	∞†	∞†
Exposure to cattle	6 (29)	∞†	∞†
Exposure to other farm animals (hens, goats, sheep)	7 (33)	11.1 (1.4–92.4)	5.9 (1.2–28.8)
Provided antimicrobial drugs to animals	10 (48)	∞†	∞†
Contact with farm workers	16 (76)	5.2 (1.4–19.3)	∞†
Contact with farmer	14 (67)	3.2 (1.0–10.6)	∞†
Contact with veterinarian	7 (33)	6.3 (1.3–30.7)	6.6 (1.4–31.8)
Lived in countryside	13 (62)	7.2 (1.5–33.8)	5.2 (1.4–18.9)
Had cat in home	11 (50)	3.2 (1.0–10.6)	3.4 (1.1–9.9)
Used manure in garden	7 (33)	3.2 (0.9–11.0)	6.6 (1.4–31.8)
Visited farm, zoo, or stables	12 (57)	0.8 (0.3–2.4)	4.7 (1.3–17.4)
Medical-related exposures			
Admission to hospital in 12 mo before diagnosis	13 (62)	6.8 (1.9–24.4)	1.5 (0.4–5.2)
Someone in household with chronic condition	12 (57)	3.8 (1.2–12.5)	2.0 (0.8–9.3)
Antimicrobial drug use in 12 mo before diagnosis	9 (43)	3.4 (1.0–11.5)	2.6 (0.8–9.2)
Contact with person with skin sore or other skin infection	5 (26)	8.6 (1–74.9)	0.6 (0.2–1.9)

*MRSA, methicillin-resistant *Staphylococcus aureus*; CC, clonal complex.
†p<0.01, by unmatched analysis.

owning or having contact with dog(s) or horse(s), preference for eating pork, and being born outside Denmark.

Multivariable Analysis

Logistic regression models were applied separately to the case–control and case–case studies. In both studies, the first model only included farm and animal-related exposures: lived or worked on a farm with animals, worked with animals or meat, exposed to pigs, exposed to cattle, exposed to other farm animals, provided antimicrobial drugs to animals, owned cat(s), and had contact with any farm workers. Other farm and animal exposures were excluded because of colinearity. In both case–control and case–case studies, living or working on a farm with animals remained the independent association in this first model. A second model combined living or working on a farm with animals with medical-related exposures: contact with a person having a skin sore or other skin infection, history of hospital admission in the 12 months before diagnosis, antimicrobial drug use in the 12 months before diagnosis, and someone in the household with a chronic condition.

Living or working on a farm with animals was an independent risk factor for CC398 in the case–control study (MOR 35.4, 95% confidence interval [CI] 2.7–469.8) and the case–case study (MOR 14.5, 95% CI 2.7–76.7). A history of hospital admission in the 12 months before diagnosis was associated with an increased risk in the case–control study (MOR 11.4, 95% CI 1.4–94.8) but not in the case–case study.

Farm Investigations

Nine pig farms and 2 cattle herds, with which 10 case-patients had contact, were identified. One case-patient had contact with 2 pig farms, and 2 case-patients had contact with the same pig farm. No controls or non-CC398 case-patients had direct contact with a pig or cattle farm. The owners of 5 pig farms and of the 2 cattle herds agreed to participate in the study. The length of time between date of patient diagnosis and farm sampling was 2–24 months.

Microbiologic Analysis

All but 1 of 31 human isolates were nontypeable by PFGE after digestion with *Sma*I. Twenty-nine isolates had *spa* type t034, including the isolate typeable by PFGE, and the other 2 were related variants of t034 (t108 and t1793). Because of the strong correlation between *spa* typing and MLST, all isolates could be assigned to CC398. One isolate (PFGE nontypeable, *spa* type t034) was typed by MLST and confirmed to be ST398. SCC*mec* typing showed that 24 isolates harbored SCC*mec* type V. SCC*mec* type IV was also found in 2 isolates (PFGE nontypeable, *spa* type t034). Three isolates were *ccr*AB2 positive, which indicated either a type II or type IV variant, but the *mec* class could not be determined. Two isolates were nontypeable. Isolates from the 21 case-patients were *spa* types t034 (SSCmec IV, n = 2, SSCmec V, n = 16, and a type II or type IV variant, n = 1, typeable by PFGE), t108 (SSCmec V, n = 1), and t1793 (SSCmec V, n = 1). These isolates showed considerable variation in antimicrobial drug resistance patterns; most isolates were resistant to tetracycline and erythromycin. All isolates from case-patients who reported exposure to pigs were tetracycline resistant and PVL negative. Two isolates were PVL positive; these were from case-patients who reported a connection to China. All isolates tested for exotoxin showed negative results for all toxins examined.

Twenty-three (46%) of 50 pigs on 4 of 5 sampled farms carried CC398 *spa* type t034. All isolates were resistant to tetracycline and trimethoprim. Pig isolates were indistinguishable or only differed by 1 additional antimicrobial drug class when compared with isolates from case-patients who had contact with them (Table 2). MRSA was not detected in the 2 cattle herds.

Discussion

This study provides compelling epidemiologic and microbiologic evidence that persons living or working on farms in Denmark, particularly pig farms, are at increased risk of being colonized or infected with MRSA CC398. We provide evidence for pigs being a substantial reservoir of human MRSA CC398 in Denmark, as appears to be the case in other European countries such as the Netherlands and France (*3*,*4*,*7*,*12*), and in Canada (*6*).

In the case–case analysis, only animal and farm-related exposures were associated with being a case-patient, which indicates that these exposures are the major factors associated with CC398 acquisition. Furthermore, comparison of results from the case–control study (where farm/animal and medical-related variables remained associated) indicates that medical-related exposures are risk determinants for community-detected MRSA in general but not specifically for subtype CC398. This finding can be deduced because the design of the case–case analysis controls for exposures common to both groups, which means that these exposures will not be identified as a risk or might be underestimated (*20*).

Because of evidence of prolonged MRSA carriage (*21*), questions related to exposures referred to a period of 1 year before patient diagnosis. This lengthy recall period is a limitation of this study. However, all questions related to memorable activities and lifestyle choices; any bias introduced is therefore thought to be minimal.

Our finding that living or working on a farm with animals is associated with CC398 acquisition reinforces results of studies in France, the Netherlands, and Canada that indicated that CC398 is transmissible from animals to humans (*3*,*4*,*6*,*7*). Also in the Netherlands, a CC398 prevalence of 3.9% in 179 veterinarians has been de-

Table 2. Isolate characteristics for human case-patients and contact pig herds sampled, Denmark, March 2007*

Case-patient no.	Date of diagnosis	Resistance pattern	*spa* type	Contact herd	No. isolates	Resistance pattern	*spa* type
1	2005 Mar	tet, ery, cli, str, tmp	t034	A	9	tet, ery, cli, str, spe, tmp	t034
2	2005 Oct	tet, tmp	t034	B	5	tet, tmp	t034
					4	tet, str, tmp	t034
					1	tet, str, tmp	t034
3	2006 Oct	tet, ery, cli, str, spe, tmp	t034	C	1	tet, ery, cli,str, spe, tmp	t034
					2	tet, str, spe, tmp	t034
4	2006 Nov	tet, kan, str, spe, tmp	t034	D	1	tet, ery, cli, kan, str, spe, tmp	t034

*spa, staphylococcal protein A; tet, tetracycline; ery, erythromycin; cli, clindamycin; str, streptomycin; tmp, trimethoprim; spe, spectinomycin; kan, kananmycin.

scribed; all positive persons had recent or regular contact with pigs and cows (*22*). Screening participants from 38 countries at a veterinary conference in Denmark in 2006 found a CC398 prevalence of 9.6% (26/272); the highest prevalence was in German and Dutch delegates (*23*). In comparison, the prevalence of CC398 among delegates attending several national animal conferences in Denmark was 0.7% (4/576) (*24*).

Pig isolates from contact herds were indistinguishable, or only differed by 1 additional antimicrobial drug class, from the isolates from human case-patients who worked or lived on farms where these pigs were located. These samples were obtained months, in some cases years, after the human case-patient's infection was diagnosed, findings that lend weight to the hypotheses that CC398 carriage in animals is unlikely to be transient and that animals are a reservoir of CC398. Although we isolated CC398 from 23 pigs on 4 of 5 pig farms, but not from cattle farms, this type may not be exclusive to pigs. CC398 has been shown to have a prevalence of 39% in pigs in the Netherlands (*25*). However, it has also been found in horses and dogs in Germany and Austria (5) and in cattle and poultry in the Netherlands (*26*,*27*). These findings are particularly interesting because *S. aureus* is usually host specific (*28*).

Our study has highlighted other important human epidemiologic aspects of CC398. The clinical picture for CC398, with ≈50% of case-patients having had an infection and all reporting skin and soft tissue infections, is similar to that seen in community-acquired MRSA isolates in general (*29*,*30*). There was a serious invasive infection with MRSA reported in a man in Denmark after surgery on his knee; arthritis and multiorgan failure also developed (*31*). Serious complications from CC398 have also been described in other reports including ventilator-associated pneumonia (*5*) and endocarditis (*32*). Because no statistical association was found with travel abroad, this finding indicates that CC398 is endemic among pigs in Denmark. Nevertheless, there was an overrepresentation of case-patients or their family members who have had contact with another country, 2 with adopted children from China and 3 case-patients with relatives from the Netherlands. Likewise, in the Netherlands, a child adopted from China was found to be an MRSA CC398 carrier in 2004 (*12*). Three family clusters were identified in the present study, which indicates that CC398 can be transmitted from person to person. This finding is not surprising because of the adaptability of MRSA. Its potential for transmission between humans has also been observed in the Netherlands (*4*,*7*). MRSA has been isolated from dairy products, beef, chicken and pork (*33*–*37*) and although foodborne transmission is plausible (*34*), the risk is thought to be low. Preference for eating pork was not associated with being a CC398 case-patient in our study.

A high degree of variability in the types of CC398 (resistance patterns, *spa* types, and SCC*mec* types) suggests that this type is either rapidly evolving or emerging from a hitherto unrecognized reservoir. In the latter case, CC398 must have been introduced into Denmark on more than 1 occasion or by various routes to explain the high degree of variance. When one considers the rapid adaptability of MRSA, it may only be a matter of time before we see an increased prevalence of CC398 in humans, including those in hospitals as has been recently reported in the Netherlands (*12*). A high prevalence of tetracycline resistance in CC398 patients in contact with pigs has also been observed in the Netherlands; this finding suggests that use of tetracyclines and possibly other antimicrobial drugs in food animals is selecting these multidrug-resistant bacteria (*25*). Two case-patients who were positive for PVL had direct connections to China. To our knowledge, there are no published reports of CC398 patients in China but isolates from pigs have recently been reported from Singapore, with Indonesian origin (*38*).

In conclusion, transmission of CC398 from a zoonotic reservoir to humans could undermine existing MRSA control programs. We therefore recommend increased awareness among healthcare professionals that animals are a possible source of MRSA infection and that the potential for person-to-person spread exists. To limit further spread, pig farmers may warrant screening and isolation on admission to hospitals as has been implemented in the Netherlands (*39*). However, further studies are required to better understand the human and veterinary epidemiology of this emerging zoonosis. Areas of study should include size of the reservoir in pigs, whether other animals constitute a res-

ervoir of CC398, and how frequently CC398 is transmitted from animals to humans and from humans to humans. The European Union baseline survey on the prevalence of MRSA in breeding pigs, initiated in January 2008, is an important step in addressing the first of these points (*40*).

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Ms Lewis is a fellow with the European Programme for Intervention Epidemiology Training at the Statens Serum Institut in Copenhagen. Her primary research interest is intervention epidemiology, particularly in relation to zoonoses.

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Pediatric Parapneumonic Empyema, Spain

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Pediatric parapneumonic empyema (PPE) has been increasing in several countries including Spain. *Streptococcus pneumoniae* is a major PPE pathogen; however, antimicrobial pretreatment before pleural fluid (PF) sampling frequently results in negative diagnostic cultures, thus greatly underestimating the contribution of pneumococci, especially pneumococci susceptible to antimicrobial agents, to PPE. The study aim was to identify the serotypes and genotypes that cause PPE by using molecular diagnostics and relate these data to disease incidence and severity. A total of 208 children with PPE were prospectively enrolled; blood and PF samples were collected. Pneumococci were detected in 79% of culture-positive and 84% of culture-negative samples. All pneumococci were genotyped by multilocus sequence typing. Serotypes were determined for 111 PPE cases; 48% were serotype 1, of 3 major genotypes previously circulating in Spain. Variance in patient complication rates was statistically significant by serotype. The recent PPE increase is principally due to nonvaccine serotypes, especially the highly invasive serotype 1.

Pleural effusions occur in at least 40% of children hospitalized with bacterial pneumonia. Occasionally, the infectious agent invades the pleura to cause pediatric parapneumonic empyema (PPE) (*1*), characterized by the presence of pus. Although rarely associated with fatalities in industrialized countries, PPE often results in prolonged hospitalization and surgical intervention, and patients are at risk for serious and long-lasting illness (*2*,*3*).

An increasing incidence of PPE has been reported in several countries since the mid-1990s (*2*–*6*), but it is not clear why. *Streptococcus pneumoniae* is the most frequently found microorganism in most recent reports. However, conventional microbiologic culture methods have low sensitivity, usually because of antimicrobial pretreatment before sterile-site sampling. Consequently, the contribution of antimicrobial drug–susceptible serotypes might be higher than reported estimates. Molecular and antigen detection-based techniques, including direct molecular typing of culture-negative pleural fluid (PF) samples (*7*), can be useful adjuncts in defining the contributory role of different microorganisms and pneumococcal serotypes to PPE etiology (*4*,*8*).

Our study's goal was to prospectively investigate the molecular epidemiology of pneumococcal PPE among children admitted to 3 of the largest tertiary-care pediatric hospitals in Spain. There were 4 objectives: 1) identify the serotypes and multilocus sequence typing (MLST) genotypes causing PPE and determine whether a temporal change in the circulating genotypes could explain the recent increase; 2) determine whether the causal genotypes were only associated with PPE or also caused other invasive pneumococcal disease (IPD) in the same population, or were carried by healthy children; 3) compare serotypes and genotypes recovered from northern and southern Spain in the context of regional differences in 7-valent pneumococcal conjugate vaccine (PCV7) uptake; and 4) identify any differences between highly invasive serotypes and more opportunistic serotypes with respect to epidemiology and inflammatory markers.

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Methods

Prospective and Retrospective Identification of PPE Cases

PPE cases involving all children <18 years of age at Sant Joan de Deu Hospital Barcelona, Spain, were prospectively enrolled beginning October 1, 2003. PPE patients <14 years of age admitted to Virgen del Rocío Children's Hospital (VRCH) in Seville and Carlos de Haya Children's Hospital (CHCH) in Malaga were prospectively enrolled beginning January 1, 2005. The study period extended to June 30, 2006, in all locations for molecular analyses and through December 2006 for trend analyses. Eligible patients were identified through notification by attending physicians or by clinical microbiology laboratories when a sterile site sample was pneumococcal culture or pneumolysin (*ply*) positive. Participating centers served a pediatric referral population of 607,796 (9% of the corresponding Spanish population).

PPE was defined according to published criteria (*6*). Requirements for thoracocentesis, decortication, administration of fibrinolytics, and antimicrobial drug therapy were determined according to usual clinical practice. Patients with PPE not requiring thoracocentesis or surgical decortication were excluded, as were cases with PF analysis consistent with a transudate. Thoracocentesis was performed by pediatric surgeons, except for acutely ill patients who were tapped in the emergency department or intensive-care unit. PF specimens were sent for routine microbiologic culture and biochemical analysis; remaining fluid was frozen at –80ºC for further molecular testing. Clinical, demographic, and outcome data were collected by using a standardized case report form.

To identify temporal trends, we also retrospectively identified PPE cases at VRCH and CHCH during 1998–2004 using International Classification of Diseases, 9th Revision, codes for empyema (510.0 or 510.9) and chart review. PPE definition and patient exclusion criteria were the same as those used in the prospective study.

Nasopharyngeal Carriage Study

From January 2005 through June 2006, nasopharyngeal (NP) swab specimens were obtained from 635 children 6 months to 6 years of age attending 4 primary healthcare centers for well-child visits and 2 hospital emergency rooms in Seville. Exclusion criteria were chronic medical condition, moderate to severe acute process including fever >39ºC, lower respiratory tract infection, vomiting, dehydration, or other ill appearance. The study was conducted according to World Health Organization recommendations (*9*)

Informed Consent

Written informed consent was obtained from parents or legal guardians of participating children before thoracocentesis or nasopharyngeal swabbing. Hospital ethics committees approved the studies.

Retrospective Analysis of IPD Cases

IPD cases from patients at VRCH and CHCH during 2001–2006 were retrospectively ascertained from microbiology department databases of both centers and confirmed by chart review. Viable pneumococcal isolates were serotyped (70% of cases) by the Spanish Reference Laboratory of Pneumococci and genotyped by MLST (61% of cases).

Testing of PF Samples

Pneumococci were identified by using microbiologic and molecular genotyping methods; susceptibility testing was performed by agar dilution (*10*), and Clinical Laboratory Standards Institute interpretive criteria were used to define susceptibility (*10*). Culture-negative PFs were assayed for the presence of the pneumolysin (*ply*) gene, by using a real-time PCR in Barcelona adapted from Corless et al. (*11*) and a published assay (*12*) in Seville. Conventional serotyping using the Quellung method was performed where possible; culture-negative or incompletely genotyped PFs were serotyped by using a real-time PCR that targets different capsular locus genes (*13*). DNA was extracted from PFs by using 20% wt/vol Chelex-100 resin (Bio-Rad Laboratories, Hercules, CA, USA) in Barcelona and the Nucleospin kit (Clontech Laboratories, Inc., Mountain View, CA, USA) in Seville.

Molecular Genotyping

MLST was performed by using standard methods (*14*), with the exception of a change in PCR primers for the *gdh*, *recP*, *xpt* genes when genotyping PFs directly; in the PCR amplification step, first-round primers from a nested PCR (*15*) were substituted for standard MLST primers to increase sensitivity. Allele and sequence type (ST) designations were made by using the MLST website (www.mlst.net).

Statistical Analyses

Statistical analyses were performed by using SPSS for Windows version 14.0 (SPSS, Inc., Chicago, IL, USA). Reported p values were 2-tailed, and the level of significance was set at 0.05. Analysis of categorical variables was performed with the χ^2 test and Fisher exact test, as appropriate. Continuous variables were compared by analysis of variance followed by a Bonferroni test for multiple comparisons. When data were not normally distributed, we used the Kruskal-Wallis test and conducted posterior comparison between individual groups using the Mann-Whitney U test with the Bonferroni correction. IPD potential was estimated by using a standard odds ratio and Mantel-Haenszel confidence intervals (*16*).

Results

Overall PPE Trends

In Seville and Malaga, the annual number of PPE cases increased 13-fold (5 to 66 cases) during 1998–2006 (Figure 1). In Barcelona, the annual number of PPE cases increased from 11 cases in 2004 to 62 cases in 2006 (data before October 2003 were not available). Over these study periods, no obvious changes in referral patterns, overall pediatric population, guidelines for evaluating children with fever, pneumonia or PPE, or recommendations for performing diagnostic thorachocentesis in children with PPE were found. Table 1 describes the demographic characteristics of the 208 PPE patients prospectively enrolled during the molecular analysis study period (n = 98, Seville and Malaga; n = 110, Barcelona). There were no deaths.

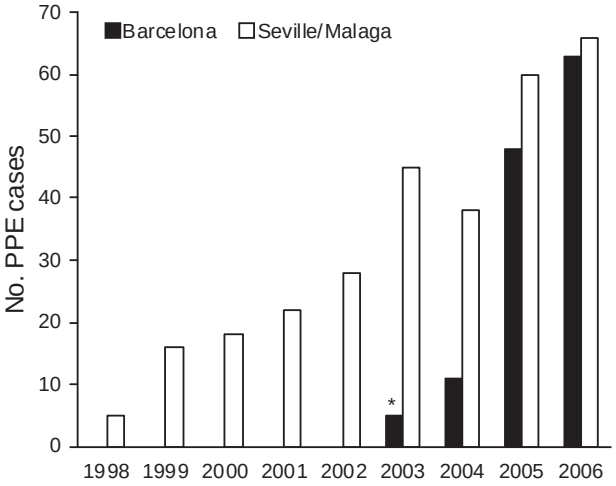

Figure 1. Annual number of pediatric parapneumonic empyema (PPE) cases among children <14 years of age admitted to Seville and Malaga hospitals from 1998 to June 2006 (combined prospective and retrospective data) and among children <18 years of age admitted to a Barcelona hospital from October 2003 through June 2006. *October 1, 2003, through December 31, 2003.

Microbiologic Evaluation

Sixty-seven (32%) patients had positive blood and/or PF cultures for any pathogen, and *S. pneumoniae* was isolated from 53 (79%) of these cases (Figure 2). In 51 of these, a pneumococcal serotype could be identified via the conventional Quellung reaction. Evidence of pneumococcal infection in 99 (84%) of 118 culture-negative PF samples was found on the basis of *ply* or *wzg* gene detection. PPE cases, diagnosed only by *ply* or *wzg* PCR, were significantly more likely to have received antimicrobial drug therapy before PF aspiration than patients with culture-positive pneumococcal PPE (92% vs. 53%; p<0.0001). Of the 99 culture-negative PF samples, 67 (65 *ply*-positive/*wzg*-positive and 2 *ply*-negative/*wzg*-positive) had a sufficient sample to enable serotype testing by PCR. In 52 of these samples, a serotype could be identified. Thus, a pneumococcal serotype was identified in 103 PF samples (Figure 2).

In addition, a predicted serotype based on MLST genotyping was established for 2 cases with negative PCR results and 6 cases for which neither conventional nor PCR-based serotyping was possible (Figure 2). Such predictions were possible because there is a strong relationship between serotype and MLST genotype for most genotypes (*16–18*; www.mlst.net), with the exception of a small number of well-known genotypes that are associated with different serotype variations.

Eighty-one PF samples were fully genotyped, and 18 were partially genotyped (≥4 alleles), by MLST. An ST was identified for 31 of the 99 culture-negative/*ply*-positive PPE. Among these 31 cases, there was full concordance between MLST data and PCR results for confirmation of predicted serotypes (Figure 2). Eighteen PF samples were partially genotyped by MLST: 2 were presumptive serotype 1 pneumococci based on 5–6 loci matching ST228; 1 was a presumptive serotype 5 based on 5 loci matching ST1223; 7 were genotyped at ≥4 loci and serotyped by PCR (serotype 1, n = 5; serotype 7F and 19A, n = 1 each); and 8 samples were partially genotyped at ≥4 loci (indicating presence of a pneumococcus), but PCR serotyping was either negative or not performed. Samples with predicted serotypes based on incomplete genotyping data were not included in further analyses.

Serotype Distribution

Ten serotypes were identified among the 111 PPE cases with tentatively assigned or confirmed serotyping information (Table 2). Non–PCV7 serotypes caused 96 (89%) cases of PPE, including serotype 1, which was detected in 48% of the patient samples. Although a significantly higher proportion of PPE was caused by 7F in Seville and Malaga

Table 1. Demographic characteristics of 208 patients with PPE enrolled during the molecular analysis study period*

Characteristic	Value
Age, mo, mean ± SD (range)	51.8 ± 31 (2–180)
Gender ratio, M/F	1.06
Underlying disease, %†	4
Oral antimicrobial drugs before admission, %‡	29
Antimicrobial drug free before thoracocentesis, %§	23
PCV7 ≥1 dose, %	31
Referral, %	38

*PPE, pediatric parapneumonic empyema; PCV7, 7-valent pneumococcal conjugate vaccine.
†Underlying disease included (no. patients): lymphoma (2), congenital heart disease (2), mild psychomotor retardation (2), varicella zoster infection (2) and genetic disease (1).
‡Median duration: 3 d, range 1–17 d.
§100/147 children who had not been treated with oral antimicrobial drug therapy before admission received intravenous antimicrobial drug treatment before thoracocentesis for a median of 2 d (range 1–10 d).

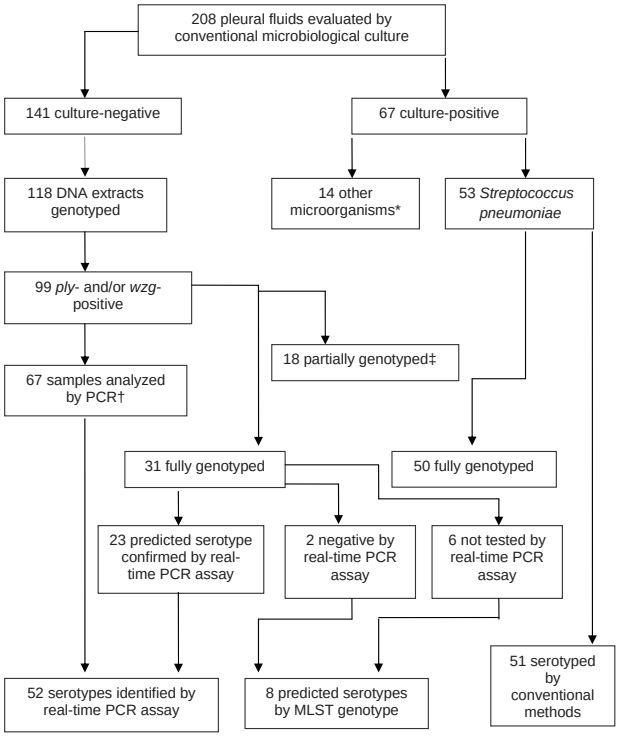

Figure 2. Microbiologic characteristics of pleural fluid specimens from pediatric parapneumonic empyema case-patients. *_Streptococcus pyogenes_ (6), _Staphylococcus aureus_ (3), _Mycobacterium tuberculosis_ (2), _Escherichia coli_ (1), _Streptococcus mitis (1), Peptostreptococcus_ spp. (1). †Pleural fluids analyzed by PCR included 2 samples that were _ply_ negative but _wzg_ positive. ‡18 partially genotyped by multilocus sequence typing (MLST) (>3 alleles), as DNA concentration was too low for reliable PCR amplification and sequencing.

than in Barcelona, the contribution of other serotypes by region was not significantly different (Table 2). PCV7 uptake among PPE patients was significantly higher in Seville and Malaga than Barcelona (40% vs. 22%, p = 0.005), but there were no significant regional differences in vaccination status among children infected with serotype 1 (28% vs. 22%, p = 0.63).

Eight (15%) of 53 cultured pneumococci were intermediately penicillin resistant and 4 (8%) were resistant at high levels. Serotype 1, 3, 5, and 7F pneumococci were uniformly susceptible to penicillin and significantly more common among culture-negative than culture-positive PF samples (89% vs. 71%, p = 0.02).

Genotyping by MLST

Eighty-one PF samples were fully genotyped; 26 STs were identified (Table 3). Three of the major serotype 1 STs (*18*), ST228, ST304 and ST306, were identified, although ST228 was only detected in Seville and Malaga, and ST304 only in Barcelona (Table 3). Serotypes 5 and 7F were represented by globally distributed genotypes ST289 (Colombia^{5}-19) and a closely related single-locus variant, ST1223; and ST191 (Netherlands^{7F}-39), respectively.

Six of 7 serotype 14–positive PF samples were ST156 (Spain^{9V}-3). Genotypic diversity among the serotypes in this study was greatest for serotype 19A; 5 unrelated STs were detected, including ST81 (Spain^{23F}-1). Such variants of ST81 have also previously been detected.

IPD and Nasopharyngeal Carriage in Seville and Malaga

During 2001–2006, 180 cases of IPD involving children <14 years of age were diagnosed with IPD at Seville and Malaga hospitals, and 126 isolates were available for serotyping; 110 of these isolates were also genotyped. Twenty-three percent (29/126) of all IPD was due to serotype 1. Over this period, there was a statistically nonsignificant increase in the proportion of IPD cases due to serotype 1: 17% (2001–2003) vs. 27% (2004–2006), p = 0.19.

Twenty-four serotypes were identified; 10 serotypes caused both PPE and other IPDs (Table 4), and 14 serotypes caused only other IPDs (6B, 11, 13, 15A, 16, 18C, 22, 23A, 23B, 23F, 24, 33, 34, and 38). Serotype 1 isolates were almost exclusively associated with pulmonary disease, including bacteremic pneumonia (12/29, 41%) and PPE (15/29, 52%). The 3 major serotype 1 PPE genotypes were also found among this collection of serotype 1

Table 2. Pneumococcal serotypes identified among pleural fluid samples

Serotype*	Barcelona, no. (%), n = 56	Seville/Malaga, no. (%), n = 55	Total, no. (%), n = 111	p value
1	27 (48)	26 (47)	53 (48)	0.92
7F	3 (5)	11 (20)	14 (13)	**0.02**
3	5 (9)	7 (13)	12 (11)	0.56
5	6 (11)	3 (5)	9 (8)	0.28
14	4 (7)	5 (9)	9 (8)	0.74
19A	6 (11)	2 (4)	8 (7)	0.27
9V	2 (4)	0	2 (2)	0.50
6A	2 (4)	0	2 (2)	0.50
8	0	1 (2)	1 (0.9)	1
19F	1 (2)	0	1 (0.9)	1

*7-valent pneumococcal conjugate vaccine serotypes include 4, 6B, 9V, 14, 18C, 19F, 23F. Pleural fluid samples were collected in Barcelona from October 1, 2003, through June 30, 2006, and in Seville and Malaga from January 1, 2005, through June 20, 2006. Serotypes were determined by Quellung reaction or PCR testing or predicted based on complete multilocus sequence typing, as described in the text. **Boldface** represents a statistically significant result.

Table 3. Sequence types and serotypes among 81 pneumococci detected in pleural fluid

Serotype*	Total	Sequence type (n)
1	43	$306_{\text{and SLVs}}$ (23)† , 228 (11)‡, $304_{\text{and SLVs}}$ (8)†§, 2373 (1)
5	9	$289_{\text{and SLV}}$ (9)†
3	8	180 (5), 260 (2), 2590 (1)§
14	7	156 (6), 17 (1)§
19A	6	276 (2), 81 (1)§, 202 (1)§, 1201 (1)‡, 2013 (1)§
7F	4	191 (4)
6A	2	135 (1)‡, 2377 (1)§
9V	2	838 (2)§

*Included are 8 strains that were culture and PCR negative, or not serotyped, but whose full genotyping by multilocus sequence typing (www.mlst.net) enabled serotypes 1, 5, and 7F to be predicted. These serotypes were predicted because in each case the sequence type (ST) was identical or closely related to a known genotype for serotypes 1, 5 or 7F that have never been identified with anything other than those respective serotypes. These included ST (no.): 306 (2), 228 (1), 2373 (1), 2378 (1), 2561 (1), 1223 (1), and 191 (1).
†SLV, single locus variant (i.e., differs at only 1 MLST locus and thus is a closely related genotype). Major serotypes 1 and 5 ST groups included (no. strains): ST306 (18) and SLVs 2375, 2376, 2378, and 2561 (1 each); ST304 (5) and SLVs 2374 (2), 2371 (1); ST289 (5), and SLV 1223 (4).
‡Recovered only in Sevilla/Malaga.
§Recovered only in Barcelona.

IPD isolates, although ST304 was no longer detected after 2002 and ST306 was first detected in 2003. A retrospective analysis of serotype 1 invasive isolates submitted to the Spanish National Reference Laboratory since 1990 showed ongoing circulation of ST228 and ST304, but ST306 was only detected once before 2000 (1998; unpub. data).

Serotype 14 was the second most common IPD-causing serotype, with an overall prevalence of 17% (23% in 2001–2003 and 12% in 2004–2006; p = 0.12). The major serotype 14 genotype (ST156) identified in PF samples was also detected throughout the entire 2001–2006 period among carriage isolates and in culture-positive IPD cases, mainly causing pulmonary disease (Table 4). Ten (8%) cases of culture-positive IPD were due to serotype 7F, 9 of which were detected after 2004. ST191 was the only serotype 7F genotype in IPD and NP carriage.

Serotype-Specific Differences in Clinical Epidemiology, Inflammatory Markers, and Outcome

PPE-associated serotypes were divided into 3 groups: 1) serotypes 1, 5, 7F, and 14, consistently associated with the highest estimates of serotype-specific high invasive disease potential (HIDP) (*16*,*17*,*19*); 2) serotype 3 alone; and 3) serotypes 6A, 9V, 19A, and 23F, which have a low invasive disease potential (LIDP) (*16*,*17*,*19*). Odds ratio estimates of invasive disease potential demonstrate as much as 60- to 120-fold variation between the most invasive (1, 4, 5, 7, 14, 18C) and the least invasive (3, 6A, 15, 23F) serotypes/serogroups (*16*,*19*).

PPE cases with HIDP serotypes were older than those with LIDP serotypes (median ages 56 and 24 months, respectively; p = 0.0001) (Table 5). Among HIDP PPE cases, 74% were due to serotypes 1 (n = 53) and 5 (n = 9) and comprised children >36 months of age, whereas serotype 14 (n = 9) only caused PPE in patients ≤36 months of age (data not shown; p = 0.0001). Serotype 3 PPE was associated with significantly more complications than PPE caused by HIDP and LIDP serotypes combined (p = 0.004). No other characteristics differed significantly between individual groups (Table 5).

Discussion

In this study, we used molecular techniques to sensitively evaluate PPE epidemiology among a large number of patients in geographically diverse locations of Spain. There was evidence of pneumococcal infection in most of the culture-positive and culture-negative cases of PPE, which was mainly associated with nonvaccine serotype 1 followed by 3, 5, 7F, and 19A, as well as vaccine serotype 14. Serotypes 1, 3, and 14 in particular are well-known PPE-associated serotypes (*2*,*4*,*7*,*20*,*21*). Antimicrobial drug–susceptible serotypes 1, 3, 5, and 7F were overrepresented in culture-negative PF samples, pointing to an important potential bias in PPE surveillance when surveillance is based solely on conventional microbiologic culture methods. Infection with serotype 3 was a risk factor independently associated with PPE complications, a finding also seen in a US study (*22*).

Serotype 1 has also been the most prevalent IPD serotype among Spanish children <14 years of age, representing 5%, 11%, and 27% of all culture-positive pediatric IPD isolates sent to the Pneumococcal Reference Laboratory in 1997, 2003, and 2006, respectively (*23*). However, the increase in serotype 1 disease cannot easily be explained by a vaccine effect, in part because PCV7 coverage was relatively low in both regions for much of the study period. Registered in Spain in June 2001, PCV7 had a low initial uptake that increased to a reported coverage of 34%–45% in 2004–2005 (*24*,*25*).

In addition, increased PPE incidence largely caused by serotype 1 was reported in the United States and the United Kingdom in the decades before PCV7 introduction in 2000 and late 2006, respectively (*4*,*6*,*20*). Previous studies have suggested that the high year-to-year variability of serotype 1 and 5 disease may represent large-scale outbreaks of a cyclical nature (*26*–*28*).

However, the observation in this study that 2 of the 3 MLST genotypes of serotype 1 (ST228 and ST304) had been "resident" in Spain at least since 1990 indicates that serotype 1 PPE increases in Spain were likely not due to a recent introduction of a specific clone. In general, MLST analyses demonstrated that the recent increase in PPE was mainly due to pneumococcal STs previously described to be present in Spain and other European countries for some years (*18*,*27*,*29*–*31*).

Our study has several limitations. First, the limited study period did not enable a longer-term analysis of PPE epidemiology. Second, our analyses relied exclusively on serotype identification and MLST genotyping, neither of which detects differences in virulence factors apart from the serotype. Genetic factors independent of the capsule have been associated with invasiveness and disease severity (*17*,*32*,*33*). Third, other factors that may also modulate the epidemiology of PPE (e.g., differences in case ascertainment between the retrospective and prospective studies, viral infections, or climatic patterns [*34*]) were not evaluated. Fourth, it remains difficult to evaluate the PCV7 impact because reliable written immunization registries detailing the number of administered doses are lacking, and thus vaccine coverage figures mainly come from parent reporting. Finally, the results obtained here may not apply to less severe pneumonia cases, whose etiology may be qualitatively different.

Unfortunately, PCV7 has a serotype coverage of only 11%–14% (including the cross-reactive 6A) in the population of PPE patients. However, conjugate vaccines containing serotypes 1, 5, and 7F, such as the newly developed 10-valent pneumococcal *Haemophilus influenzae* protein D conjugate vaccine candidate (*35*), could increase the

Table 4. Contribution of PPE-associated serotypes and STs to IPD, Seville and Malaga, 2001–2006, and nasopharyngeal carriage in children <6 years of age, Seville*†

Serotype	No. (%) patients with IPD, n = 126	STs detected: diseases detected (no. patients), n = 111	Carriage, no. (%) patients, n = 194	STs detected in carriage (no. patients)	OR (95% CI)
1	29 (23)	**228**: P (7), PPE (6), A (1), B (1) **306**‡ : PPE (7), P (3) **304**§: PPE (1)	1 (1)	**306** (1)	**57.7 (7.7–429.9)**
14	22 (17)	**156**: P (7), PPE (4), M (2) 9: PPE (1) 62: P (1) 124: PPE (1) 2204: M (1)	15 (8)	**156** (12) 409 (1) 1684 (1) 2607 (1)	**2.5 (1.3–5.1)**
7F	10 (8)	**191**: PPE (4), P (3), M (1), B (1)	3 (2)	**191** (3)	**5.5 (1.5–20.3)**
19A	10 (8)	**276**: PPE (2), M (2), P (1) **202**: B (1) **1201**: PPE (1)	13 (7)	**276** (2) **202** (3) **1201** (1) 199 (2) 433 (2) 392 (1) 2109 (1) 2609 (1)	1.2 (0.5–2.8)
3	6 (5)	**260**: PPE (1), P (1), M (1) **180**: PPE (1)	7 (4)	**180** (3) **260** (2) 2200 (2)	1.6 (0.5–4.6)
6A	5 (4)	1150: M (1) 1668: S (1) 1876: M (1)	24 (12)	338 (8) 386 (5) 1876 (3) 224 (2) 327 (1) 392 (1) 448 (1) 473 (1) 2201 (1) 2611 (1)	**0.29 (0.1–0.79)**
5	3 (2)	**289**: PPE (2) **1223**: P (1)	2 (1)	**289** (1) 1540 (1)	2.3 (0.39–14.2)
19F	3 (2)	87: C (1), B (1) 88: M (1)	8 (4)	**81** (3) 87 (1) 179 (2) 63 (1) 2615 (1)	0.57 (0.15–2.2)
9V	1 (1)	**838**: B (1)	3 (2)	**838** (3)	0.5 (0.05–5)
8	1 (1)	53: M (1)	0		–

*Culture-positive isolates only for IPD. **Boldface** indicates a statistically significant result and represents STs that were identified among pleural fluids during the study period in Seville, Malaga, and Barcelona. An OR demonstrating the potential for each serotype to cause invasive disease, relative to its prevalence in nasopharyngeal carriage, was also calculated (*16*). Serotypes 6A, 19F, and 9V were associated with PPE in Barcelona (but not Seville and Malaga) and are included here for a complete list of invasive serotypes with any association to PPE among the 3 locations; however, data presented here are only from Seville and Malaga. Serotypes identified in IPD cases but not among children with PPE: 6B, 11, 13, 15A, 16, 18C, 22, 23A, 23B, 23F, 24, 33, 34, 38.
†PPE, pediatric parapneumonic empyema; IPD, invasive pneumococcal disease; ST, sequence type; OR, odds ratio; CI, confidence interval; A, arthritis; B, occult bacteremia; P, pneumonia; M, meningitis; S, sepsis; C, orbital cellulitis.
‡First detected in 2003.
§First detected in 2002.

Table 5. Characteristics of children hospitalized with PPE, by serotype category, excluding patients with serious underlying disease (n = 3)*

Characteristic	HIDP serotypes, n = 84	Serotype 3, n = 11	LIDP serotypes, n = 13	p value
Median age, mo (range)	55.6 (2–180)	37.9 (9–71)	24 (2–36)	0.0001†
Median hospital stay,‡ d (range)	13 (4–38)	15 (9–29)	10 (6–24)	0.042§
Complications, % patients¶	10	45	0	0.004#

*PPE, pediatric parapneumic empyema; HIDP, high invasive disease potential; LIDP, low invasive disease potential. HIDP serotypes: 1, 5, 7F, and 14; LIDP serotypes: 6A, 9V, 19A, and 19F (*16,17,19*). All results shown were statistically significant (p<0.05). There were no significant differences between groups for the following variables: median days febrile preadmission; preadmission antimicrobial therapy; intensive care unit admission; mean leukocyte count; mean C-reactive protein; mean pleural fluid glucose; mean pleural fluid pH; mean lactate dehydrogenase; median days to thorachocentesis; referral; primary fibrinolytics or thoracoscopy; or oxygen requirement >4 d.
†HIDP was compared with LIDP by post hoc analysis.
‡Since being admitted to first center.
§No significant differences between individual groups by post hoc analysis (p = 0.023 for comparison between serotype 3 and LIDP)
¶Complications included (no. patients): bronchopleural fistula (3), pyopneumothorax (2), pneumatoceles (4), lung abscess (1), mechanical ventilation >48 h (2), severe anemia requiring blood transfusion (2), severe hypoalbuminemia requiring seroalbumin replacement (1).
#Serotype 3 compared withHIDP and LIDP groups combined.

serotype coverage for PPE up to 80%; the subsequent addition of serotypes 3 and 19A in vaccine candidates currently in development would add an additional 18% of coverage (*35*). Finally, continued epidemiologic surveillance with molecular diagnostic techniques will be crucial to understanding this serious pediatric disease.

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Circulation of 3 Lineages of a Novel Saffold Cardiovirus in Humans

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Cardioviruses cause serious disease, mainly in rodents, including diabetes, myocarditis, encephalomyelitis, and multiple sclerosis–like disseminated encephalomyelitis. Recently, a human virus isolate obtained 25 years ago, termed Saffold virus, was sequenced and classified as a cardiovirus. We conducted systematic molecular screening for Saffold-like viruses in 844 fecal samples from patients with gastroenteritis from Germany and Brazil, across all age groups. Six cardioviruses were identified in patients <6 years of age. Viral loads were 283,305–5,044,412,175 copies/g of stool. Co-infections occurred in 4 of 6 children. No evidence for outbreak-like epidemic patterns was found. Phylogenetic analysis identified 3 distinct genetic lineages. Viral protein 1 amino acids were 67.9%–77.7% identical and had a distance of at least 39.4% from known cardioviruses. Because closely related strains were found on 2 continents, global distribution in humans is suspected. Saffold-like viruses may be the first human cardiovirus species to be identified.

The family *Picornaviridae* comprises 9 genera with >142 species and 200 serotypes, many of which are highly pathogenic for humans and animals. The genus *Cardiovirus* contains 2 animal-pathogenic species— *Encephalomyocarditis virus* and *Theilovirus*—that occur mainly in rodents and swine. The type species is *Encephalomyocarditis virus*, which includes strains of murine encephalomyocarditis virus (EMCV), Mengo virus, and Maus Eberfeld virus. The species *Theilovirus* is represented by Theiler's murine encephalomyelitis virus (TMEV, also known as mouse poliovirus) and rat encephalomyelitis virus. Both species show clinical association with encephalomyelitis in mice, and EMCV shows an additional association with myocarditis (*1*). EMCV is used in laboratory mice to model the symptoms and pathogenesis of human type I diabetes and viral myocarditis (*2*,*3*). TMEV comprises strains of differing neuropathogenicity, which constitute accepted mouse models of either human acute poliomyelitis or disseminated encephalomyelitis. The latter is indistinguishable from multiple sclerosis in humans (*4*,*5*).

No human-pathogenic cardiovirus is recognized today. Isolation of EMCV-like viruses from mammals other than rodents and pigs has been reported in the past (*6*–*9*), but the clinical relevance of these sporadic findings has been doubted, especially findings involving humans. TMEV-like cardiovirus may have been involved in an apparently infectious neurodegenerative disease in persons living in Vilyuisk, Siberia (*10*). A virus related to TMEV, named Vilyuisk virus, was isolated from a laboratory mouse that had been injected intracerebrally with blood and cerebrospinal fluid (CSF) of a symptomatic patient (*11*,*12*). However, serum antibodies to Vilyuisk virus were found only in some but not all Vilyuisk encephalitis patients by a mouse neutralization assay (*13*–*15*). Therefore, controversy remains on whether the virus really circulates in humans or whether the isolate may have resulted from mouse passage.

Recently, the genome of another cardiovirus, the Saffold virus, was characterized (*16*). This virus was isolated in 1982 from a stool sample of a child with fever of un-

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known origin. No mouse passage was involved, but the original stool sample had been passaged several times in Wistar Institute–38, human fetal diploid lung–645, and human fetal diploid kidney cells. No associated study has been conducted to determine whether this singular cell culture isolate had any clinical meaning. Most recently, Abed and Boivin reported that a cardiovirus similar to Saffold virus was identified from a cell culture showing cytopathic effects but reacting only weakly with antienterovirus serum pools (*17*). Specific screening identified the same or a closely related virus from ≥2 cell cultures. All cultures had been injected with respiratory secretions from children with respiratory disease. The report summarized 3 clinical cases but did not address prevalence, disease association, or molecular–epidemiologic aspects of the virus.

In this study, we used broad-range nested reverse transcription–PCR (RT-PCR) targeted at domains conserved between the Saffold prototype virus, various TMEV strains, and EMCV. We screened 844 patients from all age groups with acute enteritis, including 39 controls, in 3 independent cohorts from 2 countries (Germany and Brazil) on 2 continents. Viral loads were determined by specific real-time RT-PCR. Phylogenetic analysis showed 3 independent lineages of circulating Saffold-like viruses (SafVs). Analysis of amino acid identities and considerations regarding transmission patterns suggest that SafV most likely constitutes a new cardiovirus that is associated with humans worldwide.

Materials and Methods

Patients and Samples

Cohort 1

From January through December 2004, 538 stool samples were collected from patients in urban areas in northern Germany in a prospective study on acute, community-acquired diarrhea. All patients were outpatients who had been examined by general practitioners; 96 (17.8%) were ≤6 years old. Diarrhea in these patients was defined as excretion of at least 2 loose and malodorous stools during 24 hours for breastfed infants and of at least 2 loose stools in a 24-hour period for all other patients. Patients were excluded if they had inflammatory bowel disease, celiac disease, cystic fibrosis, food intolerance, or a known malignant disease. The cohort included stool samples from 39 control patients of compatible ages with conditions other than enteritis. Written informed consent was obtained from all patients or their parents. The study protocol and data handling were approved by the local ethics committee. In both groups, patients with norovirus, adenovirus, enterovirus, astrovirus, or rotavirus infection were excluded.

Cohort 2

Archived stool samples from 118 patients with acute enteritis were obtained from the routine diagnostic laboratory of a municipal health service in Hamburg, Germany. This cohort contained 3 subcohorts classified with regard to patient age and context of sampling: 1) children sampled during childcare center outbreaks (n = 51); 2) adults sampled in the context of outbreaks of gastroenteritis mainly in association with catering and canteen food (n = 35); and 3) senior citizens sampled because of outbreaks of enteritis in retirement homes (n = 32).

Cohort 3

This cohort contained 188 samples from infants and children in Brazil with acute diarrhea, defined as >3 watery stools in the previous 24 hours and within 13 days before admission. Patients were seen as outpatients or were hospitalized because of severe dehydration from February through December 2006 at the University Hospital Professor Edgar Santos in Salvador de Bahia, Brazil. Informed consent was obtained from the mothers of all patients enrolled in the study. The study was approved by the institutional ethics committee. All analyses were performed at the Infectious Disease Research Laboratory, University Hospital Professor Edgar Santos.

Co-infection in the Brazil cohort was assessed by using recently published methods of real-time RT-PCR for norovirus, rotavirus, enterovirus, parechovirus, adenovirus, and astrovirus (*18*–*23*). For the Germany cohorts, testing was done with the IDEIA rotavirus, adenovirus, and astrovirus antigen enzyme immunoassays (DakoCytomation, Ely, UK) and nested RT-PCRs as described before (*23*). All samples had been stored at –20°C and thawed a few times before this study.

Preparation of Stool Samples for RT-PCR

Stool samples stored at –20°C were extracted by using the QIAamp DNA Mini Stool Kit or the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Both protocols used an input of ≈200 mg of stool prediluted 1:10 in phosphate-buffered saline. Suspensions were vortexed and centrifuged, and 200 or 140 μL of supernatant, respectively, was extracted according to the manufacturer's instructions.

Nested RT-PCR for Cardiovirus Screening

Primers (Table 1) were designed upon aligning the Saffold virus (GenBank accession no. EF165067) (*16*) with genomes of EMCV and TMEV strains. Saffold virus served as the template sequence. Formulations of both rounds of amplification are shown in Table 1. Although the first round alone was sufficient for amplification of an 800-bp fragment in samples with an apparently high viral load,

Table 1. PCR oligonucleotides and formulations for cardiovirus screening*

ID no.	Sequence (5′ → 3′)	Position†	Orientation	Usage
CF188	CTAATCAGAGGAAAGTCAGCAT	188–209	+	Nested RT-PCR, 1st round‡
CF204	CAGCATTTTCCGGCCCAGGCTAA	204–226	+	Nested RT-PCR, 2nd round§
CR718	GCTATTGTGAGGTCGCTACAGCTGT	718–742	–	Nested RT-PCR, 2nd round§
CR990	GACCACTTGGTTTGGAGAAGCT	990–1011	–	Nested RT-PCR, 1st round‡
CF723	TGTAGCGACCTCACAGTAGCA	723–743	+	Real-time PCR¶
CR888	CAGGACATTCTTGGCTTCTCTA	888–909	–	Real-time PCR¶
CP797	FAM-AGATCCACTGCTGTGAGCGGTGCAA-BHQ1	797–821	+ (probe)	Real-time PCR¶

*ID, identification; RT-PCR, reverse transcription–PCR; FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1.
†Relative to Saffold virus EF165067 genome.
‡25-μL reactions used the QIAGEN OneStep RT-PCR kit (QIAGEN, Hilden, Germany), with 400 nmol/L each of 1st-round primers CF188 and CR990, 1 μL enzyme mix, 1 μg bovine serum albumin, and 5 μL RNA extract. Amplification involved 30 min at 50°C; 15 min at 95°C; 10 cycles of 20 s at 94°C, 30 s starting at 60°C with a decrease of 1°C per cycle, and 50 s at 72°C; and 40 cycles of 20 s at 95°C, 30 s at 54°C, and 50 s at 72°C with a final elongation step of 5 min at 72°C.
§50-μL reactions used 1 μL of 1st-round PCR product, with 1x Platinum Taq buffer (Invitrogen, Karlsruhe, Germany), 200 μmol/L deoxynucleoside triphosphates each, 2.5 mmol/L $MgCl_2$, 400 nmol/L each of 2nd-round primers CF204 and CR718, and 1 U Platinum Taq polymerase. Amplification involved 3 min at 94°C and 45 cycles of 20 s at 94°C, 30 s at 60°C, and 40 s at 72°C.
¶25-μL reactions used 3 μL of RNA extract, 1x reaction buffer and enzymes from the QIAGEN OneStep RT-PCR kit, 600 nM of primer CF723, 400 nM of primer CR888, and 160 nM of probe CP797. Cycling in an Applied Biosystems 7700 SDS instrument involved the following steps: 55°C for 15 min, 95°C for 15 min, and 45 cycles of 95°C for 15 s/58°C for 30 s.

only the nested protocol was able to amplify all samples with lower viral load and possible PCR inhibition (500-bp fragment).

SafV Real-time Quantitative RT-PCR Assay

Various combinations of primers and probes were designed manually upon inspection of the SafV prototype sequence EF165067 (*16*) and the newly sequenced SafV isolates from this study. Optimal primer and probe combinations and reaction conditions were determined experimentally. The final formulation is shown in Table 1. For the calculation of absolute virus RNA concentrations in stool samples, efficiencies of RNA recovery for both RNA purification kits were evaluated by spiking known amounts of RNA in vitro transcripts into different cardiovirus-negative stool samples and comparing the quantification results with those obtained from direct usage of the unextracted in vitro transcripts. Correction factors were 1/5 for the Viral RNA kit (i.e., 20% RNA recovery) and 1/250 for the DNA stool kit, indicating poor RNA recovery with the latter. The projected equivalent amount of stool tested per PCR vial, receiving 3 μL of RNA eluate, was 0.3 mg or 0.3 μL (see description of nucleic acid extraction).

P1 Gene Amplification and Sequencing

Based on the published genome of Saffold virus EF165067 and the 5′ untranslated region sequences obtained from our positive samples (nested PCR), primers spanning the complete viral protein 1 (VP1) gene were designed. cDNA was produced by using the Superscript III Kit (Invitrogen, Karlsruhe, Germany) and an ≈4-kb fragment was amplified by using the Expand High Fidelity Plus Kit (Roche, Penzberg, Germany). This PCR product was sequenced directly from both sides by using primer walking. All primer sequences are available upon request.

In Vitro Transcribed RNA Standard

The 800-bp 5′-noncoding region fragment from sample BR/118/2006 was ligated into pCR 2.1 (Invitrogen) and TOPO-cloned. Plasmids were purified, sequenced, and reamplified with plasmid-specific primers. Reamplification products were transcribed into RNA with a MegaScript T7 kit (Ambion, Austin, TX, USA). After DNase I digestion, RNA transcripts were purified with QIAGEN RNeasy columns and quantified photometrically. Sensitivity of real-time RT-PCR was determined to be in the single-copy range when purified and quantified in vitro transcripts were amplified.

Cardiovirus Strains and Accession Numbers

The following sequences were used for analysis and primer design: Saffold virus (EF165067), TMEV strain DA (M20301), TMEV strain GDVII (M20562), TMEV strain BeAn (M16020), Vilyuisk virus (M94868), Theiler-like virus of rats NGS910 (AB090161), Mengo virus (L22089), and EMCV (X87335). Several other subgenomic sequences of TMEV and EMCV were added in alignments for PCR primer design. At the time of preparation of this article, the polyprotein sequence of a Canadian virus isolate related to Saffold virus was described, AM922293 (*17*). This sequence was added to the phylogenetic analyses. The complete P1 sequences from 4 of the SafVs identified in this study could be determined and are available at GenBank under accession nos. EU681176–EU681179.

Virus Isolation

Virus-positive samples from Germany were subjected to virus isolation on a range of cell cultures as described earlier (*23*). However, no virus isolates were obtained. It was suspected that the stored samples had been frozen and thawed too many times because isolation of co-detected adenoviruses and enterovirus was also unsuccessful.

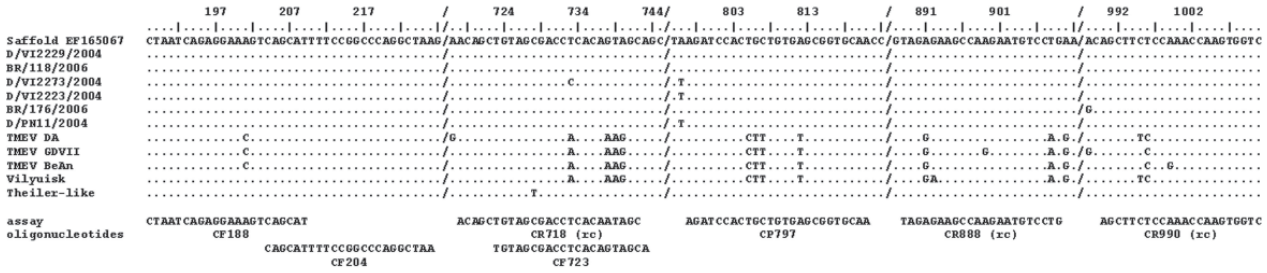

Figure 1. Nucleic acid alignment of the hybridization sites of diagnostic reverse transcription–PCR oligonucleotides. Oligonucleotides are shown below the alignment panel. The base count in the top line is based on Saffold virus, which also serves as the comparison sequence in the alignment. Dots represent identical bases in compared sequences; deviations are spelled out. A slash (/) represents a gap in the alignment; (rc) means that the reverse complementary sequence is shown for the antisense primer.

Results

A nested RT-PCR was designed on the basis of a recently published sequence of a prototype human cardiovirus, the Saffold virus. The 5′-noncoding region of this sequence was aligned with that of other cardioviruses, including TMEV, EMCV, and Mengo virus. Primers were placed in regions conserved among the original Saffold virus sequence and various theiloviruses (Figure 1). Because several members of the *Picornaviridae* family are transmitted by the fecal–oral route, the search for human cardioviruses was focused on patients with gastroenteritis. Samples from pretested cohorts of patients from 2 continents were examined (Table 2).

In the first cohort, 538 stool samples were tested from 538 outpatients of all ages who had acute enteritis in absence of common enteric virus infections. Stool samples from 39 asymptomatic patients served as controls. All patients were observed by practitioners in northern Germany and were not selected for an association with outbreaks of gastroenteritis. Samples from neither patients nor controls yielded virus. A second cohort from Germany comprised 118 patients from all age groups sampled in the context of outbreak investigations by a municipal health center (Table 2). Samples from 4 children yielded cardioviruses in a subcohort of 51 children from childcare centers. No virus was found in subcohorts of 35 adults sampled because of catering kitchen outbreaks, and 32 patients from retirement homes, respectively. In the third cohort, 188 children (1–60 months of age) from an outpatient clinic in Salvador de Bahia, Brazil, were examined; 2 (1.1%) and had positive test results for a cardiovirus.

All patients with positive results for cardiovirus were retested by a quantitative real-time RT-PCR for SafV, which was designed after sequencing of the 5′-noncoding regions of all viruses. Clinical information and the resulting viral load data are summarized in Table 3. All cases occurred in children ≤6 years of age who had symptoms of gastroenteritis. Both cases in Brazil occurred during the rainy season, when rainfull was frequent and temperatures were 20°–26°C. All case-patients in Germany were seen in November, when temperatures were ≈5°C and rainfall was frequent.

A broad range of viral loads was observed (283,305-673,009,359 copies/mL or gram of stool). Co-infections with enteric viruses occurred in both patients from Brazil and in 2 of the 4 patients from Germany. Average viral loads in patients with and without co-infections were 1.7×10^8 and 2.5×10^9 per mL or gram of stool, respectively. The difference was not significant at the 95% confidence level (1-way analysis of variance, $p = 0.19$).

Only 1 child had concomitant respiratory symptoms. A nasopharyngeal aspirate from this child taken at the same time as the positive stool specimen was tested by real-time RT-PCR for SafV; results were negative.

To appreciate the genetic range of cardioviruses in our patients, we first sequenced an 800-bp fragment containing 80% of the viral 5′-noncoding region from all 6 samples. The complete P1 gene could then be sequenced from 4

Table 2. Characteristics of samples obtained from gastroenteritis patients, 2004 and 2006

Location	Origin of samples	Patient age range	No. patients	Cardiovirus prevalence, %
Brazil	Hospital outpatient department	1–60 mo	188	1.1
Germany	General practitioners	1–98 y	538*	–
	Kindergartens	1–144 mo	51	7.8
	Catering kitchens	16–65 y	35	–
	Retirement homes	74–98 y	32	–

*This cohort contained no patients with predetected enteric viruses (refer to Materials and Methods section); 39 healthy controls were included.

Table 3. Characteristics of cardiovirus-positive patients, Germany and Brazil, 2004 and 2006*

Patient ID	Sampling date	Sampling site epidemiologic context	Patient sex	Patient age at sampling	Clinical symptoms	Viral co-infections†	Virus concentration‡
BR/118/2006	2006 Aug 2	Salvador, Brazil	M	6 wk	Gastroenteritis	Adenovirus, norovirus	33,373,329
BR/176/2006	2006 Oct 2	Salvador, Brazil	F	4 y	Gastroenteritis, URTI§	Norovirus	283,305
D/VI2273/2004	2004 Nov 9	Outbreak, childcare center, Altona, Hamburg Germany	M	2 y	Gastroenteritis	Adenovirus	673,009,359
D/VI2223/2004	2004 Nov 2	Single case, pediatric outpatient, Hamburg Germany	M	2 y	Gastroenteritis	None	59,687,364
D/VI2229/2004	2004 Nov 1	Single case, kindergarten, Bergedorf, Hamburg Germany	F	4 y	Gastroenteritis	None	5,044,412,175
D/PN11/2004	2004 Nov 15	Family outbreak, Bergedorf, Hamburg Germany	M	6 y	Gastroenteritis	Enterovirus	3,093,024

*ID, identification; URTI, upper respiratory tract infection.
†All samples were tested for norovirus, rotavirus, adenovirus, astrovirus, parechovirus, and enterovirus.
‡Viral RNA copies in ≈1 g/1 mL of stool.
§Respiratory tract sample testing negative for Saffold-like virus.

samples. Samples D/PN11/2004 and BR/176/2006, which showed the lowest virus concentrations, did not yield P1 gene PCR products on several trials.

In addition to the lineage containing the prototype Saffold virus (hereafter referred to as the Saf-1 lineage), ≥2 genetic lineages were identified (Figure 2). A second lineage (Saf-2 lineage) comprised the strain from Germany, D/VI2229/2004, the strain from Brazil, BR/118/2006, and the isolate from Canada, AM922293. A third lineage (Saf-3) was clearly differentiated from Saf-1 and Saf-2. It comprised the viruses D/VI2223/2004, D/VI2273/2004, and D/PN11/2004, although the last viruscould be sequenced only in the 5′-noncoding region (the tree for the 5′-noncoding region is not shown because it provides little additional information for virus classification).

In several genera of *Picornaviridae*, the degree of nucleotide and amino acid identity in the P1 protein gene or in VP1 alone is used as a criterion of taxonomic classification. Table 4 shows amino acid identities of strains of SafV, theilovirus, and EMCV in VP1. The degree of identity between theilovirus and EMCV was the same as that between encephalomyocarditis virus and SafV, ≈50%. The lowest degree of identity was seen between Saf-3 and EMCV at 46.7%. The maximum degree of identity between strains of theilovirus and SafV was up to 60.6%. Within the 2 established cardiovirus species, the lowest degree of identity between strains was observed between Vilyuisk virus and Theiler-like virus of rats, at 69.6%. The lowest degree of identity between SafV strains was 67.9%, as observed between both representatives of Saf-3 and the original Saffold virus (Saf-1). Lineages Saf-1 and Saf-2 were 77.3%–77.7% identical in their P1 protein genes.

Discussion

In parallel with a recent report on the detection of SafV cardioviruses in 3 children (*17*), we investigated in this study the prevalence of these agents in defined patient cohorts. We gained evidence that cardioviruses circulate in the human population and that they are genetically diversified at a level similar to recognized cardiovirus species. They can be subdivided in 3 types and may constitute a novel cardiovirus species.

On the basis of the initial isolation of the prototype Saffold virus (*16*) from fecal material, we analyzed 844 stool samples from Brazil and Germany by broad-range nested RT-PCR. Cumulative prevalence in all age groups was 0.71%. However, both in Germany and Brazil no virus was detected in patients >6 years of age. Virus prevalence in all children up to 6 years was 1.84%. This age spectrum was in concordance with the 4 case-patients reported in earlier studies, who were 8 months, 19 months, 23 months, and 4 years old (*16*,*17*).

This age distribution is consistent with epidemiologic patterns seen for other picornaviruses that have comparably low antigenic variability and high attack rates, e.g., certain enteroviruses and human parechoviruses (*25*,*26*). These viruses infect a large part of the young human population and rarely infect adults. Adaptive immunity rather than conditions of exposure (sanitation, food safety) likely determines probability of infection, making exposure conditions less relevant in outbreak settings (*23*). Consistently, we did not observe a different prevalence between Germany and Brazil (where hygienic conditions and food safety are supposedly inferior). Moreover, even though all 4 viruses from Germany were obtained from samples taken within a 10-

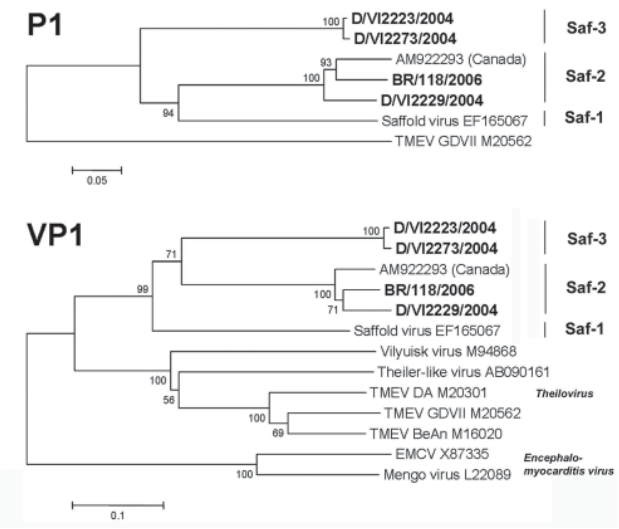

Figure 2. Phylogenetic relationships in the P1 and viral protein 1 (VP1) genes. Analysis was done by using a neighbor-joining method with pairwise deletion for gaps, and 1,000 bootstrap reiterations for confidence testing. Bootstrap confidence values are depicted next to root points. Branch lengths are proportional to the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses were conducted by using MEGA version 4 (*24*). For cardiovirus isolates from GenBank, accession number is shown after isolate identification number. For economic reasons, only for VP1 is the whole cardiovirus genus depicted. New strains from this study are shown in **boldface**. TMEV, Theiler murine encephalomyelitis virus; EMCV, murine encephalomyocarditis virus. Scale bars indicate number of substitutions per site.

day period in a single city, they were clearly distinct from each other and belonged to different genetic lineages. Thus, no evidence of outbreak-like transmission was found. All viruses in our study were isolated from samples obtained in the cold or rainy season, when low UV irradiation and the crowding of persons favor virus transmission. This supports the notion that transmission of SafV from person to person may be more relevant than transmission through food or water.

Stool samples of 4 of 6 children with SafV showed at least 1 viral co-infection with typical enteric viruses, indicating that SafV may not have been the only cause of the observed gastroenteritis. This conclusion is also supported by the fact that patients with single infection had no higher viral loads than patients with co-infection. It remains to be determined whether the enteric tract might be more important for replication and shedding of virus than for primary pathogenesis; at least for now, any such conclusion would be premature. Nonetheless, the high viral load observed in stool samples of our patients suggests a role of the fecal–oral route for transmission. Further studies are clearly needed to investigate disease association of SafV. Such studies would greatly benefit from the inclusion of a control group without clinical symptoms of diarrhea, and, if possible, of greater size than the group included in our study. Moreover, studies on virus prevalence should be complemented by serologic surveys that use neutralization tests, as soon as these become available.

Notably, in 3 cases reported recently from Canada, virus was isolated from respiratory specimens from children with respiratory symptoms (*17*). In our study, only 1of 6 patients exhibited symptoms of upper respiratory tract infection, and no cardiovirus could be detected by PCR from nasal secretions. Future studies should address systematically whether SafV is associated with respiratory disease.

The molecular ecology of SafV seems especially relevant in view of the diversified and strain-dependent pathogenetic changes caused by the related TMEV in rodents. Neurovirulent strains, such as GDVII, cause an acute encephalomyelitis in mice, resulting in a high proportion of deaths. Persistent strains like BeAn and DA cause a chronic demyelinating disease that provides an experimental animal model for multiple sclerosis in humans (*4*,*27*–*30*). These drastically different disease patterns seem to be determined by conformational changes in the outermost structures of VP1 and VP2 (*31*). Even minimal genetic alterations may affect disease attenuation (*30*,*32*,*33*). Analyzing the genetic diversity of cardioviruses in humans appears highly relevant.

Our study shows that 3 different genetic lineages of SafV are circulating, which suggests nonrecent virus diversification in humans. These findings indicate that a true virus–host relationship exists, rather than sporadic or accidental spillover of a virus that resides in another animal (as observed with EMCV in humans; *8*). Support of a genuine human association is also provided by the occurrence of closely related members of the same lineage (Saf-2) in Brazil and in Germany. Such wide distribution requires efficient transmission of virus from human to human. In combination with findings of the virus in the United States (*16*) and Canada (*17*), a proposal that the distribution of SafV is global seems reasonable.

The level of diversification between SafV genetic lineages is clearly higher than the 20% amino acid distance in the VP1 protein, which resembles the distance between serotypes of enteroviruses or types of human parechoviruses (*1*,*34*,*35*). In analogy with human parechoviruses, one could thus look at the SafV lineages defined in this study as types (SafV-1 to 3 in analogy to human parechovirus types 1 to 6). We suspect that these types may also be discriminated by differential cross-neutralization properties as soon as neutralization tests become available. Types could then be redefined to serotypes.

The amino acid distance between isolates from the 2 established cardiovirus species, *Encephalomyocarditis virus* and *Theilovirus*, was ≈50%, whereas the distance between theilovirus and SafV isolates was 40%. However, genetic

Table 4. VP1 amino acid identity between sequences*†

Taxon/species	Strain	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]
Saf-1	[1] Saffold original													
Saf-2	[2] SafV Canada	77.3												
	[3] D/VI2229/2004	77.3	98.5											
	[4] BR/118/2006	77.7	97.8	98.2										
Saf-3	[5] D/VI2273/2004	67.9	73.4	73.1	73.1									
	[6] D/VI2223/2004	67.9	73.4	73.1	73.1	100.0								
Theilovirus	[7] TMEV GDVII	57.9	58.3	58.3	58.3	56.7	56.7							
	[8] TMEV DA	59.1	61.0	60.6	60.6	57.8	57.8	90.9						
	[9] TMEV BeAn	57.2	58.7	58.7	59.0	56.7	56.7	92.8	93.1					
	[10] Rat Theiler-like	59.0	57.9	57.9	57.9	55.9	55.9	72.8	73.7	74.3				
	[11] Vilyuisk	60.3	56.6	56.6	57.0	57.4	57.4	71.7	71.5	71.7	69.6			
Encephalomyocarditis	[12] EMCV	48.4	49.3	49.3	49.3	46.7	46.7	49.4	50.2	49.4	49.1	49.6		
virus	[13] Mengo	49.1	50.7	50.7	51.1	47.4	47.4	50.9	51.3	50.9	49.8	50.4	95.7	

*VP1, viral protein 1; SafV, Saffold-like virus; TMEV, Theiler's murine encephalomyelitis virus; EMCV, murine encephalomyocarditis virus.
†The percentage of amino acid identity per site from analysis between sequences is shown. All results are based on the pairwise analysis of 13 sequences (pairwise deletion option). Analyses were conducted by using MEGA version 4 (*24*). The final dataset contained a total of 287 positions. GenBank accession numbers are given in the Materials and Methods section.

distance is not the only criterion for classifying cardiovirus species. The clear subdivision into types and, most critically, the likely association with a different host (human instead of rodent), makes it appear not unlikely that SafV may be classified as a new cardiovirus species in the future. However, more genetic, ecologic, and functional analysis must be done before such a conclusion can be reached.

In recent years, several novel viruses have been discovered in humans, mostly by advanced molecular screening (*36*–*38*). Despite intensive clinical study, some of these viruses still cannot be associated with clinically relevant disease. Our study shows that SafV is circulating in humans, but we cannot prove any clinical relevance from our data. However, 2 facts suggest that it may be rewarding to look for SafV disease associations in specifically selected cohorts of patients. First, 2 groups independently have isolated the virus on cell cultures, which suggests that the agent may replicate in a range of human tissues (*16*,*17*). Notably, most recently identified viruses that show no overt disease association do not grow in culture (*36*–*38*). Second, the murine cardioviruses, and especially TMEV, the closest relative to SafV, display a range of clinical associations that are dependent on strain properties (*4*,*27*–*30*). The existence of high and low pathogenic variants most likely provides advantages in the interplay between host population density, herd immunity, and viral replicative fitness. The overall genetic range of SafV observed in this preliminary genetic characterization seems to exceed that of both species, *Theilovirus* and *Encephalomyocarditis virus.* Research into the human disease association of SafV should therefore receive high priority in the clinical virology community.

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Excretion of Transmissible Spongiform Encephalopathy Infectivity in Urine

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The route of transmission of most naturally acquired transmissible spongiform encephalopathy (TSE) infections remains speculative. To investigate urine as a potential source of TSE exposure, we used a sensitive method for detection and quantitation of TSE infectivity. Pooled urine collected from 22 hamsters showing clinical signs of 263K scrapie contained 3.8 ± 0.9 infectious doses/mL of infectivity. Titration of homogenates of kidneys and urinary bladders from the same animals gave concentrations 20,000-fold greater. Histologic and immunohistochemical examination of these same tissues showed no indications of inflammatory or other pathologic changes except for occasional deposits of disease-associated prion protein in kidneys. Although the source of TSE infectivity in urine remains unresolved, these results establish that TSE infectivity is excreted in urine and may thereby play a role in the horizontal transmission of natural TSEs. The results also indicate potential risk for TSE transmission from human urine–derived hormones and other medicines.

Transmissible spongiform encephalopathies (TSEs) are fatal neurologic diseases. In humans, a long asymptomatic incubation period is followed by a progressive clinical course that typically lasts a few months to a year. TSE infectivity and pathologic changes are concentrated in the nervous system; however, much of the transmission risk results from parenteral exposure to the much lower concentrations of infectivity found in tissues outside the nervous system. Thus, despite the very low concentration of TSE infectivity in blood (*1*,*2*), 4 human cases of transmission of variant Creutzfeldt-Jakob disease through blood transfusions have been documented (*3*,*4*). If TSE infectivity were excreted, human urine, which is a source of injectible fertility hormones and other drugs (*5*,*6*), could also pose a risk for transmission. Infected urine might also account for the horizontal transmission of sheep scrapie and might contribute to the natural spread of chronic wasting disease in deer and elk.

Early attempts to transmit Creutzfeldt-Jakob disease by cross-species inoculation of rodents and primates with urine from diseased patients failed (*7*,*8*). More recent attempts in which urine from infected hamsters was injected back into hamsters have produced variable results (*9*,*10*). Two other studies have reported infectivity in urine (*11*) and infectivity with disease-specific prion protein (PrP^{d}) in kidneys of mice with simultaneous scrapie and nephritis but not in those with scrapie alone (*12*). To resolve these discrepancies, we used a highly sensitive and precise method of measuring low concentrations of TSE infectivity, which we have successfully used for quantitation of TSE infectivity in blood (*1*,*2*), to measure the concentration of TSE infectivity in urine of scrapie-infected hamsters.

Materials and Methods

Urine Collection and Processing

Urine was collected from a cohort of 22 Syrian hamsters (Harlan Sprague-Dawley, Haslet, MI, USA) that had been infected by intracranial injection with 10% (wt/vol) scrapie brain homogenate (263K strain) and from a cohort of 8 age-matched, noninoculated control animals. At the time of urine collection, the scrapie-infected hamsters showed clear clinical evidence of disease but were still able to drink and eat (67–74 days postinoculation). Hamsters were placed 2 at a time for 24 hours in metabolism cages in which they had access to water but not food. Food was

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withheld to prevent contamination of the urine. Urine was maintained at 4°C during collection. Separate metabolic cages (Rat metabolic cage no. 2100-R; Lab Products, Seaford, DE, USA) were used for each cohort. The urine produced daily was stored at –80°C. The individual collections were then combined into clinical and control pools of ≈60 mL and ≈125 mL, respectively.

Limiting Dilution Titration of Urine

We used the limiting dilution method of titration developed in our laboratory to measure the concentration of TSE infectivity in urine (*1*,*2*). In this method, a relatively large volume of low-titer sample is injected intracerebrally, 50 μL at a time, into a large cohort of weanling hamsters. Immediately before animal inoculation, aliquots of the clinical and control urine pools were thawed and sonicated on ice with separate sterile ultrasonication probes for each pool. Sonication was for 4 cycles of 15 s on and 10 s off for 1 min of total sonication, using a microtip probe at 40% amplitude (Vibra-Cell 750W; Sonics & Materials, Newtown, CT, USA). Two milliliters of control urine was injected undiluted into 40 hamsters. Clinical urine (urine from hamsters showing clinical signs of disease) was diluted 1:3 with inoculation buffer (phosphate-buffered saline [PBS] supplemented with 1% fetal calf serum and 1× penicillin and streptomycin) to remove concentration-related toxicity. Five milliliters from the clinical urine pool was diluted to 15 mL, and the entire volume was injected into 300 hamsters, 50 μL/animal. Soon after inoculation, 8 animals inoculated with urine from the infected animals died, which left 292 animals in the study. All inoculations were conducted under anesthesia with pentobarbital (40–90 mg/kg). At each step the control urine was processed before the infected urine.

All animals were assessed weekly for early signs of scrapie. At the first signs of disease, animals were separated from their cage mates, observed daily for disease progression, and euthanized after disease was confirmed clinically. After 559 days postinoculation all remaining animals were euthanized. Brains were collected from all animals in the study and assayed for infection-specific, proteinase K–resistant prion protein (PrP^{res}) by Western blot or ELISA, using a dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) developed in our laboratory (described below). The infection status of each animal was tabulated, and the probabilities of infection and titer were computed as described (*1*,*2*; Table 1).

Tissue Collection and Processing

Kidneys and urinary bladders were harvested from each of 12 infected animals that donated urine either 71 or 76 days postinoculation. Animals were euthanized by asphyxiation with CO_2. The bladder was removed first and immediately frozen in liquid nitrogen. The kidneys were collected next; the renal capsule was removed before freezing the tissue in liquid nitrogen. Both tissues were dissected aseptically with a clean, sterile set of instruments for each animal and each organ; particular care was taken to not touch other organs or tissues. The tissues (12 bladders and 19 kidneys) were pulverized with a cryomill by using separate cryo-capsules for each tissue (Cryogenic Sample Crusher, Model JFC-300; JAI, Tokyo, Japan). The tissue powder was stored at –80°C until use.

End-Point Dilution Titration of Tissues

Pooled bladder powder (1.65 g) and pooled kidney powder (0.64 g) were separately mixed with homogenization buffer (PBS, pH 7.2) to make 10% (wt/vol) tissue suspensions before sonication at 40% amplitude, using separate sterile microtip probes for each homogenate. The kidney homogenate was prepared according to the same schedule of sonication used for the urine pools. The bladder homogenate was sonicated for 10 s, repeated 2 times (20 s total sonication time) at room temperature. Longer sonication times or delays in the injection of the bladder homogenate caused the sample to solidify, which made it impossible to dilute and inject. Immediately after sonication the homogenates were serially diluted 10-fold in inoculation buffer, and each dilution was injected into hamsters in 1 to 5 cages (4 hamsters/cage) for titration by end-point dilution (Table 2).

All dilutions were by weight. The study was terminated at 426 days postinoculation, and the infection status of each animal was confirmed by Western blot of the brain for PrP^{res}. The titers were calculated by the methods of Reed and Muench (*13*), Pizzi (*14*), and Spearman and Karber (*15*).

PrP^{res} Detection Procedures

Immunoblotting

Individual brains were homogenized in PBS, pH 7.2, to 10% (wt/vol) by using a FASTH homogenizer (Consul AR; Villeneuve, Switzerland) according to the manufacturer's instructions. To test for PrP^{res}, brain homogenate

Table 1. Titer of urine from scrapie-infected hamsters

Hamster	Volume assayed, mL	Fold dilution	Volume inoculated, mL	Total no. hamsters	No. infected hamsters	Titer, ID/mL*	SD†
Infected	4.87	3	14.6	292	18	3.8	0.9
Noninoculated	2	None	2	40	1	–	–

*ID, infectious dose. Titer = –ln(P(0)) × (1/v), where P(0) = (noninfected animals)/(total animals inoculated) and v = inoculation volume, 0.05 mL.
†SD = square root (titer/V), where V = 4.87 mL, the total volume of the undiluted urine inoculated (*1*).

Table 2. End-point dilution titration of urinary bladder and kidney from scrapie-infected hamsters

Dilution	Total/no. infected	
	Bladder	Kidney
10^{-1}	19/19	4/4
$10^{-1.3}$	8/8	20/20
$10^{-1.7}$	8/8	8/8
10^{-2}	4/4	8/8
10^{-3}	4/4	4/3
10^{-4}	4/2	4/1
10^{-5}	4/1	4/0
10^{-6}	4/0	4/0
Titer ($\log_{10}$ ID_{50}/g)*	5.5	5.0
Standard error	0.5	0.4

*ID_{50}, 50% infectious dose. Titers calculated by the Reed and Muench method (*13*); standard errors by the Pizzi method (*14*).

was digested with proteinase K at 0.1 mg/mL final concentration as described by Gregori et al. (*1*). Sample buffer containing 2% sodium dodecyl sulfate was added, and the samples were heated at 100°C for 10 min and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blots of the samples were developed by using anti-PrP 3F4 monoclonal antibody (Covance, San Diego, CA, USA) for PrP detection (*1*).

ELISA

After proteinase K digestion and heat denaturation as described for immunoblotting, the samples were diluted 100-fold in assay buffer (DELFIA Assay Buffer; PerkinElmer, Waltham, MA, USA). They were then assayed for PrP concentration by DELFIA by using a Wallac Victor V instrument (PelkinElmer) for signal detection, with purified 3F4 monoclonal antibody (Covance) as the capture antibody and purified 7D9 monoclonal antibody (Covance) labeled with Europium according to the manufacturer's instructions (PerkinElmer) as the detection antibody. The molar ratio of Europium:7D9 antibody was 7.4:1 (*2*).

Histologic and Immunologic Tissue Preparation

Formalin-fixed brains were cut and divided on the midline; 1 hemisphere was cut in the sagittal plane; the other was cut coronally at the anterior basal ganglia, the middle of the thalamus, and the brainstem with cerebellum. Spleens, kidneys, and bladders were divided in the middle. All blocks were embedded in paraffin and processed for conventional staining with hematoxylin and eosin and Luxol fast blue/nuclear fast red (for brain) as well as for immunohistochemical detection of PrP with monoclonal anti-PrP antibody 3F4 (1:1,000; Covance). For detection of PrP^{d}, sections were pretreated with 30 min of hydrated autoclaving at 121°C followed by 5 min in 96% formic acid. Immunostained sections were counterstained with hematoxylin.

Animal Husbandry and Decontamination Procedures

Animals were maintained in a Biosafety Level 3 (BSL-3) animal facility at the Veterans Affairs Medical Center in Baltimore, Maryland, USA. Standard operating procedures specifically designed for TSEs, including TSE select agents, were followed. The operation of this facility has been described in detail (*16*). Animal cages were changed once a week, and cages and bedding were decontaminated by autoclaving for 1 h at 134°C. The sonicator probes and dissection instruments were decontaminated by autoclaving for 2 h at 134°C immersed in 2 N NaOH, followed by cleaning, repackaging, and sterilizing. All laboratory surfaces were decontaminated before use with either 2 N NaOH or LpH (Steris Corporation, Mentor, OH, USA) (*16*).

Results

Urine Titration

Urine collections from infected and control animals were combined into separate pools. Pools minimized the possibility of an idiosyncratic measurement from an individual and serve as a resource for future experiments once the titer has been determined. Clinically affected animals consumed lower amounts of water and produced 4–5-fold less urine than control animals. This resulted in slightly elevated specific gravity, proteins, glucose, and ketones as measured with a standard urine dipstick. Elevated urine ketones may also have been caused by fasting. The higher concentration of the urine pooled from infected animals resulted in a toxicity that required a 3-fold dilution in buffer before it could be injected.

TSE developed in 18 of the 292 animals that survived the injection of the 3-fold diluted infected pool. Incubation times are shown in Figure 1. As observed in other studies (*1*,*2*), scrapie incubation times for animals infected with low-titer samples begin at ≈150 days and rarely extend past 500 days. None of the animals from either the infected or noninfected cohorts that survived to the end of the experiment were positive by DELFIA. None of the 24 animals that died during incubation without clinical evidence of scrapie were positive for scrapie infection by Western blot. Only those animals with clinical scrapie had the typical PrP^{res} signal in the brain as assessed by Western blot. The infectivity titer of the urine as calculated from the Poisson distribution was 3.8 ± 0.9 infectious doses (ID)/mL (Table 1).

Scrapie developed (at 425 days postinoculation) in 1 of the 40 hamsters inoculated with control urine. Because none of the control donor animals contracted scrapie and because their brains were negative for PrP^{res}, it is clear that this infection resulted from contamination. However, the contamination was unlikely to have been environmental. Our BSL-3 is managed under a strict regimen of continuous decontami-

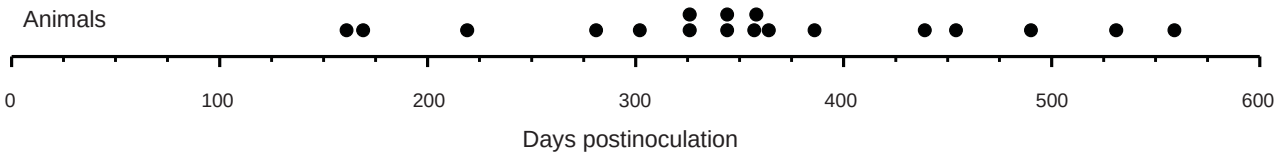

Figure 1. Distribution of incubation times of hamsters infected by injected urine. Each dot represents 1 animal with clinical scrapie that was euthanized at the corresponding day postinoculation. The 22 additional animals that died during the incubation period and the 252 animals that survived to the end of the experiment (559 days) showed no clinical or immunochemical evidence of scrapie and were scored as scrapie negative.

nation and precautionary cleaning (*16*). As evidence of the effectiveness of these measures, we have conducted several titrations, involving hundreds of animals each, in which there were no infections at all during ≥540 days of incubation. One such study was ongoing during the titration of the urine pools reported in this study (*2*). If there are environmental sources of infectivity, the concentration is below the level of detection by the data accumulated in infection-free titrations to date. Instead, after an intensive review of our procedures, we concluded that the most likely source of this contamination was a technical lapse during collection of the urine pools. The level of contamination (1 infection/2 mL of control pool injected vs. 18 infections/4.87 mL of clinical pool injected) is consistent with a pooling error at the time of collection. Nevertheless, had it been an environmental contamination, the associated titer (0.51 ID/mL SD = 0.50 ID/mL) would have had a negligible effect on the value determined for the infected urine.

Tissue Titrations

The concentration of scrapie infectivity in hamster urine is similar to that in plasma of scrapie-infected hamsters at the same stage of disease, which suggests plasma as a possible source of the infectivity. To investigate other possible sources, we also measured the concentration of TSE infectivity in separate pools of kidneys and bladders collected from the same donor animals. The titrations were by the end-point dilution method. The titers calculated by the methods of Reed and Muench (*13*) and Pizzi (*14*) were $10^{5.5 \pm 0.5}$ 50% infectious doses (ID_{50})/g of bladder and $10^{5.0 \pm 0.4}$ ID_{50}/g of kidney. The Spearman and Karber method gave almost identical values (*15*).

Histologic and Immunohistochemical Examination of Tissues

Others have reported TSE infectivity in the urine of scrapie-infected mice with nephritis but not in infected mice without nephritis (*11*,*12*). In contrast, our hamster colony in general, and the animals in this experiment, showed no evidence of inflammation, as indicated by clinical assessments or urine parameters. Nitrates were within normal limits, and no leukocyturia was noted. Proteinuria in the clinical hamsters was likely the consequence of low-volume urine excretion. To further assess whether hamsters infected with scrapie were also affected by kidney inflammation or other abnormalities of the urinary system, we examined the kidneys and the urinary bladders of 8 scrapie-affected hamsters at 84 days postinoculation and 4 preclinically infected hamsters at 49 days postinoculation for PrP^d by immunohistochemical and histologic methods (Figure 2). We also examined control tissues from 10 age-matched uninoculated animals as well as brain and spleen tissues from infected and control animals.

All tissues were evaluated for signs of inflammation and for the pattern of PrP^d immunoreactivity; brains were also examined for spongiform change. No inflammatory changes were found in any tissue examined. In 9 infected animals (clinical and preclinical), we noted nidus formation in the lumina of the bladder with a few neutrophilic granulocytes. However, leukocytes had not invaded the wall of the bladder. Nidus formation is often associated with dehydration.

PrP immunoreactivity was not observed in the bladder wall of scrapie-infected or control animals (data not shown). Spongiform change and deposition of PrP^d was lacking in control animal brains (Figure 2, panel A) and was noted to various extents, according to the stage of the disease, in all scrapie-infected animal brains (Figure 2, panel E). We observed fine synaptic PrP^d immunoreactivity with focal patchy or plaque-like appearance in gray matter structures, but we also noted ependymal, subependymal, perivascular, and white matter PrP^d deposits (data not shown). PrP^d immunoreactivity was observed in the germinal centers of the spleen of all scrapie-infected animals (Figure 2, panel F) but not in those of controls (Figure 2, panel B). None of the control animals exhibited immunoreactivity for PrP^d in the kidneys (Figure 2, panels C, D). PrP^d immunostaining showed fine granular deposits in the collecting tubules of the medulla (Figure 2, panels G, H) in 4 (50%) of 8 animals in the clinical stage of scrapie and in 3 (75%) of 4 animals in the preclinical stage, for a total of 7 (58.3%) of 12 scrapie-infected animals.

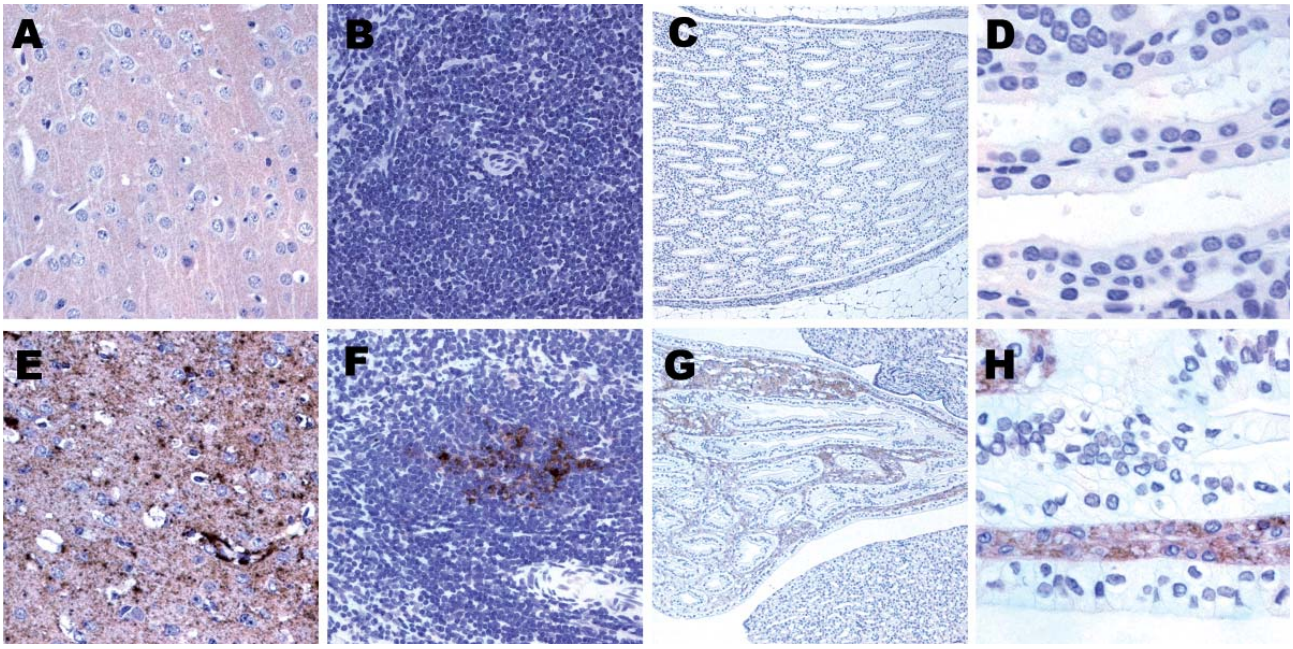

Figure 2. Immunostaining for prion protein (PrP) in control and scrapie-infected hamsters. Deposition of disease-associated PrP is lacking in the brain (A), spleen (B), and kidneys (C,D) of control hamsters. Fine synaptic and plaque-like PrP immunoreactivity in the frontal cortex (E), granular immunoreactivity in the germinal center of spleen (F) and in the collecting tubules of kidneys (G,H) in a representative scrapie-infected animal. Original magnification ×200 for panels A, B, D, E, F, and H and ×40 for panels C and G.

Discussion

Anticipating that the titer of scrapie infectivity in excreted urine would be low, we measured concentration by using limiting dilution titration, a method with which we have extensive experience quantitating TSE infectivity in blood and blood components. In a limiting dilution titration, all animals in the bioassay are inoculated with the highest concentration of inoculum that is tolerated by the intracranial (most efficient) route. Infectivity assorts randomly into the inoculated animals; provided that at least some, but not all, of the animals are infected, the concentration can be calculated from the Poisson distribution of the infections (*1*). The method is highly sensitive and far more precise than other methods of TSE titration. We considered concentrating the urine before bioassay, but to circumvent uncertainties about the recovery of endogenous infectivity, we decided to inject the urine as collected.

We found TSE infectivity in the urine of hamsters that had no evidence of kidney or bladder inflammation. In contrast, Seeger et al. did not detect infectivity in the urine of scrapie-infected mice (*11*) unless the mice were also affected by nephritis, in which case they found low levels of infectivity. Whether the bioassay they used was capable of detecting infectivity at the concentration we observed for hamsters is not clear. If it was not capable, then detection of infectivity in mice with nephritis implies a higher concentration of infectivity in urine excreted by a nephritic kidney. In another study, urine and feces from deer with chronic wasting disease failed to demonstrate infectivity when orally given to the same susceptible species (*17*). Although usually an inefficient route of inoculation, the oral route did successfully transmit chronic wasting disease infectivity in saliva. The authors identified several possible reasons for the unsuccessful transmission by excreta, including incubation time, genotype, or sample size.

In our experiments, cross-contamination by feces can not be excluded as a source of infectivity. Although the metabolism cage effectively separated urine and feces, some contact is possible because of the anatomy of the hamster.

Protein misfolding cyclic amplification uses sonication to generate PrP^{res} and infectivity in vitro. Although we routinely disperse all samples by ultrasonication before injection, our conditions are much harsher than those used to generate PrP^{res} de novo (*18*) and do not support protein misfolding cyclic amplification of PrP^{res}, or presumably infectivity (L. Gregori and R.G. Rohwer, unpub. data).

The kidney and bladder titers were far greater than expected compared with findings of historical studies in which, with only rare exceptions (*19–21*), most attempts at transmission have been unsuccessful. These titers cannot be explained by the infectivity in residual blood (10 ID/mL) (*1*,*2*). In addition, we observed PrP^{d} in the kidneys of scrapie-infected animals that had no indications of tissue inflammation. Heikenwalder et al. found PrP^{d} staining within

follicular infiltrates only in kidneys of mice affected by nephritis and not in control mice with noncomplicated scrapie (*12*). These data together with those by Seeger et al. (*11*) suggested that renal inflammation might be a prerequisite for TSE infectivity in renal tissue and its excretion in urine. In contrast, our results indicate that renal inflammation is not necessary for the deposition of PrP^d in kidneys or for excretion of infectivity. One interpretation is that nephritis enhances the accumulation of PrP^d at sites of inflammation, consistent with the excretion of higher levels of infectivity inferred above for this same condition (*11*).

Two studies of scrapie in naturally and experimentally infected sheep reported PrP^d depositions in the renal papillae (*22*) and in the intraepithelial cortex, medulla, and papillae (*23*). Similar to our findings, both studies indicated that not all scrapie tissues examined were positive for PrP^d. In chronic wasting disease, PrP^d staining was uniquely localized in the ectopic lymphoid follicle of the kidney of a whitetail deer (*24*). All studies indicated either no changes (*22*,*24*) or mild to no inflammatory changes of the kidney (*23*). Thus, our histologic and immunohistochemical results for scrapie-infected hamsters are consistent with results found for sheep and deer and suggest that under normal conditions TSE diseases do not have concomitant inflammatory changes in the kidney.

That urine titer is similar to that of plasma suggests that urine infectivity may originate from blood (*25*), but how the infectivity would be excreted is not clear. In general, proteins >40 kDa are not excreted and smaller proteins crossing the glomeruli are reabsorbed in the renal tubule and returned to the blood. If TSE infectivity is particulate (>40 kDa), its presence in urine might indicate abnormalities in renal filtration, perhaps related to the accumulation of PrP^d in the collecting tubules of the medulla. The accumulation of immunoglobulins in the urine of TSE-infected hamsters and humans may also indicate malfunction of the urinary system (*9*,*26*). Excretion of a small C-terminal fragment of the normal cellular form of the prion protein in urine of infected and noninfected animals has been reported (*27*), but PrP^{res} or PrP^d forms can only be inferred from the presence of infectivity. Nevertheless, excretion of proteins similar to PrP^{res} or PrP^d forms has been documented. Follicle-stimulating hormone is a glycosylated protein of 203 amino acids organized mostly as a β-sheet, which bears some remarkable similarities to β-rich forms of the prion protein. Follicle-stimulating and several similar hormones are excreted in urine at great enough concentration to be extracted commercially. Alternatively, TSE infectivity may be excreted by processes analogous to those responsible for the low-level virurias that occur during infections of the nervous system by mumps, measles, and West Nile virus (*28*–*30*).

To the extent that results from the hamster model can be generalized to other TSE infections (and it has so far proven highly predictive), then even the very low concentrations of infectivity measured here could result in substantial environmental contamination. Several liters of urine and several thousand doses of TSE infectivity may be excreted daily over the course of the illness; even higher titers might be excreted by an animal with nephritis. The high stability of TSE infectivity would account for its persistence in pasture years after infected animals are removed (*31*). Recent studies have shown that infectivity that is adsorbed and immobilized by soil minerals (*32*) can still infect hamsters by oral exposure 29 months later (*33*). Our study also warns of a possible risk from TSE contamination to fertility hormones and other medicinal products extracted from human urine.

Acknowledgments

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Spatial Analysis of Tuberculosis Cases in Migrants and Permanent Residents, Beijing, 2000–2006

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To determine the role of the migrant population in the transmission of tuberculosis (TB), we investigated the distribution and magnitude of TB in permanent residents and migrant populations of Beijing, People's Republic of China, from 2000 through 2006. An exploratory spatial data analysis was applied to detect the "hot spots" of TB among the 2 populations. Results, using the data obtained from 2004–2006, showed that people who migrated from the western, middle, and eastern zones of China had a significantly higher risk of having TB than did permanent residents. These findings indicate that population fluctuations have affected the rate of TB prevalence in Beijing, and interventions to control TB should include the migrant population.

Tuberculosis (TB) is a reemerging infectious disease and a substantial public health problem in metropolitan Beijing, People's Republic of China. The case notification rate and mortality rate of TB have ranked third and first, respectively, among rates for 37 notifiable infectious diseases since 2000 (*1*–*5*). The proportion of cases in migrants is increasing year by year. In 2006, the migrant population accounted for 1,638 of 4,088 cases, a proportion that is 80% of the total number of cases of the permanent residents. Prevention and control of TB among the migrant population are now great challenges in Beijing (*1*–*3*).

"Migrant population" is a characteristic concept in China, resulting from the *hukou* system. *Hukou* is a household registration system in which a permanent residency permit for 1 place is issued by the government to each family. Every family has a *hukou* booklet that records information about the family members, including name, birth date, relationship with each other, marriage status (and to whom if married), employer, and residence address. Everyone has a *hukou* in China, which is assigned to a baby at birth, according to the residence of his or her parents. To move *hukou* from 1 place to another is usually difficult (*6*).

Before 1980, *hukou* was extremely important for citizens of China. They were required to stay at the small area where their *hukou* was until they died. They could travel, but, if they did, they had no access to jobs, public services, education, or even food. It was just like visiting other countries with a B-1 (business) visa. After 1980, many circumstances changed with the reform of the Chinese economy. In practice, *hukou* does not play as important role as before, which has led many farmers to leave their homeland and go to cities to seek jobs. A migrant population has thus arisen.

For this migrant population to be managed, migrants are required to register for a temporary residence permit (TRP) in the Department of Migrant Population Management of the local public security bureau if they are >16 years of age and stay in a place for >1 month (*7*). Employers are responsible for registering TRP for their workers in the district where the work place is located. For persons without a job, the community they reside in has to super-

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vise their registration to obtain a TRP. The period of validity for TRP is usually 1 year. If the migrant changes his or her job or residence before the TRP expires, the person must register a new TRP in the new location and cancel the old one at the same time (*7*).

By law, migrants have the same rights as permanent residents. However, in terms of economic status, many differences exist between migrants and permanent residents. For example, the migrant population is restricted in access to public health and welfare services in the host areas because the local financial allocation is mainly based on the number of registered *hukou*, and the local governments only provide for the persons with *hukou*. Additionally, because migrants are usually employed at a lower income, they are unlikely to be covered by social medical insurance and thus are unlikely to seek healthcare promptly when they are ill. All of these factors indicate that the migrant population usually holds a lower economic status in host areas.

Economic development and urbanization in China have increased in recent years. In 2001, Beijing had ≈2 million migrants and 11 million permanent residents (*8*). By the end of 2006, the migrant population exceeded the 5 million mark and accounted for one third of the total population in Beijing (*9*). Moreover, most migrants come from the rural areas, where prevalence of TB is high (*10*). They travel between their hometowns and metropolitan Beijing and, consequently, bring the disease to Beijing.

Previous studies indicated that the reemergence of TB seemed to be associated with the mass migrant population (*10–14*). However, detailed assessments of the potential effects of the migrant population are hampered by limited information about the reported cases from this population. In the current study, we analyzed the spatial distribution of the patients with diagnosed TB cases in both migrants and permanent residents using geographic information system (GIS) techniques (*15*). We also attempted to identify the "hot spot" areas in the 2 populations. Finally, GIS-based multilevel extra Poisson regression models analysis was conducted to clarify the impact of the migrant population on the reemergence and transmission of TB in Beijing (*16*).

Materials and Methods

Data Collection and Management

The data on all the TB cases reported in Beijing from 2000 through 2006 were obtained from the Beijing Institute for Tuberculosis Control, which specializes in TB prevention and research and is responsible for supervision of TB control in 18 districts of metropolitan Beijing. The cases that met the diagnostic criteria of TB issued by Ministry of Public Health in 2003 (*17*) were included in the analyses. The data include information on age, origin, current address, and date of TB onset. To assess the contribution of the migrant population from different areas, the case origins were divided into 4 zones, according to economic status and geography, i.e., western zone (including Shanxi, Gansu, Qinghai, Ningxia, Inner Mongolia, Xinjiang, Tibet, Sichuan, Chongqing, Guizhou, Guangxi, Yunnan Provinces, or other administrative regions), middle zone (including Heilongjiang, Jilin, Shanxi, Henan, Anhui, Hubei, Hunan, Jiangxi Provinces), eastern zone (including Liaoning, Hebei, Shandong, Jiangsu, Zhejiang, Fujian, Guangdong, Hainan Provinces), and 2 municipalities (Tianjin and Shanghai) (Figure 1). The zonal classification corresponded to that of the Report on Nationwide Survey on Epidemiology of Tuberculosis in 2000 (*18*) and thus was easily used for comparison. The case data have been stratified by age, gender, origin, and onset date of TB; age was divided into 3 groups: 1) 0–14 years, 2) 15–64 years, and 3) ≥65 years. All the TB cases were coded according to the address where they resided (geo-coded) and matched to a 1:100,000 digital map of Beijing by using ArcGIS version 9.1 software (ESRI Inc., Redlands, CA, USA).

The demographic data of permanent residents and migrant population for each district were obtained from the 2000–2006 censuses, provided by Beijing Municipal Public Security Bureau (*8*,*9*,*19–23*). The 18 districts of Beijing, covering a total surface area of ≈16,800 km^2, had 11,976,900 permanent residents and 5,475,000 migrants in 2006 (*9*). On the basis of these data, the population densities of each district in different years were calculated and displayed on the digital map of Beijing (Figure 2).

The permanent residents in our study were defined as those who reside in Beijing with registered *hukou* in Beijing, and the migrant population was defined as those who had been residing in Beijing >1 month but whose *hukou* were still held in their homelands. Persons originally from other countries were beyond the scope of our

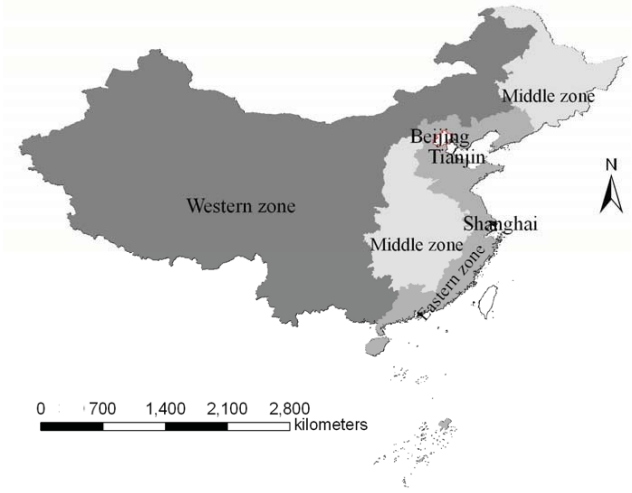

Figure 1. The 4 zones of China. Divisions were made on the basis of economic and geographic factors.

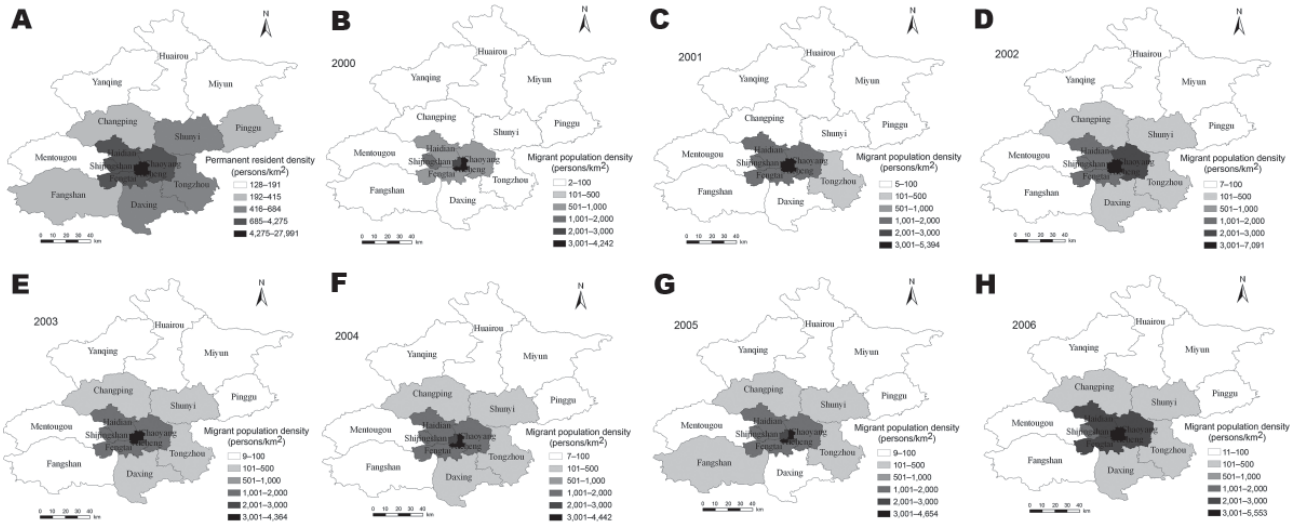

Figure 2. Population density of permanent residents and migrant population in Beijing, 2000–2006. Because the population density of permanent residents showed little change from 2000 through 2006, we showed the mean density of permanent residents in panel A. Panels B through H denote the density of migrant population from 2000 through 2006, respectively.

current study. A shape file of property boundary data of 18 districts in Beijing 2003 obtained from the Ministry of Water Resources of the People's Republic of China was used to generate visual presentations with 1:100,000 scale by using ArcGIS 9.1.

Statistical Analysis

The dynamic changes in population densities and the TB case notification rate of both migrant population and permanent residents from 2000 to 2006 were displayed by district on the digital map of the Beijing municipality (Figures 2, 3). Global Moran's *I* statistics with z score test and Getis's G_i^* statistics, which specify 10 km as the threshold of distance, have been used to detect the spatial distribution and the hot spots of TB in the 2 populations (*24*,*25*). Global Moran's *I* is used to discern spatial autocorrelation of TB cases in the study area and disclose the spatial pattern of disease with z score at the district level. A statistically significant (z score $\geq$1.96) estimate of *I* indicates that neighboring districts (within 10 km) have a similar prevalence rate of TB and that the cases are likely to cluster at the district level (*24*). Getis's G_i^* statistics only assess positive spatial autocorrelation and are used to detect hot spots in the study area. A calculated value of $G_i^* \geq 1.96$ indicates that district *i* and its neighboring districts (within 10 km) have a TB prevalence rate that is statistically significantly different (higher) than other districts. District *i* is the center of the area with the higher TB prevalence rate, and is defined as a TB hot spot (*25*).

Model 1

To detect the difference of prevalence of TB among 18 districts, we constructed two 2-level statistical models (extra Poisson regression model) (*26*,*27*). The first level consisted of the following equation (details in the online Technical Appendix, available from www.cdc.gov/EID/content/14/9/1413-Techapp.pdf):

Case rate per unit of population = constant

The second level (equation) of Model 1 examined the estimated coefficient for the constant and the statistical significance of the error term. If the error term is statistically significant, then it indicates that there are differences in the prevalence rate of TB at the district level (but does not identify which districts are different and the degree of difference).

Model 2

Our second statistical model assessed whether the migrant population was significantly associated with any differences between districts. Model 2 was also a 2-level statistical model, with the first level consisting of the following equation (details in the online Technical Appendix):

Case rate per unit population = constant
+ origin of case (zone) + age + gender

The second level (equation) of Model 2 examined the statistical significance of the error terms associated with the coefficients for both the constant and the zone of case

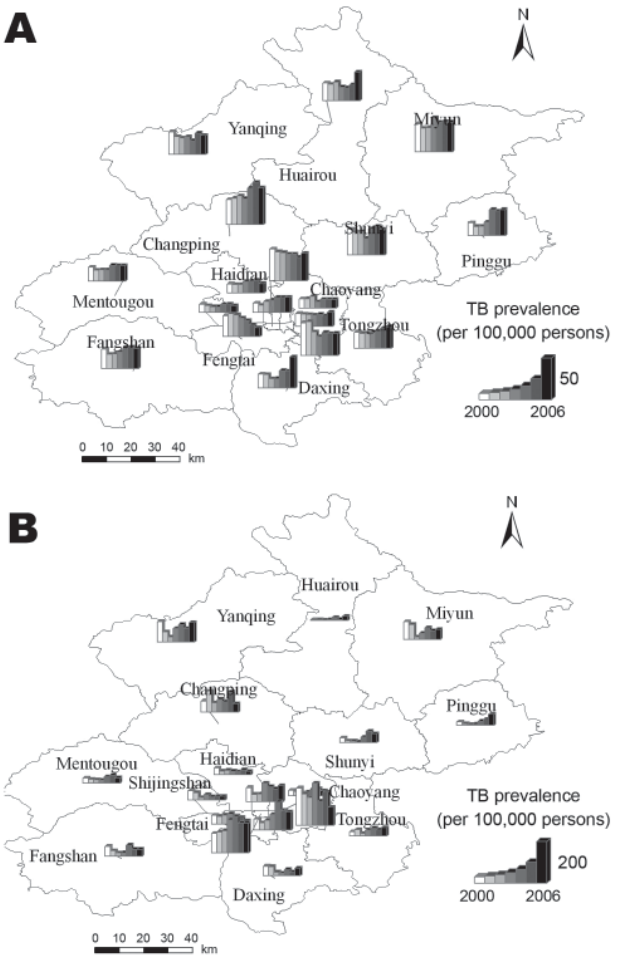

Figure 3. The prevalence rate of tuberculosis (TB) among the permanent residents and migrant population in Beijing, 2000–2006. The district graph unit consists of 7 bars, which denote the prevalence rate of TB from 2000 through 2006, respectively. A) Change in TB prevalence among permanent residents. B) Change in TB prevalence among migrant population.

origin. If the error term of the constant is statistically significant, then it indicates that there are differences in the prevalence rate of TB at the district level. If the error term for the zone of case origin is statistically significant, then it indicates that there are differences between resident and migrant populations.

Results

Our study included 15,078 cases among the permanent residents and 7,948 cases among the migrant population diagnosed from January 1, 2004, through October 31, 2006, in Beijing. Of the migrant population during this time, 61.6% were male; 16.8% came from the western zone, 41.9% came from the middle zone, 40.5% came from the eastern zone, and 0.8% came from Tianjin and Shanghai.

Figure 2 shows the distribution of the permanent residents and migrant population among 18 districts from 2000 through 2006. During these 7 years, the permanent resident population and the migrant population were most dense in Xuanwu, Dongcheng, Chongwen, and Xicheng. The permanent resident population was stable and tended to decline, and the migrant population fluctuated and slightly increased. Figure 3 shows the prevalence rate of TB in the 2 populations. Except in Fengtai, the prevalence rate of TB in the permanent residents has tended to increase since 2004, which is in accordance with the prevalence in the migrant population.

When the 2 populations were compared among the 18 districts, a highly spatial cluster (z>1.96) of TB was shown at the district level (Table 1). The hot-spots among the migrant population persisted in Xuanwu, Chongwen, Xicheng, and Dongcheng from 2000 through 2006 (online Appendix Table, available from www.cdc.gov/EID/content/14/9/1413-appT.htm). For the permanent residents, however, global clusters were not detected, and just 1 hot spot, Miyun, was observed, in 2003 (online Appendix Table). This implies that the epidemic of TB is dominated by the migrant population in Beijing.

The results from Model 1 indicate that statistically significant differences exist in the prevalence rates of TB at the district level (Table 2) The results from the first level of Model 2 show statistically significant difference in TB prevalence by district (the constant term), origin of cases (zone), gender, and age (Table 3). In the second level of this model, the error term associated with the origin (zone) of cases was significant, but the error term associated with district was not (Table 3).

Discussion

Previous studies have indicated that the migrant population affects the prevalence of TB in Beijing (*9*–*13*). However, because of the limitation of the techniques used in the analysis of previous studies, the hot-spot distribution has not been displayed at the district level, and the potential association with the migrant population could not be analyzed quantitatively. In our study, we used GIS-based spatial analysis to elucidate the spatial distribution of TB and highlight the hot spot areas.

Population data showed that the 4 largest migrant populations live in the 4 suburban districts: Chaoyang, Haidian, Fengtai, and Changping (*8*,*9*,*19*–*23*). However, our study indicated that Chongwen, Xuanwu, Xicheng, and Dongcheng, the 4 central districts, were the hot spots of TB among the migrant population from 2000 through 2006. These central districts may be hot spots because the population densities of the migrants and the permanent residents in the 4 central districts are much higher than that in the suburban districts (Figure 2). In fact, ≈20% of the popu-

Table 1. Moran's *I* analysis on TB cases in the migrant population and permanent resident population, Beijing, 2000–2006*

	TB cases among Beijing permanent residents		TB cases among the migrant population	
Year	*I* statistic	z score	*I* statistic	z score
2000	–0.46	–1.51	0.17	0.94
2001	–0.43	–1.42	0.11	–0.25
2002	–0.27	–0.82	0.84	3.62†
2003	–0.14	–0.34	0.99	4.24†
2004	–0.34	–1.14	0.13	4.64†
2005	–0.24	–0.72	0.74	3.09†
2006	–0.36	–1.25	0.71	2.91†

*Moran's *I* statistics with z score test value to detect the spatial distribution of tuberculosis (TB) in the 2 populations. A statistically significant (z score >1.96) estimate of *I* indicates that nearby districts (within 10 km) have a similar prevalence rate of TB and the cases are likely to cluster at the district level.
†Statistically significant, p<0.05 as measured by z score >1.96.

lation crowd into the 4 center districts, which cover only 0.50% of the surface area (*8*,*9*,*19*–*23*), resulting in a high risk for transmission of TB from the migrant population to the permanent residents in these areas. Another explanation may be related to the education and the occupations of the migrant population. Data from Beijing economic census yearbook 2004 showed that most migrants were employed in commercial services in the 4 central districts (*28*), where they were likely to come into contact with the permanent residents. On the other hand, the migrants in the 4 suburban districts were mostly college students, construction workers, and technicians. These migrants usually resided in a given region and formed a special social group, which indicated that they would have less chance of coming into contact with persons outside their group. We are conducting further studies to investigate the effects of economic factors on TB prevalence, in particular, housing, life span, healthcare, gross domestic product, income, education, insurance, social welfare, and traffic.

The spatial analysis showed that TB is distributed randomly among permanent residents and that it tends to cluster in the migrant population; the correlation reached its peak in 2004 and declined in the next 2 years. The analytical result was consistent with the trend of TB in Beijing. During the previous 90 years, the incidence of TB had been controlled to 7 cases per 100,000 population and approached that of industrialized countries (*1*,*2*,*5*). With mass migrant populations pouring into Beijing, TB has reemerged in recent years. According to the report from the Beijing Municipal Health Bureau, annual new registered active cases held steady at ≈2,500 and slightly increased among the permanent residents from 2000 through 2006 (*1*) (Figure 3, panel A). To bring TB in the migrant population under control, some new control measures, such as free therapy (*29*) aimed at the migrant population, were carried out in Beijing, and the increase in disease was effectively halted. Our study also found that the migrant population made different contributions to the prevalence of TB. The national survey showed that TB prevalence in the western zone, with 451 active cases and 136 positive cases per 100,000 population, was ≈1.7× higher than the TB prevalence in the eastern zone and slightly higher than TB prevalence in the middle zone, with 438 active cases and 148 positive cases per 100,000 populations. The western zone was the area where TB was most serious, whereas the 3 municipalities (Beijing, Tianjin, and Shanghai) were the areas of lowest prevalence (*18*). A special study on socioeconomic factors attached to the national survey further disclosed that the differences in the epidemic of TB mainly originated from the economic inequalities among the 4 zones. The socioeconomic study pointed out that the income per person in 80% of families with patients was lower than that in the local population (*18*).

The results from our statistical models indicated that the effects of the migrant population on TB prevalence differ between districts. We can conclude that the migrant population is distributed asymmetrically in 18 districts and has different living conditions. Furthur investigation is needed to determine the underlying causes of TB in each district.

Our work was subject to several limitations. The first was that we performed a retrospective analysis. A prospec-

Table 2. Results from Model 2: differences in prevalence rate of TB among the districts*

Parameter	Estimate	Standard error	χ^2 value	p value
Level 1				
Constant†	–8.280	0.096	7,378.941	<0.001
Level 2				
Error term associated with TB prevalence‡	0.150	0.056	7.229	0.0071

*TB, tuberculosis.
†In this statistical model, the constant term represents the rate of TB by district.
‡If the error term is statistically significant, it indicates that there are differences in the prevalence rate of TB at the district level (but does not identify which districts are different and the degree of difference).

Table 3. Results from Model 3: differences in TB prevalence by district, origin of cases, gender and age of cases*

Parameter	Estimate	Standard error	χ^2 value	p value
Level 1				
Constant	–9.823	0.117	6,993.521	<0.001
Zone	0.795	0.144	30.397	<0.001
Age	0.948	0.035	737.1151	<0.001
Gender	0.501	0.031	269.385	<0.001
Level 2				
Error terms combined	0.200	0.069	8.330	<0.005
Error term associated with TB prevalence†	–0.067	0.068	0.965	0.3259
Error term associated with origin (zone) of cases‡	0.342	0.124	7.603	<0.005

*TB, tuberculosis.
†If the error term is statistically significant, it indicates that there are differences in the prevalence rate of TB at the district level (but doesn't identify which districts are different and the degree of difference).
‡If the error term is statistically significant, it indicates that there are differences in the prevalence rate of TB by origin (zone) of cases (but doesn't identify which the degree of difference between origins).

tive study would have been worthwhile for predicting the impact of the migrant population on the epidemic of TB. Businesspersons or travelers can become the transmitter of TB if they have been infected by *Mycobacterium tuberculosis.* The role of public transportation in the transmission of TB has been mentioned in many reports (*30*,*31*). However, the potential impact of businesspersons and travelers was not assessed in our study because we could not trace them and obtain reliable information about them. We have also noted that when the individual cases were aggregated to the district level, individual differences could be missed, and the effect of the individual on the disease was not taken into account. However, in the current study, we emphasized the effect of the whole migrant population on the epidemic of TB in each district. The delay or lag time between the initial outbreak of disease in the migrant population and the permanent residents was expected to affect the number of the permanent residents infected with *M. tuberculosis* (*32*) (data not shown).

In summary, our study confirmed that the migrant population contributed to the prevalence of TB and the differences among the 18 districts in Beijing. Our findings suggest that TB control measures should incorporate the migrant population, particularly persons from the western and middle zones. Our study also implied that further research is necessary on the correlation between TB and the economy.

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We Are Legend

Julian W. Tang

We are HIV. Our family is ancient.
Out of Africa,
Monkey to man,
From the trees and forests,
To the towns and cities.
We are here.

For we are HIV, we are legion.
Our children are billions,
Our home, in your defenses,
In your blood, your brain,
Your saliva, your semen.
We are everywhere.

For we are HIV, we are immortal.
We are part of you,
And you of us,
We live with you, but
May not die with you.
We go on.

For we are HIV, we are travelers.
From lover to lover,
Mother to baby,
Donor to blood bank,
Blood bank to patient,
We follow you.

For we are HIV, we evolve.
NRTIs, NNRTIs, PIs, INIs,
New designs, new drugs,
Bring it on, bring it on,
Q151M, K103N, L90M.
We adapt, we survive.

We are HIV. We consume.
Your resources, your time,
Your hope, your lives,
Your new drugs are easy.
Where are your vaccines?
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Bluetongue Virus Serotype 8 Reemergence in Germany, 2007 and 2008

Bernd Hoffmann, Michael Saßerath, Sabine Thalheim, Claudia Bunzenthal, Günter Strebelow, and Martin Beer

Reemerging bluetongue virus serotype 8 (BTV-8) in Germany was detected first in May 2007 in a sentinel cow and in February 2008 in an export heifer. Reemergence was confirmed by retesting the samples, experimental inoculation, fingerprinting analysis, and virus isolation. Overwintering of BTV-8 and continuous low-level infections are assumed.

Bluetongue virus (BTV) is a double-stranded RNA virus of the genus *Orbivirus.* BTV is transmitted to its hosts by the bite of *Culicoides* spp. midges. It causes a noncontagious, arthropod-borne disease of domestic and wild ruminants and camelids (*1*); disease can be serious, particularly in sheep (*2–4*). BTV had never been reported in any European country north of the Alps until August 2006, when outbreaks of BTV serotype 8 (BTV-8) were almost simultaneously discovered in the Netherlands, Belgium, Germany, and France (*5–7*). In 2006, a total of 893 cases were detected in Germany, but the source of initial virus introduction remains unknown. Subsequently, BTV-8 overwintered in the region, spread over most of the country, and led to almost 21,000 new cases in 2007 in Germany. BTV-8 infections spread to additional European countries, e.g., the United Kingdom, Switzerland, the Czech Republic, Denmark, and Spain (*8–11*).

To detect reemerging BTV-8 in Germany, a sentinel animal program was started in March 2007. Each month, the program tested 150 BTV-naive cattle per 2,025 km². Screening was performed by ≈30 regional laboratories located in the 16 federal states of Germany. Diagnostic testing was performed with commercially available BTV-blocking-ELISAs (Pourquier ELISA Bluetongue Serum, Institut Pourquier, Montpellier, France; Bluetongue Virus Antibody Test Kit, VMRD, Pullman, WA, USA: ID Screen Bluetongue Competition ELISA kit, ID Vet, Montpellier, France; INGEZIM BTV, INGENASA, Madrid, Spain) and validated BTV real-time PCRs (*12*; B. Hoffmann et al., unpub. data). All positive results were confirmed by the National Reference Laboratory for BTV at the Friedrich-Loeffler-Institut, Insel Riems.

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The Cases

On May 3, 2007, the first sentinel animal in the program (4-year-old Holstein Friesian cow, North-Rhine Westphalia, Germany) tested positive for BTV antibodies and genomes in a regional laboratory (Krefeld, Germany). This animal had tested negative for BTV on March 3 and April 3. After the positive test result, the original samples as well as new samples were transferred to the reference laboratory for further investigation. The sample collected on May 3 and all further samples of this animal tested positive by BTV ELISA as well as by BTV real-time reverse transcription–PCR (RT-PCR). According to Deutscher Wetterdienst, available from www.dwd.de, the daily temperatures in the region of North-Rhine Westphalia during this period ranged from minimums of 13.5°C to –1.3°C to maximums of 9.5°C to 28.1°C. A period with daily maximum temperatures >22°C from April 12 to April 16 suggests increased likelihood for activity of *Culicoides*. Similar prognoses for the reemergence of BTV-8 were published by Gloster et al. (*13*).

Samples taken on May 3 had positive ELISA titers of 8–16, and the cycle threshold (Ct) values of the real-time RT-PCR analysis were 25.6–27.9 (Figure, panel A). Therefore, new BTV-infection of a BTV-naive animal was suggested. To confirm the presence of infectious BTV, fresh EDTA-blood samples from the positive animal were experimentally injected into 2 bovids (40 mL intravenously and 10 mL subcutaneously). To mimic natural exposure by the bite of a midge, only 20 μL was injected subcutaneously into a 3rd bovid (Table). Both animals inoculated with 50 mL had positive real-time PCRs at day 2 postinoculation and reached Ct values of 22.9 and 24.3 by day 13. In contrast, the animal that had received the smaller volume remained negative for BTV antibodies and genomes. In addition, 1 of the animals that had received the larger volume showed late seroconversion according to BTV-blocking ELISA at day 30 (Table). Furthermore, the virus was typed as BTV-8 by using the BTV-8–specific primers BT8/101/F (*14*) and BT8/765/R (5′-CCACTGTCTATGTG CGTAA-3′, kindly provided by P. Mertens, Institute for Animal Health, Pirbright, UK) to amplify a 1,993-bp fragment of the VP2 gene by conventional RT-PCR (Figure, panel B). Testing of 80 contact animals from the same herd showed 38 antibody-positive cattle but no additional RT-PCR–positive case.

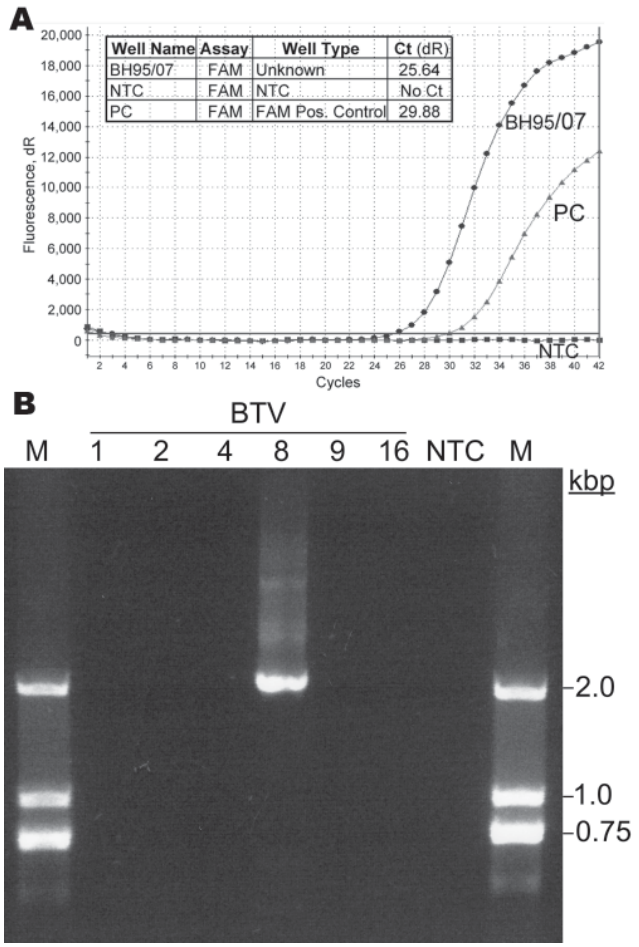

Figure. A) Bluetongue virus (BTV) serotype 8 genome in a field sample collected May 3, 2007 (BH95/07), detected by using real-time reverse transcription–PCR with a 6-carboxy fluorescein-labeled probe (FAM). The magnitudes of the fluorescence signals per PCR cycle are shown for the sample (Unknown), the no-template control (NTC), and the positive control (PC). Ct, cycle threshold. B) Confirmation of serotype 8 for the isolated virus by serotype-specific PCR and agarose gel analysis. Additionally tested serotypes (BTV 1, 2, 4, 9, 16) were negative. A DNA ladder was used as marker (M).

In February 2008, a 2-year-old Holstein Friesian heifer, destined for export from Schleswig-Holstein, tested positive for BTV antibodies and genomes during quarantine. Testing was done by a regional laboratory (Frankfurt Oder, Germany). Whereas a first blood sample collected on January 22, 2008, was negative according to real-time RT-PCR; a subsequent sample taken on February 15 was positive by real-time RT-PCR and had a Ct value of 25.13. Both test results were confirmed by the German National Reference Laboratory for BTV. For the positive EDTA blood sample, a Ct value of 25.1 and an ELISA inhibition value of 85.7% (positive >50%) were ascertained. Furthermore, fingerprinting analysis (StockMarks Cattle Genotyping Kit, Applied Biosystems, Foster City, CA, USA) confirmed that both the negative and the positive samples were from the same animal (data not shown). The meteorologic data from January 20 to February 15 showed unsuitable weather conditions for BTV replication in outdoor midges at the time of BTV transmission; daily minimum temperatures were 7.2°C to –6.3°C, and daily maximum temperatures 3.4°C–13.4°C. Finally, BTV was isolated after inoculation of Vero cell cultures with the real-time PCR-positive blood samples.

Conclusions

According to the dates of sampling and subsequent seroconversion, genome detection, and Ct values, 1 animal became infected during April 1–25, 2007, and the other became infected during January 20–February 12, 2008. According to meteorologic data, infection between April 15 and 25 is likely, whereas the suggested infection time in 2008 was not associated with weather suitable for BTV transmission. Therefore, we have drawn several conclusions. First, BTV-8 reemerged in Europe in 2007 and 2008 unexpectedly early. This timing makes it unlikely that a real vector-free period exists in northwest Europe. Second, the detection of both cases was fortuitous. Because of the low number of sentinel animals in 2007, or double-tested cattle destined for export in January and February 2008, substantially more new infections may have occurred in winter 2007 and early spring 2008. Therefore, overwintering of BTV-8 appears to be efficient, and under the climatic conditions of winter and spring 2007 and 2008 in Germany, continuous infections occurring at a low level must be assumed. However, the detailed mechanisms of overwintering remain unclear. In addition, the meterologic data connected to the early case in 2008 suggest indoor survival of infected midges. Third, sentinel animals proved to be useful for the early detection of recirculating virus. However, establishing and conducting an efficient sentinel program are complex and expensive endeavors, and, as shown in 2008, positive cases might be detected earlier by chance.

On the basis of our experience, we recommend that all samples that could represent new infections in a given region or season should be confirmed by a reference laboratory that as a minimum standard confirms identity of the origin of all samples. This step would avoid misleading interpretations due to, for example, a mix-up of samples or animals. Also, depending on future climate in the BTV-8–infected regions in 2008, the first BTV-8 case could indicate the need for early protection, especially of sheep and goats, by BTV-8–specific vaccination.

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Table. Results of rRT–PCR and bluetongue virus competition ELISA of samples from experimentally inoculated animals*

Day postinoculation	Animal no., dose					
	415, 40 mL IV and 10 mL SC		418, 40 mL IV and 10 mL SC		419, 20 μL SC	
	rRT-PCR, Ct	ELISA	rRT-PCR, Ct	ELISA	rRT-PCR, Ct	ELISA
2	>42.0	–	>42.0	–	>42.0	–
4	37.7	–	37.2	–	>42.0	–
6	32.1	–	28.7	–	>42.0	–
9	30.9	–	26.6	–	>42.0	–
13	24.3	–	22.9	?	>42.0	–
16	24.3	–	22.8	+	>42.0	–
21	26.7	–	24.5	+	>42.0	–
30	27.0	+	27.2	+	>42.0	–

*rRT-PCR, real-time reverse–transcription PCR; IV, intravenous; SC, subcutaneous; Ct, cycle threshold (Ct values >42 were defined as negative); –, negative; +, positive; ?, in doubt.

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Ecoepidemiology of Cutaneous Leishmaniasis Outbreak, Israel

Shepherd Roee Singer, Nitsa Abramson, Hanna Shoob, Ora Zaken, Gary Zentner, and Chen Stein-Zamir

A total of 161 cases of cutaneous leishmaniasis caused by *Leishmania tropica* occurred in the Jerusalem district during 2004–2005; 127 (79%) cases were in a town just outside Jerusalem. Environmental models suggest that in the context of global warming, this outbreak has the potential to extend into Jerusalem.

Leishmaniasis is a zoonotic infection in which parasites of the genus *Leishmania* are transmitted from rodents and small mammals to *Phlebotomus* species sandfly vectors. Humans may inadvertently enter the zoonotic cycle and contract cutaneous leishmaniasis (CL). This disease manifests as a chronic ulcer, potentially leaving unattractive scars.

CL incidence throughout the 1990s remained at a relatively stable 0.5–2.5 cases/100,000 (*1*). *Leishmania major* is found in low-lying arid and semiarid deserts and has been responsible for most cases in Israel (*2*). *L. tropica,* typically anthroponotic, is more common in suburbs and villages, although in hilly rural areas, mammals may act as reservoirs (*3*). The sandfly *Phlebotomus papatasi* is vectorially competent for *L. major* only (*4*), and *Ph. sergenti* is specific for *L. tropica* (*5*). Sandflies are usually found within 200 m of their source.

Since the 1990s, *L. tropica*, either alone or in conjunction with *L. major*, has been implicated in several outbreaks of CL in the western regions of the Jordan Valley (*6*). We report a large outbreak of CL caused by *L. tropica* in a town on the outskirts of Jerusalem.

The Study

Jerusalem is located atop the Judean Hills, on the edge of the Judean Desert. The desert drops off steeply to the east, falling 1,200 m over a course of 20 km to a nadir of –400 m on the shores of the Dead Sea, the lowest point on land on Earth. On the edge of this region is Ma'ale Adumim, 5 km east of Jerusalem (population ≈33,000). The town is built along narrow ridges that fall away to deep ravines inhabited by wildlife. Houses on the periphery are often just meters from desert crags and crevices; none is more than 500 m from the wilderness.

All cases of leishmaniasis in Israel are required to be reported to the district health office, and a weekly national report is published by the Department of Epidemiology, Israeli Ministry of Health (*7*). We confirmed suspected cases by using stained smears, culture, or serologic analysis. Cases were plotted on the Ministry of Health geographic information system (Environmental Systems Research Institute, Redlands, CA, USA), adapted specifically for the Informatics and Computation Division of the Ministry of Health (Systematics Technologies Ltd., Tel Aviv, Israel). National population data were derived from the Israeli Central Bureau of Statistics. Local population data were supplied by the town municipality, and neighborhood incidence rates were derived and averaged for 2004–2005. Patients were interviewed by using a standardized national CL questionnaire (online Technical Appendix, available from www.cdc.gov/EID/content/14/9/1424-Techapp.pdf). Simple rate ratios (RRs) were calculated where applicable.

A total of 161 cases of CL were reported in the Jerusalem district in 2004 (n = 71) and 2005 (n = 90) compared with 1 or 2 cases in each previous year. Of the cases reported in 2005, microscopic examination was positive for 74 (82%). Forty-eight (53%) had positive cultures; 20 (41.6%) of these were serologically positive for *L. tropica*, and none was positive for *L. major*.

Average annual incidence of CL in Israel (excluding the Jerusalem district) increased from 0.95/100,000 in 1999–2003 to 1.61/100,000 in 2004–2005 (RR 1.63). Over the same period, however, rates for the Jerusalem district increased from 0.13 to 9.7/100,000 (RR 74.7). Rates for the Jerusalem district were lower than those for the rest of Israel during 1999–2003 (RR 0.14) but substantially higher during 2004–2005 (RR 6.1). In 2006, rates for the Jerusalem district decreased, and national incidence continued to increase; 2007 showed a trend to an increased incidence in the Jerusalem district (Figure 1).

Of the case-patients in the Jerusalem district, 54 (76%) in 2004 and 73 (81%) in 2005 occurred in Ma'ale Adumim, where the incidence was 214/100,000 in 2004–2005, compared with an annual average of 2/100,000 in preceding years. However, cases were not distributed evenly. The epicenter of the outbreak was a small neighborhood of 1,040 residents (A in Figure 2) in which 52 cases occurred over the 2-year period (attack rate 50/1,000); this attack rate was greater than in any other neighborhood. The second most affected neighborhood had 24 cases among 2,251 residents (attack rate 10.7/1000 for the 2-year period). All but 6 case-patients lived within 200 m of the ravines that encompass the town, and 3 of these case-patients had occupational

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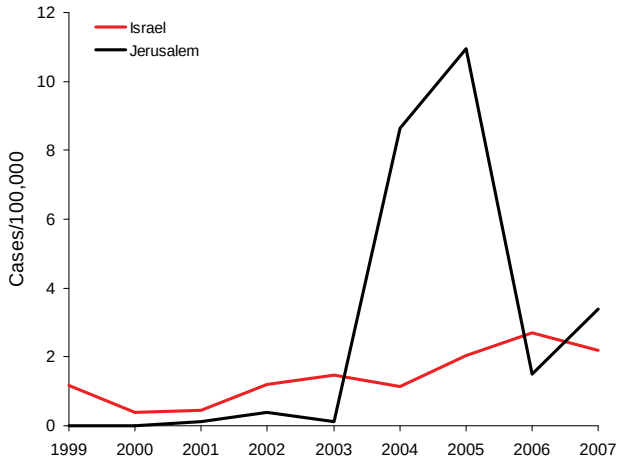

Figure 1. Incidence rates of cutaneous leishmaniasis for the Jerusalem district and Israel, 1999–2007. Rates for Israel do not include cases reported in the Jerusalem district.

exposure. In 2006 and 2007, 13 and 29 cases, respectively, were reported in the Jerusalem district; 5 (38%) and 9 (31%) of these case-patients, respectively, were infected in Ma'ale Adumim. The remaining cases were widely dispersed.

Age distribution of patients was not different from that of uninfected persons in the town (Table). More than one third of the patients (37.5%) reported a family member who was infected with CL in the same period. Most patients (62.2%) had >1 lesion, 70% reported having a private garden, 57.1% lived adjacent to public parks, 52.7% had a home that faced a wilderness area, 45.8% reported a construction site near their home, and 65.1% had intact insect screens on their windows. Eight patients (14%) (6 in neighborhood A and 2 in neighborhood B) reported seeing hyraxes near their homes (Figure 2).

Conclusions

We report a large outbreak of CL in Israel caused by *L. tropica* that was centered on a town just outside Jerusalem. This outbreak was observed in the context of increasing rates of CL in Israel. During the outbreak, highly visible environmental intervention and active surveillance were undertaken, which may have introduced detection bias, but it is unlikely that these alone could explain the dramatic increase in incidence.

The association between CL outbreaks and urban development has been noted repeatedly, and construction waste and soil humidity are considered intermediaries (*8*,*9*). A study in Colombia found that habitat degradation negatively affected phlebotomine populations but that medically important sandfly species were able to exploit modified environments (*10*). Ma'ale Adumim has undergone rapid development and expansion over the past decade, and sandflies are abundant in the area. A 2005 study collected 80,000 sandflies near the town, of which 85% were *Ph. sergenti* (*11*), the vector for *L. tropica*. Hyraxes were sighted most frequently in the worst CL-affected neighborhoods. Although environmental investigation is ongoing, we suspect that hyraxes infesting building sites were the source of this outbreak. This hypothesis is in accordance with current knowledge that associates *L. tropica* with urban outbreaks and hilly terrain. However, the pattern of this outbreak supports a zoonotic rather than anthroponotic source.

In 2006, Chaves and Pascual reported that climate was a valuable covariate in predicting incidence of CL (*12*). A 1996 computerized model examined the effect of warming of 1°C, 3°C, and 5°C on the likelihood of CL transmission at 115 southwest Asian sites. Sandflies are not known to reproduce in Jerusalem, but Cross and Hyams suggest that Jerusalem could support endemic transmission if the average temperature increased by 1°C (*13*). Since that model was proposed, the average temperature in Jerusalem has increased by ≈1°C (*14*). The spate of recent outbreaks sug-

Figure 2. Geographic distribution of cutaneous leishmaniasis cases in Ma'ale Adumim, Israel, 2004–2005. Each star represents 1 case. In dense areas, some marks may be missing because of technical limitations. Wilderness ravines (white areas) reach within meters of houses at the periphery of the neighborhood in all directions. A and B indicate neighborhoods with the highest incidence rates.

Table. Age distribution of cutaneous leishmaniasis case-patients in the Jerusalem district, Israel, 2004–2005

Age group, y	No. (%)
<1	4 (2.5)
1–4	12 (7.5)
5–14	40 (24.8)
15–44	46 (28.6)
45–64	29 (18.0)
≥65	19 (11.8)
Unknown	11 (6.8)
Total	161 (100)

gests that *L. tropica* is no longer an emerging pathogen but rather that it is an established pathogen in Israel. The proximity of the outbreak to Jerusalem, in light of the trend toward global warming (*15*), makes an outbreak of CL in Jerusalem a real and disturbing prospect.

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Highly Pathogenic Avian Influenza Virus (H5N1) Isolated from Whooper Swans, Japan

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On April 21, 2008, four whooper swans were found dead at Lake Towada, Akita prefecture, Japan. Highly pathogenic avian influenza virus of the H5N1 subtype was isolated from specimens of the affected birds. The hemagglutinin (HA) gene of the isolate belongs to clade 2.3.2 in the HA phylogenetic tree.

Wild birds have been affected by the highly pathogenic avian influenza virus (HPAIV) of H5N1 subtype since 2002. Wild birds are a natural reservoir of type A influenza viruses, which generally cause asymptomatic infection. However, outbreaks of HPAIVs of the H5N1 subtype among residential and migratory birds occurred in the New Territories of Hong Kong Special Administrative Region, People's Republic of China, in late 2002 (*1*). Furthermore, thousands of migratory birds of several species were affected by HPAIV (H5N1) at Qinghai Lake in western China in 2005 (*2*,*3*). Viruses similar to the ones that caused the outbreak in China eventually spread to Europe (*4*) and Africa (*5*). These events raised concern that migratory birds may play a role in transmission of HPAIVs. The whooper swan (*Cygnus cygnus*) is considered to be a highly susceptible species among wild birds. Infections with HPAIV (H5N1) of this species were reported from China (*6*) and Mongolia in 2005; Iran, Germany (*7*), France, Denmark, the United Kingdom, and Mongolia in 2006; and Russia in 2007. We describe isolation of (H5N1) HPAIV from a whooper swan in Akita prefecture, in the northern part of the main island of Japan.

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The Study

On April 21, 2008, three whooper swans (2 adults and 1 juvenile) were found dead at Lake Towada, Akita Prefecture, Japan (Figure 1). Carcasses were brought into Akita Animal Hygiene Service Center for postmortem examinations. One juvenile swan was also found alive but very weak. It was taken to the Wildlife Protection Center in Akita but had to be euthanized that day in moribund status. Homogenates from the tracheas, cloacas, and internal organs of 3 swans were pooled and inoculated into embryonated chicken eggs for virus isolation.

Hemagglutinating agents from eggs inoculated with each homogenate. Agents were confirmed to be type A influenza viruses by a commercial rapid antigen assay kit and were excluded from being Newcastle disease virus by the hemagglutination inhibition test with Newcastle-specific antiserum. No pathogenic bacteria were isolated. After those tests conducted at the Animal Hygiene Service Center, viruses were brought to the National Institute of Animal Health, Tsukuba, Japan, for further analysis.

The viruses were subtyped as H5N1 with a panel of antiserum, and 1 yielded from cloaca homogenates was designated as A/whooper swan/Akita/1/2008 (WsAk08) and was further analyzed. WsAk08 was shown to be highly pathogenic to chickens by an intravenous administration of 10-fold diluted infectious allantoic fluid. All 8 inoculated chickens died by 26 hours after inoculation. This result coincides with the sequence analysis of the hemagglutinin (HA) gene, showing that the HA protein possesses a series of basic amino acids (PQRERRRKR) at the cleavage site.

Phylogenetic analysis of the HA1 region of the HA gene (Figure 2) showed that WsAk08 belongs to clade 2.3.2 and is clearly distinguishable from the HPAIVs previously isolated in Japan in 2004, A/chicken/Yamaguchi/7/2004 (clade 2.5), and in 2007, A/chicken/Miyazaki/K11/2007 (clade 2.2). Although sequence data were not found in GenBank, A/common magpie/Hong Kong/5052/2007 reportedly resides in the same clade (*9*). Antigenic analysis of WsAki08 with a panel of antiserum and monoclonal antibodies showed low reactivity against antibodies in the panel (online Appendix Table, available from www.cdc.gov/EID/content/14/9/1427-appT.htm). A ≥32-fold reduction from homologous titers of all hyperimmune serum used was noted with WsAk08. Postinfection duck serum against A/chicken/Yamaguchi/7/2004 and A/chicken/Miyazaki/K11/2007 did not react with WsAk08. None of the monoclonal antibodies against HA protein of A/chicken/Yamaguchi/7/2004 reacted with WsAk08. Thus, WsAK08 is genetically and antigenically distinguishable from the HPAIVs that caused previous outbreaks in Japan.

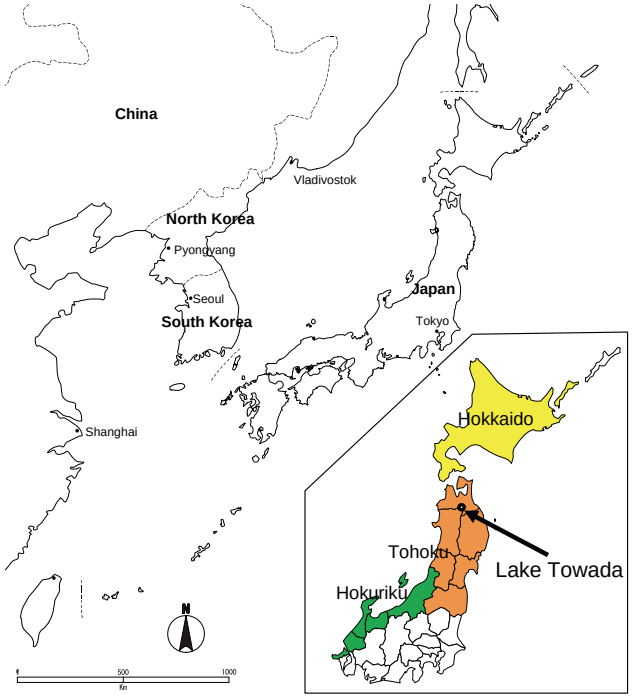

Figure 1. Map of Japan and nearby countries, with enlargement of the northern part of the country (inset) showing location of Lake Towada.

Sequencing analysis of the entire genome of WsAk08 (GenBank accession nos. AB436731–AB436738) showed that it does not contain amino acid substitutions conferring resistance to adamantane or neuraminidase inhibitors. Unlike many isolates related to Qinghai Lake strains that have spread worldwide, it does not have an E627K substitution in the polymerase basic protein 2. The neuraminidase protein has a 20-aa deletion at aa 49 to 68 in the stalk region. Nonstructural protein 1 has a 5-aa deletion at aa 80 to 84, commonly observed in currently circulating HPAIVs (H5N1) in southeastern Asia.

Conclusions

Whooper swans breed in northern Eurasia and winter in Europe and eastern Asia i.e., China, the Korean peninsula, and Japan. In Japan, ≈35,000–38,000 whooper swans spend every winter primarily in Hokkaido, Tohoku, and the Hokuriku area (*10*). In the Lake Towada area, ≈300 whooper swans arrive beginning in late October; they leave the area between late March and late April (*11*). In late March, summer birds begin to arrive. According to results of satellite tracking of 8 swans (*12*), as well as the results of banding studies since 1961 (*13*), whooper swans that winter in Japan migrate from the northern end of Honshu Island to eastern Hokkaido, by means of Sakhalin, and reach eastern Siberia, where they breed. To our knowledge, there have been no reports of whooper swans that winter on the Eurasian continent migrating north through Japan.

In light of the migratory route mentioned above, the whooper swans found dead at Lake Towada were most likely recently infected with HPAIV (H5N1) in Japan. It is unlikely that the swans were infected before they flew to Japan in autumn, maintained the virus within the flock, and then suddenly developed the disease after no apparent infections for several months. Although the susceptibility of a certain species of birds to a subtype H5N1 virus may be different depending on the virus strain (*14*), whooper swans as well as mute swans have been considered to be susceptible species to HPAIV (H5N1), as they showed a fulminant course of disease at the outbreak in Germany in 2006 (*7*).

The possibility that the swans were infected by domestic fowl is low because there has been no report of HPAI among domestic fowl in Japan since the beginning of 2008. One possible explanation is that other wild birds brought the virus from outside the country. Although it is not known whether any birds wintering on the continent migrate north through Japan, passage visitor birds such as wader birds migrate from south to north through Japan in spring; summer birds, e.g., egrets, swallows, songbirds, and some raptors, come to Japan from the south in spring for breeding. Also, the possibility of anthropogenic introduction of virus, such as by inappropriate importation of birds, meats, or materials, cannot be excluded.

In conclusion, genetic analysis demonstrates that the virus that killed the 4 swans in Japan in 2008 is geneti-

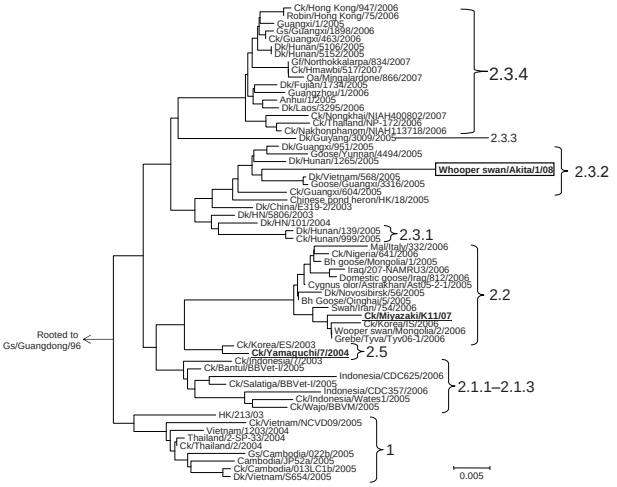

Figure 2. Phylogenetic tree constructed based on the hemagglutinin (HA) 1 region (966 bp) of the HA gene of the highly pathogenic avian influenza viruses (H5N1). Clade designation follows the criteria proposed by the World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization H5N1 Evolution Working Group (8). Representative strains of the previous highly pathogenic avian influenza outbreaks in Japan are in **boldface**. Scale bar represents number of nucleotide substitution per site.

cally distinguishable from the strains that caused previous poultry outbreaks in Japan, ruling out a possibility of resurgence of previously introduced HPAIV in Japan. After the incident we describe, 2 other whooper swan cases of HPAIV (H5N1) infection were confirmed in eastern Hokkaido in early May. Possible involvement of wild birds in the introduction of the virus to Japan requires further scrutiny.

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Neurobrucellosis in Stranded Dolphins, Costa Rica

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Ten striped dolphins, *Stenella coeruleoalba,* stranded along the Costa Rican Pacific coast, had meningoencephalitis and antibodies against *Brucella* spp. *Brucella ceti* was isolated from cerebrospinal fluid of 6 dolphins and 1 fetus. *S. coeruleoalba* constitutes a highly susceptible host and a potential reservoir for *B. ceti* transmission.

Brucellosis is a zoonotic disease of terrestrial and marine mammals. During the past 3 decades, contacts between cetaceans and humans have increased worldwide (*1*), augmenting the risk for transmission of pathogenic *Brucella* spp. from these animals to people (*2*). Indeed, *Brucella* marine strains are capable of infecting humans and livestock (*3*,*4*).

The Study

From August 2004 through April 2007, 10 live striped dolphins, *Stenella coeruleoalba* (3 female adults, 2 female juveniles, 1 female calf, 4 juvenile males), were found stranded in populated areas at the Pacific shoreline of the Puntarenas Province of Costa Rica. All animals had swimming problems compatible with neurologic disorders and died within 48 hours of being found. Corpses were kept on ice and transported to the Pathology Unit, Veterinary School, National University, Costa Rica, for sampling; necropsy; and histopathologic, immunohistochemical, and serologic studies. With exception of 1 dwarf sperm whale, *Kogia sima*, these 10 dolphins were the only cetaceans we were able to examine during this 32-month period.

Because marine *Brucella* spp. have been reported to cause intracerebral infections (*3*), we decided to perform immunohistochemical and serologic tests. For these tests, rabbit immunoglobulin (Ig) G anti–*B. abortus* lipopolysaccharide (LPS) was produced and isolated as described elsewhere (*5*). Antibodies against dolphin *Steno bredanensis* IgG were produced in rabbits, purified according to described protocols (*6*). Both rabbit antibodies were linked to fluorescein isothiocyanate and peroxidase and were assayed by using immunofluorescent and Western blot techniques, respectively (*5*,*7*). Rose Bengal agglutination test, immunofluorescent assays, and competitive ELISA were designed and used as described (*8*,*9*).

Blood was collected from the live dolphins in situ, serum was obtained, and physical and chemical examinations were performed, followed after death by necropsies and gross pathologic and histopathologic studies. Tissues were fixed in formalin, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (*10*). Organs and tissues of 5 adult females and 1 juvenile male were analyzed for bacteria (*11*); however, no samples for bacteriologic studies were available from the other dolphins that were stranded before July 2006. Identification of the bacterial isolates was performed according to standard protocols (*11*,*12*). Fresh tissue impressions or pellets from supernatants of macerated tissues were fixed with cold 3% paraformaldehyde for 15 min on ice and subjected to immunofluorescence for detection of *Brucella* spp. (*9*). Genotyping of *Brucella* isolates was performed by PCR, using 5′-GGCTGATCTCGCAAAGAT-3′ and 5′-CCAGGTCCTTGGCTTCCTTGAG-3′ primers (Invitrogen Corporation, Carlsbad, CA, USA) for the amplification of ribosomal protein L12 and PCR-*StyI* restriction fragment length polymorphism of the *omp2b* locus, as described (*11*,*12*).

No penetrating wounds or mutilations were found in the dolphins. All animals displayed neurologic disorders characterized by the inability to maintain buoyancy and by opisthotonus, tremors, and seizures (Figure 1, panel A). In all animals, parasitosis was evident and included gastric, intestinal, and pulmonary nematodes as well as subblubber and retroperitoneal cestode larvae. Some dolphins had moderately to severely congested lungs, and 1 dolphin had splenomegaly. The pregnant female had milk in her mammary glands, a 66-cm fetus, and small placental abscesses (Figure 1, panels B, C). Hyperemic meninges, congested brain, and altered cerebrospinal fluid (augmented in volume and cellularity) were evident in all dolphins (Figure 1, panels E, F).

All dolphins had antibodies against *Brucella* LPS, as determined by immunofluorescence (>1:150 dilution), competitive ELISA (>60% positivity), and Western blot. With the exception of the female calf, all dolphins displayed positive Rose Bengal agglutination. In 6 dolphins, *Brucella* organisms were demonstrated by immunofluorescence of cerebrospinal fluids and thereafter in tissue

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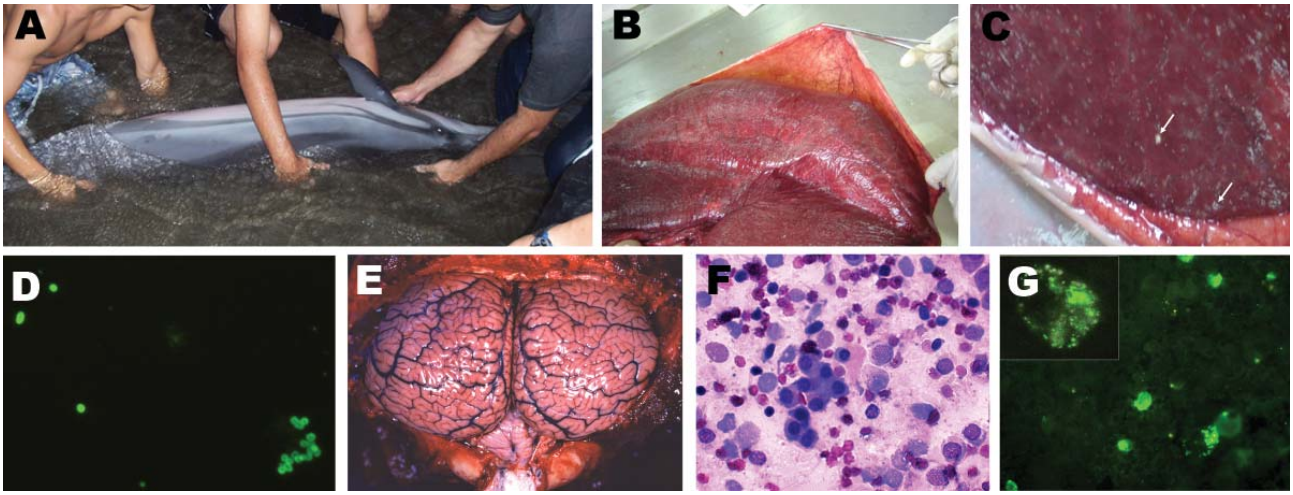

Figure 1. Clinical, pathologic, and immunofluorescence findings in stranded striped dolphin, *Stenella coeruleoalba.* A) Striped dolphin displaying swimming disorders being assisted by local persons; B) dolphin fetus within placenta; C) punctuated placental abscesses (arrows); D) immunofluorescent brucellae in impressions of placenta tissues; E) congested and hyperemic brain and cerebellum; F) Wright-Giemsa–stained mononuclear cell infiltrate in cerebrospinal fluid; G) immunofluorescent green *Brucella* spp. and *Brucella* debris within phagocytic cells infiltrating cerebrospinal fluid; the inset corresponds to an amplified phagocytic cell with fluorescent *Brucella* spp. and debris.

impressions of the brain, spinal cord, lymph nodes, spleen, liver, and kidneys. Bacteria were isolated from the same organs and fluids. The pregnant animal had bacteria in the placenta (Figure 1, panel D), umbilical cord, milk, allantoic and amniotic fluids, and fetal tissues. A large number of phagocytes infiltrating the cerebrospinal fluid of these 6 dolphins contained intracellular *Brucella* organisms (Figure 1, panel G).

Histopathologic examination of the central nervous system was performed on only the 6 dolphins from which *Brucella* organisms were isolated; tissues examined were spinal cord, medulla oblongata, cerebellum, pons, thalamic area, and the occipital and frontal cortices of the cerebrum. The most common and relevant histopathologic findings demonstrated meningoencephalitis (Figure 2) and were similar to those previously described (*13*). The predominant feature was nonsuppurative meningitis, which was more severe in the spinal cord, medulla oblongata, and cerebellum and somewhat more moderate and mild in the cerebral cortices. The meninges were hyperemic and, in most dolphins, edematous. Mild encephalitis was evidenced by a perivascular mononuclear infiltrate in the white and gray

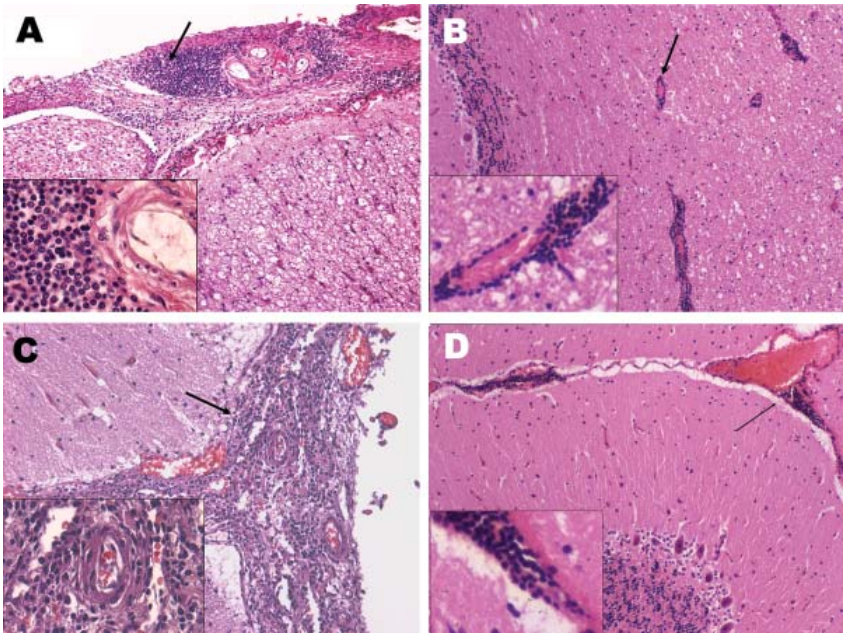

Figure 2. Main histopathologic finding of neurobrucellosis in *Stenella coeruleoalba.* A) Mononuclear infiltrates in the meninges (arrow) surrounding the spinal cord; B) mononuclear infiltrate around vessels (arrow) of the cerebellum; C) mononuclear infiltrate (arrow) in the meninges around the brain; D) hyperemic vessels and mononuclear cell infiltrate in the meninges around the cerebellum (arrow). The insets correspond to amplified sections of each figure demonstrating the mononuclear cell infiltrate.

matter of the cerebrum, cerebellum, and brainstem and by a periventricular encephalitis that was widespread surrounding the third ventricle with the same cellular infiltrate found in the meninges. A major loss of ependyma was evident; a moderate to severe mononuclear choroiditis was also present. The predominant cellular infiltrate was composed of plasma cells, small lymphocytes, and macrophages. Little or no involvement of the neural tissue was noted in areas other than the periventricular zone. Vasculitis was not found.

Organisms compatible with *Brucella* (nonmotile; urease-, catalase-, and oxidase positive; gram-negative bacteria not requiring CO_2 for growth) were isolated in blood agar or selective media from different organs, brain, and fluids from the 6 dolphins and the fetus subjected to detailed analysis. Standard bacteriologic, immunochemical, biochemical, and molecular typing indicated that the causative agent in all cases was *B. ceti* type I dolphin strain (Table).

Conclusions

This report documents the presence of marine brucellosis along Latin American shorelines. The dolphins' neurologic lesions were similar to those described previously in an infected *S. coeruleoalba* dolphin (*13*). This finding calls attention to possible increased susceptibility of this species to neurobrucellosis. Indeed, from a total of 46,826 individuals, corresponding to 28 cetacean species counted in the Costa Rican littorals, striped dolphins (inhabiting deep waters) represent only 13% of the animals sighted (*14*). From January 2004 through December 2006, the number of cetaceans reportedly stranded along the Pacific coast of Costa Rica was 31 (*15*). Of these, 14 were *S. coeruleoalba*; the other 16 comprised 11 species of odontocetes. During the 32-month period, we were able to collect samples: 10 (this study) from *S. coeruleoalba* and 1 from *K. sima*. This whale was negative for *Brucella* spp. infections. Endemic and migrating groups of *Delphinus delphis* and *Stenella attenuate* are found more frequently along the Costa Rican shorelines; however, few strandings of these species have been reported (*14*). The relatively high number of stranded *S. coeruleoalba* dolphins along the Costa Rican shorelines is in agreement with records of strandings along the European littorals (*11*). These results argue in favor of higher susceptibility of this species to neurobrucellosis.

The isolation of *B. ceti* from milk, fetal tissues, and secretions of a pregnant dolphin, and a similar discovery in European littorals, suggests that *B. ceti* is able to display tropism for placental and fetal tissues and that the bacteria may be shed, as it is with *Brucella*-infected livestock. This finding documents vertical transmission and the possibility of horizontal transmission to newborns. Moreover, the localization of the bacteria in particular organs suggests the possibility of transmission through sexual intercourse and may ensure the prevalence of both clinical and latent infections.

In terms of zoonotic transmission, we noticed that people handled and touched all these infected dolphins, mainly as an attempt to return them to the ocean. Other stranded dolphins have been transferred to privately owned swimming pools or to slaughter for use as a food source for humans and domestic animals. In this regard, susceptibility of *S. coeruleoalba* as reservoirs of *Brucella* spp. and modes of transmission must be taken into consideration.

Acknowledgments

We thank José-María Gutiérrez for assistance with preliminary diagnosis of the histologic sections and Charles Manire for advice during the development of this work. We thank Fundación PROMAR and Fundación Keto for logistic support during these 2 years of investigations.

Table. Characterization of *Brucella spp.* isolated from *Stenella coeruleoalba* and comparison with marine reference strains

	RTD phage lysis*				CO_2	Serum against†		Growth on dyes, μg/mL‡						*Omp2b*
								Thionin				Basic fuchsin	O safranin	restriction
Species, strain§	Tb	Wb	Iz	R/C	¶	A	M	10	20	40	100	20	100	pattern
B. ceti, B14/94#	–	+	+	–	–	+	+	+	+	+	+	+	+	Dolphin
								(+)	(±)	(–)	(+)	(–)	(+)	type I
B. ceti, B1/94#	–	+	–	–	–	+	–	+	+	+	+	+	+	Porpoise
								(+)	(+)	(+)	(–)	(–)	(–)	type II
B. pinnipedialis, B2/94#	–	–	+	–	+	+	–	+	+	+	+	+	+	Seal
								(–)	(–)	(–)	(–)	(–)	(–)	
B. ceti, B1 to B6**	–	+	+	–	–	+	+	+	+	–	+	+	+	Dolphin
								(+)	(–)	(–)	(–)	(–)	(–)	type I

*RTD, routine test dilution of phages Tbilisi (Tb), Weybridge (Wb), Izatnagar (Iz), and rough type Wb derivative (R/C).
†Serum against lipopolysaccharide epitopes, measured as agglutination with monospecific serum.
‡Dye concentrations expressed in μg/mL of culture medium with 10% CO_2 or, within parenthesis, raw incubation without CO_2.
§All reference and isolated strains were SH_2 negative, urease positive and amplified *Brucella spp* L12 rRNA by PCR.
¶Requirement.
#Reference strains (*11,12*).
**Isolates from Costa Rica.

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Monkey Malaria in a European Traveler Returning from Malaysia

Anu Kantele, Hanspeter Marti, Ingrid Felger, Dania Müller, and T. Sakari Jokiranta

In 2007, a Finnish traveler was infected in Peninsular Malaysia with *Plasmodium knowlesi*, a parasite that usually causes malaria in monkeys. *P. knowlesi* has established itself as the fifth *Plasmodium* species that can cause human malaria. The disease is potentially life-threatening in humans; clinicians and laboratory personnel should become more aware of this pathogen in travelers.

Traditionally, only 4 *Plasmodium* species have been known to cause malaria in humans: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*, although >26 *Plasmodium* species are known to circulate among primate populations (*1*). Some of these species have been implicated in symptomatic human malaria after experimental or accidental infection (*2*). Only a few reports of naturally acquired monkey malaria in humans are currently available (*1*,*3*–*9*). The lack of data may be because light microscopy has been used as the sole diagnostic method and an atypical *Plasmodium* species may have been misidentified as one of the 4 traditional *Plasmodium* species causing human malaria.

P. knowlesi was first described in 1931 in a long-tailed macaque imported from Singapore to India; in 1932, *P. knowlesi* was experimentally shown to be infectious to humans (*10*). The first natural infection of *P. knowlesi* in humans was reported in 1965 in a man returning to the United States after a visit to Peninsular Malaysia (*11*). Subsequently, in 1971, there was a report of a presumed natural infection in a citizen of Malaysia (*6*). Despite extensive studies in Malaysia in the 1960s (*2*), no other reports were published on naturally acquired *P. knowlesi* infections in humans until 2004, when Singh et al. studied PCR-negative *P. malariae* cases in the Kapit division in Sarawak, Malaysia (*3*). A different PCR analysis showed that *P. knowlesi* caused 58% of the 208 malaria cases studied. Further cases reported from China (*4*), Thailand (*5*), Philippines (*8*), and Singapore (*12*) show that *P. knowlesi* infections in humans are not found exclusively in Malaysia. Recently, Cox-Singh et al. reported that *P. knowlesi* is widely distributed among inhabitants of Malaysia (*7*).

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The Study

A 53-year-old Finnish man was admitted to a local hospital in Finland in March 2007 with fever after 4 weeks of travel in Peninsular Malaysia. He had not taken any antimalarial prophylaxis. In Malaysia, he spent 2 weeks in Kuala Lumpur and made a few day trips to surrounding rural areas. Thereafter, he traveled by car to the northwestern coast and stayed for 5 days in the jungle ≈80 km south of Ipoh. While in this area, he slept in a house without mosquito screens or nets and did not use any repellents; he did not report any mosquito bites. The last week of his travel was spent in the Langkawi Beach area where he stayed at a high-quality hotel. During his trip he occasionally had some minor abdominal problems, but these symptoms subsided spontaneously after his return to Finland. High fever (38.8°C axillary temperature) occurred 3 days after his return to Finland but abated quickly. On the fourth day, the fever returned and he sought medical care at a local hospital. Laboratory tests showed the following results: C-reactive protein 2.0 mg/dL (normal range <1.0 mg/dL), hemoglobin 15.2 g/dL (normal range 13.4–16.7 g/dL), leukocyte count 2.6 × 10^9/L (normal range 3.4–8.2 × 10^9/L), and thrombocytes 143 × 10^9/L (normal range 150–360 × 10^9/L). Blood smear was positive for *Plasmodium* organisms, and the causative agent was identified as *P. falciparum* with levels of parasitemia <1.0%. The patient was admitted to the hospital and given intravenous (IV) quinine dihydrochloride and oral doxycycline.

On day 2 of the patient's hospital stay, fever returned and he was transferred to the Helsinki University Central Hospital (Department of Infectious Diseases at Aurora Hospital). Blood smears obtained there showed *Plasmodium* parasites that were considered atypical, and the laboratory reported suspicion of a co-infection (*P. falciparum* and *P. malariae*) (Figure). The IV quinine dihydrochloride was replaced with oral quinine hydrochloride, and doxycycline was continued. During treatment, the patient experienced an attack of hypoglycemia (electrocardiogram and blood pressure was normal during this attack), transient mild visual and hearing loss, and transient lymphopenia (a low of 0.46 × 10^9/L). He received quinine hydrochloride and doxycycline for a total of 10 days.

Because identification of the *Plasmodium* species was difficult, a blood sample was drawn for PCR analysis on day 2 of hospitalization. First, a nested PCR was performed according to a standard protocol with rOva1 and rPLU2 primers (template DNA purified in Basel from 200 μL of erythrocytes by QIAamp DNA Mini Blood Kit (QIAGEN,

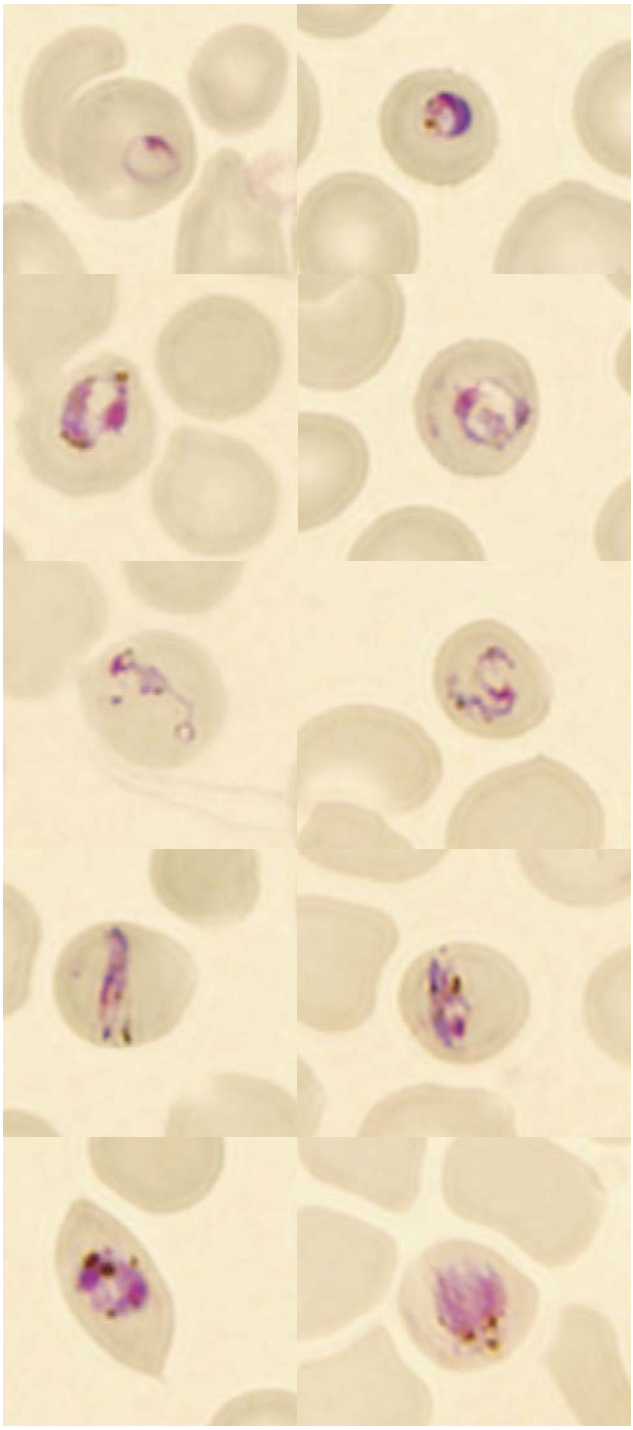

Figure. Microscopic findings in the thin blood smears of a patient with *Plasmodium knowlesi* malaria. Early ring forms are shown in the first row, later trophozoites in the second and third rows, trophozoites resembling band forms in the fourth row, and putative early gametocytes or schizonts in the fifth row. Size of the infected erythrocytes is normal. Antimalarial medications, given 8 hours before the blood shown in the smear was drawn, could have affected morphology. (Original magnification ×1,000.)

Helsinki, Finland) (*13*,*14*), but the reaction did not yield any amplification product. Nested PCR was repeated with an alternative primer pair (rPLU6 and rPLU2) (*14*) derived from a conserved region of the 18S rRNA marker gene, and an amplicon was obtained. Failure of PCR amplification has been reported for some *P. ovale* isolates (*15*); therefore, a *P. ovale* infection was suspected, and the patient was given primaquine phosphate for 14 days as an outpatient to eradicate possible liver hypnozoites. The PCR product was subjected to direct nucleotide sequencing (GenBank accession no. FJ009511) and found to be identical to 2 *P. knowlesi* sequences previously submitted to GenBank, 1 human isolate from Malaysian Borneo (AY327556) and a *Macaca mulatta* isolate from Columbia (U72542). Six other published *P. knowlesi* sequences differ from our sequence only by 1 nucleotide (99% identity). In contrast, a number of differences were seen between our sequence and the *P. ovale* sequences (*15*). The sequence from our case showed only 50% identity to the ova1e primer; therefore, we concluded that our patient was infected with *P. knowlesi*. During the 12-month follow-up period, the patient showed no signs of relapse.

Conclusions

We suggest that *P. knowlesi* infection should be considered in malaria patients who have a history of a travel to forested areas in Southeast Asia, especially if *P. malariae* malaria is diagnosed or atypical plasmodia are seen with microscopy. The asexual stages of various species of *P. knowlesi* can easily be misidentified as *P. malariae* in light microscopic examination (Figure) (*3*,*7*,*10*). Because most laboratories diagnose malaria by light microscope examination only, numerous cases of *P. knowlesi* malaria may have been misdiagnosed as ordinary *P. malariae* malaria; monkey malaria may be more widespread among humans than was previously thought. As the disease is potentially dangerous, a proper identification of the malaria species is crucial. If PCR assays for malaria detection are used, PCR primers specific for *P. knowlesi* (*3*) should be included to provide valuable diagnostic information.

P. knowlesi has established itself as the fifth species of *Plasmodium* that causes human malaria (*3*,*7*,*12*). Because the disease is potentially life-threatening in humans, laboratory clinicians and physicians (especially those taking care of travelers) should become more aware of this disease; it is easily misdiagnosed as a less severe form of malaria.

Acknowledgments

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Mycobacterium haemophilum and Lymphadenitis in Immunocompetent Children, Israel

Yishai Haimi Cohen, Jacob Amir, Shai Ashkenazi, Tal Eidlitz-Markus, Zmira Samra, Lea Kaufmann, and Avraham Zeharia

The database of a major microbiology laboratory in Israel was searched to determine the prevalence of nontuberculous mycobacterial lymphadenitis in immunocompetent children. We observed a 4-fold increase in nontuberculous mycobacteria isolates during 1985–2006, which was attributable mainly to increased detection of *Mycobacterium haemophilum* starting in 1996.

Nontuberculous mycobacteria (NTM) are a common cause of nonpyogenic craniofacial lymphadenitis in otherwise healthy children. *Mycobacterium avium* complex (MAC) is the main pathogen (*1*–*3*). *M. haemophilum* is traditionally considered a cause of NTM in immunocompromised patients (*4*–*7*), although a recent study from the Netherlands found that it is also common in immunocompetent children (*8*).

The Study

Prompted by the increasing number of *M. haemophilum* isolates identified at our tertiary medical center in Israel in the past decade, we investigated the current prevalence and clinical characteristics of NTM lymphadenitis in immunocompetent children. The database of our microbiology laboratory was searched for all NTM-positive cervical lymph node cultures of children during 1985–2006. In addition, we reviewed records of the Day Hospitalization Unit (DHU) for all patients with a diagnosis of NTM lymphadenitis from January 1996 (when *M. haemophilum* was first isolated in our laboratory) through December 2006. Data obtained were patient age and sex, *Mycobacterium* species, ethnic background (Jewish/Arab), medical history, duration of node enlargement until referral, site affected, number of infected sites, size of nodes at initial visit (measured by the clinician), discoloration of the skin overlying the lymph nodes, and maximal induration in response to purified protein derivative (PPD). Patients with *M. haemophilum* infection were compared with those with MAC infection.

All specimens were processed for direct Ziehl-Neelsen staining. From 1985 through 1995, specimens were placed on solid Lowenstein-Jensen (L-J) medium. Thereafter, liquid MB Redox broth and liquid Bactec 460 12B medium (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) were added to the L-J medium. Toward the end of 1999, Bactec medium was replaced with the liquid Mycobacteria Growth Indicator Tube system (Becton Dickinson Microbiology Systems). A hemin-containing paper strip (X-factor) was regularly placed into the 2 liquid media, which were incubated with the L-J medium at 37°C and 30°C.

Discrete variables were compared between groups by using a Pearson χ^2 test or Fisher exact test, as appropriate. Continuous variables were compared with 1-way analysis of variance. A p value ≤ 0.05 was considered statistically significant. Data were analyzed by using BMDP software (www.statsol.ie/html/bmdp/bmdp_home.html). The study was reviewed and approved by the local ethics committee.

The laboratory database contained 111 NTM isolates during 1985–2006, of which 77 (69%) were in samples from patients who visited the DHU from January 1996 through December 2006. Species distribution was as follows: MAC, 54; *M. haemophilum*, 41; others, 16. The Figure shows the increase in isolation rate of NTM and *M. haemophilum* since the initial isolation of *M. haemophilum* in March 1996.

The 77 patients managed at our DHU for NTM lymphadenitis included 38 boys (49%) and 39 girls 8 months to 15.5 years of age (median 2.4 years). *M. haemophilum* was isolated from 39 children and MAC from 29. The demographic and clinical features of these children are shown in the Table. The patients in the *M. haemophilum* group were significantly older than those in the MAC group (mean 4.7 years vs. 2.3 years; $p<0.001$); 9 patients with *M. haemophilum* infection (23%) were >7 years of age compared with none with MAC infection. Mean time to referral was significantly longer in the *M. haemophilum* group (1.5 months vs. 1.1 months; $p = 0.045$). No statistically significant differences were noted for the other parameters studied.

Conclusions

We speculate that the nearly 4-fold increase in the recovery rate of NTM from lymph nodes of immunocompetent children in the past 22 years at our center was attributable to the emergence of *M. haemophilum* as a major pathogen of craniocervical lymphadenitis starting in 1996.

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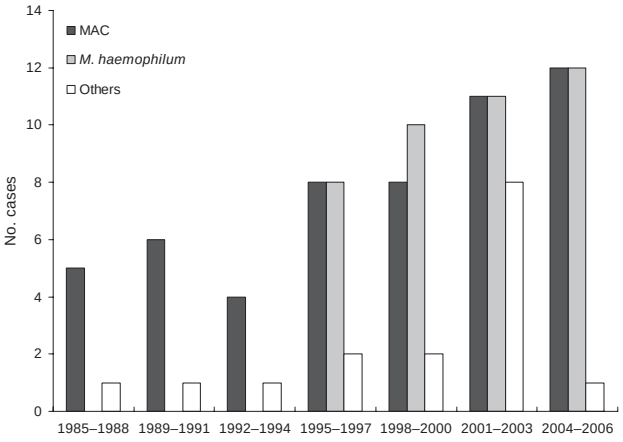

Figure. Distribution of nontuberculous mycobacteria species (*Mycobacterium avium complex* [MAC], *M. haemophilum*, and others) isolated from craniocervical lymph nodes of immunocompetent children in Israel, 1985–2006.

This assumption is supported by the only slight increase in the other NTM pathogens during the study period. The increased prevalence of NTM cervical lymphadenitis may be explained by conversion of our hospital to a tertiary pediatric center in 1991, concomitant with the growing awareness of NTM as a cause of craniocervical lymphadenitis among its physicians. Furthermore, the rate of isolation of *M. haemophilum* in this study was 51%. Another study from the Netherlands reported a similarly high prevalence rate (44%) of *M. haemophilum* lymphadenitis in immunocompetent children (*8*).

Although the distribution of NTM species may depend on local ecologic factors (*9*), given the wide geographic range of *M. haemophilum* infection in immunocompromised children (*4–7*) we would have expected to see reports of increased infection in immunocompetent children from >2 countries. We suspect that the change in our laboratory's processing procedure in 1996 to include broth with an iron supplement and incubation of the samples at 30°C in addition to 37°C (*10*) contributed to the high isolation rate. Our failure to use these conditions before 1996 could have led to an underdiagnosis of *M. haemophilum* infection; this may also be true for other laboratories (*11*).

These findings suggest that a failure to isolate a pathogen in children with suspected mycocbacterial craniocervical lymphadenitis, especially those >7 years of age, should prompt a targeted laboratory search for *M. haemophilum* by using proper culture conditions or molecular techniques (*10–12*). Identification of NTM infection has serious clinical implications because it can spare these patients, who often have a positive PPD response and cytologic results compatible with tuberculosis, unwarranted, prolonged antituberculosis therapy. The mean PPD response in our patients (>14.5 mm) confirms the lack of value of PPD in distinguishing NTM infection from tuberculosis (*13*).

The higher mean age of the children with *M. haemophilum* infection in our series compared with that of children in the MAC group is consistent with findings of a study in the Netherlands (*8*). Our finding may be explained by the younger age at which children are exposed to playgrounds, which are presumably linked to MAC infection, than to swimming pools, which are presumably linked to

Table. Demographic and clinical characteristics of children with *Mycobacterium haemophilum* and *M. avium* complex lymphadenitis*

Variable	*M. haemophilum*, n = 39	*M. avium* complex, n = 29	p value
Mean age, y (range)	4.7 (8 mo–15.5 y)	2.3 (9 mo–7y)	<0.001
Male/female, no. (%)	17/22 (44/56)	16/12 (57/43)	0.33
Ethnic origin, no. (%)			
Jewish	26 (81.2)	22 (81.5)	1.0
Arab	6 (18.8)	5 (18.5)	
Mean time to referral, mo (range)	1.47 (0.25–5)	1.1 (0.25–3)	0.045
Site, no. (%)			
Submandibular	24 (61.5)	13 (46.4)	0.32†
Neck	7 (17.9)	6 (21.4)	
Preauricular	3 (7.7)	3 (10.7)	
Occipital	–	1 (3.6)	
Cheek	–	4 (14.3)	
Bilateral submandibular	1 (2.6)	–	
Submandibular and preauricular	3 (7.7)	1 (3.6)	
Neck and preauricular	1 (2.6)	–	
No. infected sites: 1/2, no. (%)	34/5 (87/13)	27/1 (96/4)	0.72
Side: right/left, no. (%)	23/16 (59/41)	21/7 (76/24)	0.20
Mean size of lymphadenopathy, cm^2 (range)	8.7 (1.0–25)	8.9 (1.0–35)	0.93
Skin discoloration: yes/no, no. (%)	15/14 (52/48)	9/9 (50/50)	1.0
PPD: mean maximal induration, cm (range)	17.9 (0–32)	14.5 (0–45)	0.22

*PPD, purified protein derivative.
†Refers solely to the submandibular site.

M. haemophilum infection (*8*). However, the mean age of our patients was lower by >1 year than the age of the Dutch children (*8*), perhaps the result of warmer climate and of the younger age of daycare attendees in Israel, both of which are associated with longer and earlier exposure to sandpits and swimming pools.

In contrast to the results of the Dutch study (*8*), ethnicity was not a risk factor; the rates of affected Jewish and Arab children matched the distribution of these ethnicities in the general population of Israel. This finding may reflect the similar environmental conditions to which these ethnic groups are exposed.

Despite the wide variability in the interval from onset of lymph node swelling to patient referral for investigation, the mean time of ≈5 weeks is consistent with that in previous studies (*1*–*3*). The difference of ≈10 days between the 2 groups in our study, although statistically significant, was not of clinical importance. The longer interval in the *M. haemophilum* group might have been caused by less attention parents tend to pay to physical changes in older children than in infants. In contrast to the findings in the Dutch study (*8*), we noted no predilection to multisite infection in either group, and all 4 patients with extranodal (cheek) involvement were infected with MAC.

In conclusion, *M. haemophilum* is an emerging pathogen in nonpyogenic craniocervical lymphadenitis in immunocompetent children in Israel. *M. haemophilum* infection usually affects older children (>7 years of age) but is otherwise clinically similar to MAC infection.

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Obligations to Report Outbreaks of Foodborne Disease under the International Health Regulations (2005)

Martyn Kirk, Jennie Musto, Joy Gregory, and Kathleen Fullerton

Every year, Australia identifies 2–3 outbreaks associated with imported foods. To examine national authorities' obligations under the International Health Regulations (2005), we reviewed outbreaks in 2001–2007 that implicated internationally distributed foods. Under these regulations, 7 (50%) of 14 outbreaks would have required notification to the World Health Organization.

During the past 2 decades the global trade in food has increased, making outbreaks associated with internationally distributed foods more common (*1*). These outbreaks are challenging to identify and control. This is despite the involvement of multinational agencies, such as the World Health Organization (WHO), the European Centre for Disease Prevention and Control, and surveillance networks, such as PulseNet International and the European Foodborne Viruses Network, which use molecular techniques to rapidly compare infecting strains (*1–4*). Food is a silent vehicle for spreading pathogens and chemicals across country borders (*5*). Whenever agencies responsible for health, agriculture, or food safety identify contaminated foods that are imported or exported, the potential for human illness to occur in other countries exists.

WHO recently revised the legally binding International Health Regulations (IHR) to respond more effectively to the increasing spread of disease internationally (*6*). IHR (2005) are based on a risk assessment approach and came into force on June 15, 2007. Under IHR (2005), countries are required to designate or establish a National IHR Focal Point, which should be a national center for urgent communications under the regulations. These regulations include a decision-making instrument that lead National Focal Points through a series of 4 questions to assist them in making a decision to report events to WHO for international alert and response (Figure) (*7*). IHR (2005) cover events of international importance that involve contaminated food and outbreaks of foodborne disease. In 2004, WHO, in collaboration with the Food and Agriculture Organization of the United Nations, launched the International Food Safety Authorities Network (INFOSAN) to improve food safety information exchange and cooperation, including a food safety emergency component (INFOSAN Emergency; see www.who.int/foodsafety/fs_management/infosan/en). WHO has developed guidance to illustrate how INFOSAN Emergency complements processes under IHR (2005) (*8*).

In Australia, health departments in 6 states and 2 territories led multiagency teams to investigate and control ≈100 outbreaks of foodborne disease that affected 2,000–

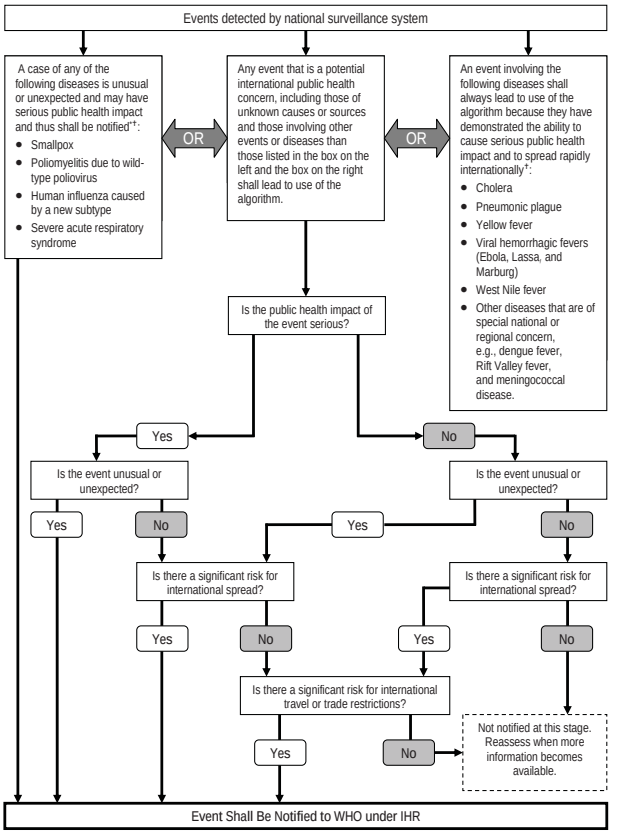

Figure. Decision instrument for the assessment and notification of events that may constitute a public health emergency of international concern under International Health Regulations (IHR) (2005). WHO, World Health Organization. *As per WHO case definitions. †The disease list shall be used only for the purposes of these regulations; adapted from Annex II of IHR (2005), reported in (*7*).

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4,000 people each year. The results of these investigations are summarized and reported to OzFoodNet, Australia's national system of foodborne disease surveillance. When outbreaks are spread across jurisdictional or country borders, OzFoodNet coordinates national responses to determine the cause of the outbreak and prevent further spread.

In this report, we assess well-characterized outbreaks of foodborne disease traced to internationally distributed foods. We used questions in the IHR decision-making algorithm to examine whether these outbreaks would be potentially reportable under IHR (2005). We also make some observations on investigating and managing these international outbreaks from recent experiences, including interaction with INFOSAN Emergency.

The Outbreaks

In the 7-year period 2001–2007, 14 (1.8%) of 768 foodborne outbreaks were associated with foods that were distributed internationally (online Appendix Table, available from www.cdc.gov/EID/content/13/9/1440-appT.htm). In total, these outbreaks affected at least 542 persons in Australia, 4.4% (542/12,423) of all those affected by foodborne disease outbreaks during the period. The median size of these outbreaks was 20 persons (range 3–230). The number of persons affected in other countries as a result of these events was unknown. Given the nature of foodborne disease, more outbreaks that we were unable to identify were likely associated with internationally distributed foods.

Several point-source outbreaks were related to each other by a common food source, even though the foods were often branded differently and supplied by different companies. The outbreaks of suspected norovirus infection (outbreaks 4, 6, and 9) were associated with individually quick frozen (IQF) oysters all harvested from the same region in Japan; this association was later confirmed after a national investigation into 3 related outbreaks (outbreaks 7, 8, and 10) (*9*). These outbreaks occurred over a 3-year period and resulted in Australia's imposing restrictions on importation of IQF oysters from this growing area.

No outbreaks was considered to be of "serious public health impact" because of their small size and moderate severity. In 4 (29%) of 14 outbreaks, the event was considered "unusual or unexpected" in Australia because of novel disease-causing agents. However, agents considered novel in Australia were common causes of disease in the country exporting the food (*10*). In 5 (36%) of the 14 outbreaks, food had been distributed to other countries, resulting in multinational food recalls; 4 more events had the potential to spread to other countries. We identified the implicated food for 2 outbreaks (outbreaks 1 and 14) because other countries rapidly published reports in Eurosurveillance Weekly. We alerted other countries to the implicated food for 3 other common-source outbreaks (outbreaks 3 and 5, by using rapid reports in the same publication and outbreak 2 through ProMED Mail) (*11–14*).

During these investigations, we attempted to identify other countries that had also received contaminated food. Before the inception of INFOSAN, we relied on diplomatic communications with the exporting country, which were often unsuccessful. In a recent incident in which persons became infected with toxigenic *Vibrio cholerae* after eating raw whitebait (outbreak 13), INFOSAN Emergency made inquiries of the exporting country and confirmed that fish had not been exported to other countries and that no outbreak was observed locally (*15*). During a multicountry outbreak of drug-resistant shigellosis (outbreak 14), INFOSAN Emergency Focal Point at WHO gained the exporting country's cooperation to trace back the produce to the facility concerned and informed other countries receiving the same batch of produce (*11*) (online Appendix Table).

Conclusions

Although IHR (2005) only came into force in June 2007, we consider that there would have been a basis for reporting 7 (50%) of 14 imported food outbreaks, with 3 of these being part of the same IQF oyster contamination event. Although National IHR Focal Points may decide not to notify or report an outbreak under IHR (2005), it is vital that they publish rapid reports involving imported and exported foods, given the potential of these foods to spread disease internationally, and consult with WHO through INFOSAN Emergency. In this report, we considered only those events that resulted in human illness, but it is important for National IHR Focal Points to consult with the INFOSAN Emergency Contact Point for their country and to consider notifying and/or reporting events in which food is contaminated in the absence of human illness. Serious, unusual, or unexpected events associated with domestic food may also trigger the criteria, even when foods are not exported. Note that under Article 9.2 of IHR (2005), public health risks associated with importation of contaminated goods may be reported to WHO independent of the event's meeting the Annex II criteria. This stipulation would allow reporting when available information is insufficient to make an adequate assessment under Annex II. We found WHO INFOSAN Emergency complementary to the management of IHR (2005). The role of WHO and other agencies in these events of potential international importance will undoubtedly continue to evolve.

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Occurrence and Clinical Relevance of *Mycobacterium chimaera* sp. nov., Germany

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Retrospective molecular genetic analysis of 166 *Mycobacterium intracellulare* isolates showed that 143 (86%) strains could be assigned to *Mycobacterium chimaera* sp. nov. Of 97 patients from whom *M. chimaera* sp. nov. was isolated, only 3.3% exhibited mycobacterial lung disease, whereas all *M. intracellulare* isolates caused severe pulmonary infections.

Bacteria of the *Mycobacterium avium* complex (MAC) play an important role among infections caused by nontuberculous mycobacteria (NTM). MAC consists of the 2 well-established species, *M. avium* (which has 4 subspecies) and *M. intracellulare*, as well as several other closely related mycobacteria (*1*). Recently, a new species derived from the group of unnamed members of the MAC has been defined. It combines features characteristic of different MAC members and has been named *M. chimaera* sp. nov. (*2*).

Based on the sequence of the 16–23S internal transcribed spacer (ITS) region, this species genetically corresponds to sequevar MAC-A and differs from *M. intracellulare* type strain, sequevar MIN-A (DSMZ 43223) by 20 nt mismatches (*2*,*3*). In contrast, the 16S rRNA gene sequence is identical, except for 1 nt mismatch, with that of the *M. intracellulare* type strain. Because sequencing of the 16S rDNA still is considered the approved standard for the identification of NTMs, *M. chimaera* sp. nov. usually has been misreported as *M. intracellulare*. Molecular genetic standard tools in clinical microbiologic laboratories do not differentiate MAC members. These tools merely provide a rough classification in *M. intracellulare* and *M. avium* and/or the MAC group as a whole. Currently, a detailed genotyping of MAC is restricted to research laboratories. Nevertheless, several studies have shown that certain serotypes or genotypes were associated with different clinical manifestations of MAC infection concerning the patient groups affected, the localization and course of disease, and the antimicrobial drug resistance patterns (*4*,*5*).

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The Study

Since available data on the epidemiology of *M. chimaera* sp. nov. are sparse, we performed a retrospective study to determine the frequency of its occurrence within the group of MAC-positive clinical specimens and its possible role in causing human disease in comparison to *M. intracellulare*. We reanalyzed mycobacterial isolates from 97 in-house patients of the Charité University Hospital that have been processed in our laboratory from 2002 through 2006. An additional 69 isolates were provided by the National Reference Center (NRC) for Mycobacteria in Borstel, Germany. All strains had previously been classified as *M. intracellulare* by 16S rDNA–based methods. In addition to the partial 16S rRNA gene, we sequenced the 16S–23S ITS region to allow for unambiguous identification. Amplification of the partial 16S rRNA gene was performed according to a standard procedure (*6*). For the amplification of the ITS, the following primers were used: Sp1 (5′-ACC TCC TTT CTA AGG AGC ACC-3′) and Mb23S.44n (5′-TCT CGA TGC CAA GGC ATC CAC C-3′) (*7*,*8*). PCR conditions and the sequencing procedure are described elsewere (*9*). The assignment to sequevars was based on the ITS sequence, according to the taxonomy introduced by Frothingham and Wilson (*3*). Laboratory analysis was performed without knowledge of the clinical course of the disease. The frequency distribution of the 166 strains according to their species or sequevar designations is presented in Table 1.

In addition, we tested the application of denaturating high-performance liquid chromatography (DHPLC) for the identification of the ITS PCR product to distinguish *M. intracellulare,* type strain, and *M. chimaera* sp. nov. DHPLC is a semiautomated, quick, and sensitive technique and has been used for the detection of genetic variations predominantly for genotyping purposes of a wide range of human diseases (*10*). Recently, it has also been introduced for the identification and genotyping of bacterial species (*11*) and yeasts (*12*). The amplified ITS gene fragments were separated on the WAVE 3500 HT System (Transgenomic, Omaha, NE, USA). Optimal separation was achieved at an oven temperature of 61.5°C and a flow rate of 1.4 mL/min on an integrated DNASep HT cartridge. Samples were loaded in 53.5% buffer A (0.1 mmol/L triethylammoniumacetate [TEAA]) and 46.5% buffer B (0.1 mmol/L TEAA in 25% acetonitrile). After 30 s, buffer B was set to 51.5%, reaching 60.5% after an additional 4.5 min. The

Table 1. Distribution of mycobacterial species and sequevars*

Species, sequevar†	Total isolates, no. (%), n = 166	Isolates from Charité,‡ no. (%), n = 97	Isolates from NRC,‡ no. (%), n = 69	Odds ratio (p value§)
MAC				
MAC-A	143 (86.1)	90 (92.8)	53 (76.8)	3.88 (p = 0.003)
MAC-C	2 (1.2)	1 (1.0)	1 (1.5)	ND
MAC-E	1 (0.6)	1 (1.0)	0	ND
Min				
Min-A	17 (10.2)	3 (3.1)	14 (20.3)	0.12 (p<0.001)
Min-C	3 (1.8)	2 (2.1)	1 (1.5)	ND

*MAC, *M. avium* complex species; MAC-A, *M. chimaera* sp. nov.; ND, not done; Min, *M. intracellulare*; Min-A, *M. intracellulare* type strain.
†Classification of MAC strains according to the taxonomy of Frothingham and Wilson (*3*).
‡Charité, Charité University Hospital, Berlin, Germany; NRC, National Reference Center for Mycobacteria, Borstel, Germany.
§χ^2 test, software package Stata version 9 (Stata Corporation, College Station, TX, USA).

column was cleaned with 100% buffer B (5.0–5.6 min) and equilibrated with 46.5% buffer B (5.7–6.6 min) before the next injection. Analysis was accomplished with Navigator software version 1.5.4 (Transgenomic).

Both reference strains, *M. intracellulare,* sequevar Min-A (DSMZ 43223), and *M. chimaera* sp. nov., sequevar MAC-A (DSMZ 44623), showed reproducible peak profiles. *M. avium* spp. *avium*, sequevar Mav-A, DSMZ 44156 (21 mismatches to *M. intracellulare*, sequevar Min-A; 18 mismatches to *M. chimaera* sp. nov.,) and *M. intracellulare*, sequevar Min-C (2 mismatches to *M. intracellulare,* sequevar Min-A; 14 mismatches to *M. chimaera* sp. nov.) served as negative run controls providing a different peak location. All clinical *M. intracellulare*, sequevar Min-A strains, and *M. chimaera* sp. nov. strains (sequevar MAC-A) could be allocated unequivocally to the 2 reference strains by their congruent peak patterns (Figure). *M. avium* complex isolates, sequevars MAC-C and MAC-E, and *M. intracellulare* isolates, sequevar Min-C, showed different peak profiles that could easily be separated from the 2 reference species. All DHPLC results could be reproduced in a second run conducted on another day.

The clinical relevance of *M. intracellulare/chimaera* sp. nov. strains isolated from respiratory specimens of 97 in-house patients of the Charité University Hospital has been assessed according to the 1997 American Thoracic Society criteria for NTB lung disease (*13*). The data have been drawn from past hospital records. Cases were subdivided into 3 categories: clinically relevant, clinically not relevant, and undetermined (Table 2). A clinical follow up of the 69 isolates provided by the NRC for mycobacteria was not possible. The characteristics of the patients with mycobacterial infection resemble already known features, such as underlying lung disease, immunosuppression, female sex, and microscopically positive respiratory samples (online Appendix Table, available from www.cdc.gov/EID/content/14/9/1443-appT.htm).

Conclusions

DHPLC was a rapid and reliable method for distinguishing *M. intracellulare* type strain from *M. chimaera* sp. nov. within a well-defined group of mycobacterial isolates. Low costs and the high degree of automation predispose this technique for epidemiologic studies. Our results show that *M. chimaera* sp. nov. accounts for most of the mycobacterial isolates formerly classified as *M. intracellulare*. The small number of clinically relevant isolates (3.3%) suggests relatively low pathogenicity. As most other studies assessing the pathogenic potential of clinical NTM isolates referred either to members of the whole MAC group (inclusively *M. avium*) or to the complete NTM spectrum, their results cannot be compared (*14*). Our observations are not concordant with those of Tortoli et al. (who suspected that *M. chimaera* sp. nov. was highly virulent), possibly

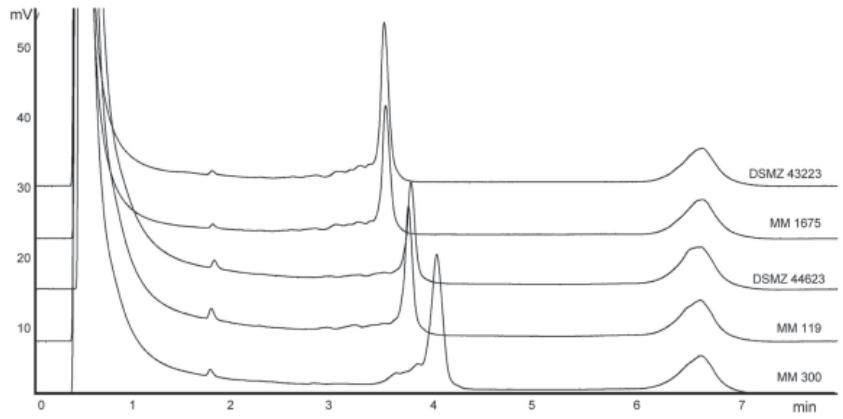

Figure. Denaturating high-performance liquid chromatography profiles after separation of PCR-amplified internal transcribed spacer regions of *Mycobacterium* spp. Strain designations from above: DSMZ 43223, *M. intracellulare*, sequevar MIN-A type strain; MM 1675, *M. intracellulare*, sequevar MIN-A, patient strain; DSMZ 44623, *M. chimaera* sp. nov., sequevar MAC-A type strain; MM 119; *M. chimaera* sp. nov., sequevar MAC-A, patient strain; MM 300, *M. intracellulare*, sequevar MIN-C, patient strain.

Table 2. Distribution of MAC isolates according to ATS criteria*

Mycobacterial species, sequevars†	Total no. (%)	Clinically relevant,‡ no. (%)	Clinically not relevant,§ no. (%)	Clinical relevance undetermined,¶ no. (%)
MAC				
MAC-A	90 (100)	3 (3.3)	82 (91.1)	5 (5.6)
MAC-C	1 (100)	0	1 (100)	0
MAC-E	1 (100)	0	1 (100)	0
Min				
Min-A	3 (100)	3 (100)	0	0
Min-C	2 (100)	0	2 (100)	0
Total	97 (100)	6 (6.2)	86 (88.7)	5 (5.2)

*ATS, American Thoracic Society; MAC, *Mycobacterium avium* complex; MAC-A, *M. chimaera* sp. nov.; Min, *M. intracellulare*. Min-A, *M. intracellulare* type strain.
†Classification of MAC strains according to the taxonomy of Frothingham and Wilson (*3*).
‡ATS criteria (*13*) for mycobacterial lung disease are fulfilled.
§ATS criteria for mycobacterial lung disease are not fulfilled.
¶ATS criteria for mycobacterial lung disease are fulfilled, but radiologic findings have been attributed to the underlying illness or insufficient sample numbers.

because of the low number of cases analyzed (12 patients) (*2*). The most striking result of our study was that all 3 *M. intracellulare,* sequevar Min-A, isolates were unequivocally associated with severe mycobacterial lung disease. Despite the low case numbers, these findings suggest that this species is more virulent and justify further epidemiologic investigations to verify this observation.

In agreement with other authors, our observations indicate that a precise differentiation of MAC isolates may provide clinically relevant data (*4*,*5*). This conclusion is in accord with a recently published review that discusses advances and future aspects of MAC genomics and points out the importance of taking into account the heterogeneity of MAC species (*15*).

If one assumes substantial differences in pathogenicity, the allocation of MAC isolates to defined species may facilitate diagnosis of mycobacterial lung disease. However, implementation of a staged identification procedure in routine microbiologic laboratories requires the availability of commercial, easy-to-use test kits. Because diagnosis of NTM infections remains a challenge and often results in indecisive situations that prolong the administration of adequate therapy, the rapid identification of MAC-related species highly predictive for mycobacterial disease would be very useful.

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Juquitiba-like Hantavirus from 2 Nonrelated Rodent Species, Uruguay

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Serologic and genetic analyses indicate that a Juquitiba-like hantavirus circulates in Maldonado, Uruguay. This virus is carried by 2 rodent species, *Oligoryzomys nigripes* and *Oxymycterus nasutus*. The same hantavirus in 2 nonrelated species can be explained by a spillover infection or a host-switching event.

Most hantaviruses (family *Bunyaviridae*) are hosted by 1 or a few closely related rodent species, including rodents of the family Cricetidae, subfamily Sigmodontinae (*1*). Sigmodontine rodents are highly diverse and comprise ≈84 genera (*2*); several hantavirus lineages associated with species belonging to 3 of its tribes—Akodontini, Oryzomyini, and Phyllotini—have been characterized (*3*–*5*).

In Uruguay, 2 closely related hantaviruses—Lechiguanas and Andes Central Plata—cause hantavirus pulmonary syndrome (HPS). Each of these viruses is carried by the yellow pigmy rice rat (*Oligoryzomys flavescens*) (*6*). We report an HPS case in Maldonado, Uruguay, and describe the serologic and genetic analysis carried out on rodents captured at the presumed site of infection.

The Study

A case of HPS was diagnosed at the Uruguayan Department of Maldonado in February 2005. The patient worked and lived in a greenhouse near Punta Ballena (34°55′S, 55°3′W).

To determine the source of the patient's infection, during March 12–15 and December 10–11, 2005, small mammals were trapped near where the patient had lived or worked during the 6 weeks before the onset of symptoms and in nearby habitats. Established biosafety guidelines were followed (*7*). Each specimen was identified in the field by using external characteristics. Taxonomic identification of seropositive rodents was corroborated by analyzing skull characteristics and by comparing mitochondrial DNA sequences with sequences available in GenBank. Voucher specimens were deposited at the Mammal Collection of the Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay.

In March 2005, during 1,100 trap nights, 133 rodents belonging to the subfamilies Sigmodontinae (family Cricetidae) and Murinae (family Muridae) were collected (trap success rate 12.1%). Nine months later, in December, 45 rodents belonging to the same subfamilies were collected (trap success rate 8.2%) (Table 1). Immunoglobulin G antibodies to Maciel hantavirus were detected by ELISA (*6*) in 5 rodents (collected in March): 4 long-nosed mice (genus *Oxymycterus*), 6% seroprevalence, and 1 black-footed pigmy rice rat (*Oligoryzomys nigripes)*, 3.2% seroprevalence. (Maciel antigen for the ELISAs was kindly provided by S. Levis, Instituto Nacional de Enfermedades Virales Humanas, Argentina.) A serum dilution was considered positive if optical density was >0.2 U after adjustment. A serum titer >400 was considered positive. Titration showed that all samples had titers >6,400.

Molecular methods corroborated the field identification of the 1 black-footed pygmy rice rat and assigned the 4 long-nosed mice to 1 of the 2 species of *Oxymycterus* inhabiting Uruguay: *O. nasutus*. All 5 specimens (3 male, 2 female) were captured in areas of human disturbance: road borders, shrublands, and artificial pine woods (*Acacia* spp. or *Eucalyptus* spp.).

Total RNA was extracted from lung tissue of seropositive rodents and from serum of the HPS patient (*6*). Nested or seminested reverse transcription–PCRs (RT-PCRs) were performed for partial small (S) and medium (M) segments (*8*,*9*). PCR products were sequenced with the same primers used in the RT-PCRs. Viral RNA was detected in 4 of 5 rodents that were seropositive for M or S segments; material was not available for viral RNA testing for 1 *O. nasutus* mouse. RT-PCR amplification attempts on the patient's serum failed for both segments.

M segment amplicons (G2 glycoprotein–encoding region) were 735 bp; S segment amplicons were 416 bp. No sequence was obtained from sample PB1011. Similarly, the sequence of the M segment from sample PB981 was not long enough to be included in the phylogenetic analyses. For sequence comparison and phylogenetic analyses, sequences of representative New and Old World hantaviruses were obtained from GenBank. Phylogenetic analyses were conducted by using Bayesian inference and maximum parsimony (Figures 1, 2).

Bayesian analysis based on partial M segment sequences (Figure 1) showed that the sequences retrieved from 1 black-footed pygmy rice rat (PB1033) and 1 long-nosed mouse (PB1002) form a strongly supported clade (posterior probability [PP] 0.97). The Uruguay clade is sister to a clade (PP

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Table 1. Rodent species captured during 2 trapping expeditions, Maldonado, Uruguay, 2005

Expedition date	Rodent species captured, no. (%)						
	Oxymycterus spp.	*Oligoryzomys nigripes*	*Scapteromys tumidus*	*Oligoryzomys flavescens*	*Akodon azarae*	*Necromys obscurus*	*Rattus rattus*
Mar	66 (49.6)	31 (23.3)	14 (10.5)	14 (10.5)	6 (4.5)	1 (0.8)	1 (0.8)
Dec	23 (51.1)	2 (4.4)	5 (11.1)	6 (13.3)	7 (15.6)	0 (0)	2 (4.4)

1.00) formed by Juquitiba (JUQ) hantaviruses retrieved from black-footed pigmy rice rats (prefix On and AY963900) in Brazil and human HPS case-patients (Hu) in Brazil. We refer to this strongly supported (PP 1.00) clade formed by Brazil and Uruguay sequences as the JUQ-like clade. This clade is sister to Maporal hantavirus. Maximum-parsimony analysis (available from A. Delfraro upon request) also recovers the JUQ-like clade (bootstrap support 100%).

Phylogenetic analysis of S-segment sequences showed a broader taxonomic coverage than that of the M

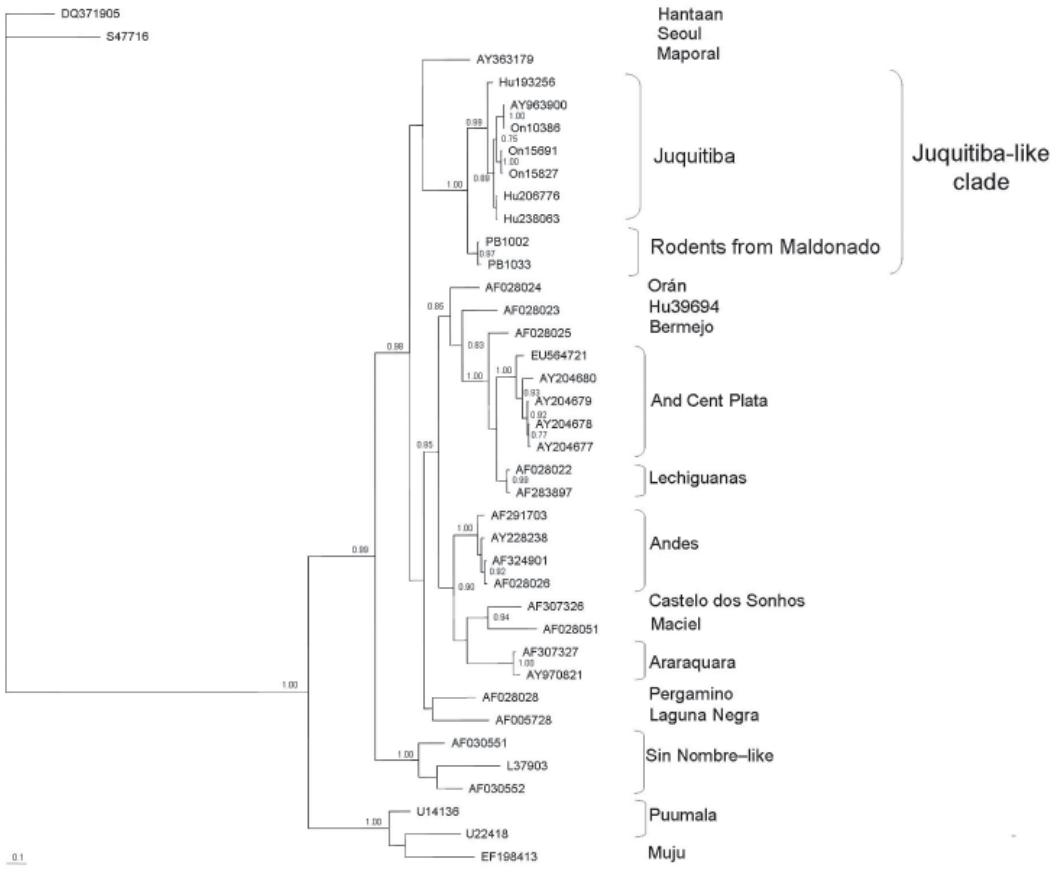

Figure 1. Majority-rule consensus tree obtained in the Bayesian analysis of sequences of the medium segment of Juquitiba-like hantavirus isolated from 2 nonrelated rodent species. Posterior probabilities >0.80 are shown at the nodes. Alignment and editing of nucleotide sequences were conducted by using BioEdit v7.0.9.0 (www.mbio.ncsu.edu/BioEdit/BioEdit.html). Sequences of Seoul and Hantaan hantaviruses were used as outgroup. Estimation of the suitable model of nucleotide substitution and phylogenetic analyses were carried out by using Modelgenerator (http://bioinf.may.ie/software/modelgenerator), MrBayes v3.1.2 (Bayesian analysis; http://mrbayes.csit.fsu.edu), and PAUP* 4.0b10 (maximum-parsimony analysis; http://paup.csit.fsu.edu). Bayesian analyses were conducted under the general time reversible + gamma + proportion invariant model. Two runs of 4 chains each (1 cold, 3 heated, temperature 0.20) were run for 3 million generations; trees were sampled every 100 generations. Convergence was assessed by using the average standard deviation in partition frequency values across independent analyses with a threshold value of 0.01; burn-in was set to 25%. Seropositive specimens from Uruguay are as follows: PB1033 (black-footed pigmy rice rat, *Oligoryzomys nigripes*) and PB1002 (long-nosed mouse, *Oxymycterus nasutus*), GenBank accession nos. EU564726 and EU564725, respectively. Analyzed hantavirus sequences are Hantaan, (DQ371905), Seoul (SA7716), Juquitiba (AY963900, On10386, On15691, On15827, Hu206776, Hu238063, Hu193256), Maporal (AY363179), Andes Central Plata (AY204678, AY204677, AY204679, AY204680, EU564721), Lechiguanas (AF028022, AF283897), Bermejo (AF028025), Hu39694 (AF028023), Orán (AF028024), Andes (AF291703, AF324901, AF028026, AY228238), Castelo dos Sonhos (AF307326), Maciel (AF028051), Araraquara (AF307327, AY970821), Pergamino (AF028028), Laguna Negra (AF005728), Sin Nombre–like (L37903, AF030552, AF030551), Puumala (U14136, U22418), and Muju (EF198413). Scale bar indicates expected changes per site.

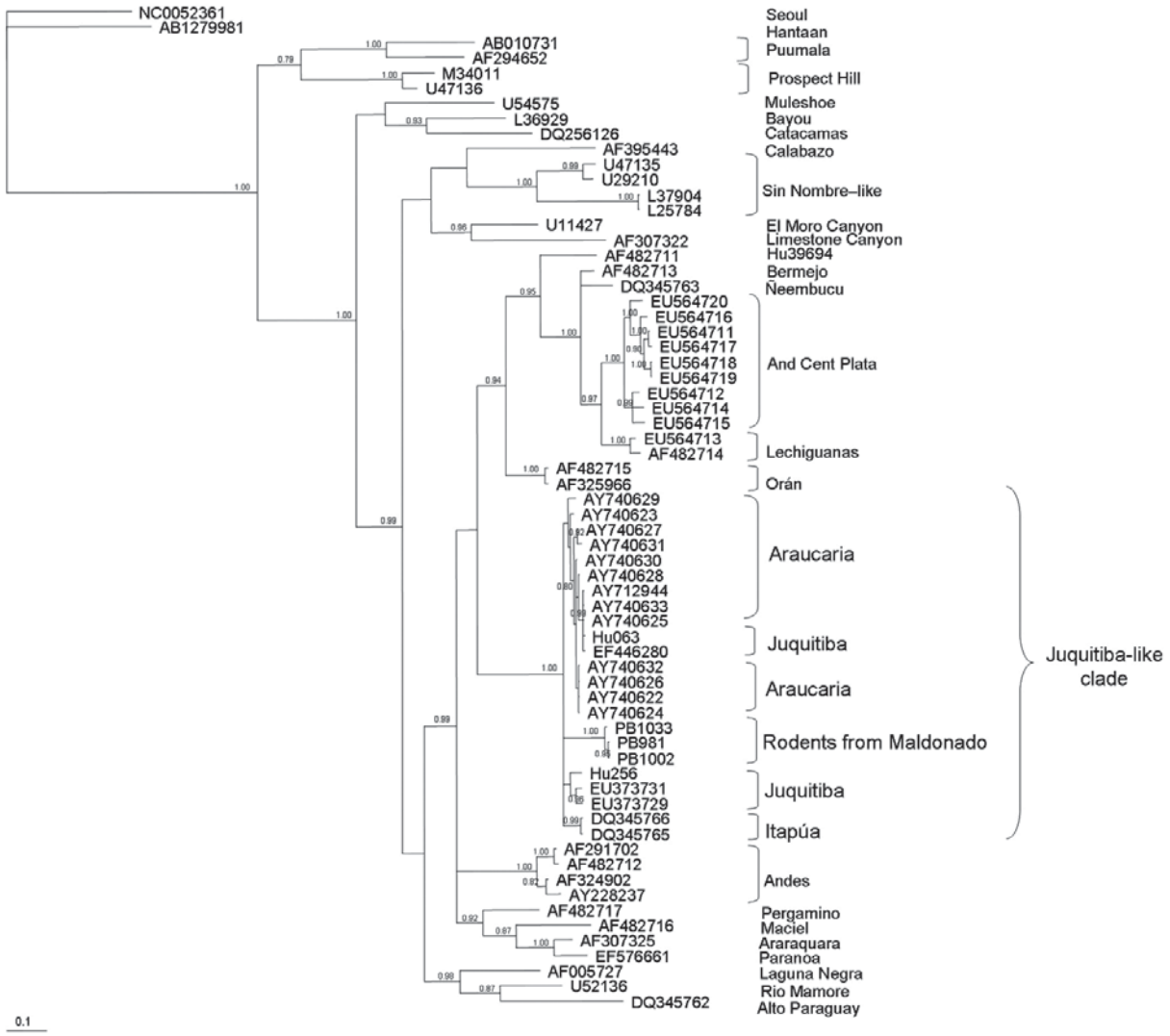

Figure 2. Majority-rule consensus tree obtained in the Bayesian analysis of sequences of the small segment of Juquitiba-like hantavirus isolated from 2 nonrelated rodent species. Posterior probabilities >0.80 are shown at the nodes. Analyses were performed as described in Figure 1. Seropositive specimens from Uruguay are as follows: PB1033 (black-footed pigmy rice rat, *Oligoryzomys nigripes*), PB981 and PB1002 (long-nosed mouse, *Oxymycterus nasutus*), GenBank accession nos. EU564724, EU564722, EU564723, respectively. Analyzed hantavirus sequences are Seoul (NC0052361), Hantaan (AB1279981), Laguna Negra (AF005727), Rio Mamore (U52136), Alto Paraguay (DQ345762), Andes Central Plata (EU564711, EU564717, EU564718, EU564719, EU564716, EU564720, EU564715), Lechiguanas (EU564713, AF482714), Bermejo (AF482713), Ñeembucu (DQ345763), Hu39694 (AF482711), Orán (AF482715, AF325966), Araucaria (AY740627, AY712944, AY740631, AY740633, AY740625, AY740628, AY740632, AY7406261, AY7406221, AY7406241, AY740630, AY740629, AY740623), Juquitiba (Hu063, EF446280, Hu256, EU373729, EU373731), Itapúa (DQ345766, DQ345765), Andes (AF291702, AF482712, AF324902, AY228237), Maciel (AF482716), Araraquara (AF307325), Paranoa (EF576661), Pergamino (AF482717), Sin Nombre–like (U47135, U29210, L37904, L25784), Calabazo (AF395443); El Moro Canyon (U11427), Limestone Canyon (AF307322), Bayou (L36929), Catacamas (DQ256126), Muleshoe (U54575), Puumala (AB010731, AF294652), and Prospect Hill (M34011, U47136). Scale bar indicates expected changes per site.

segment (Figure 2). The clade containing the viruses from Uruguay is part of a larger and strongly supported (PP 1.00) clade also formed by JUQ hantaviruses from Argentina (EU373731 from an HPS case-patient; EU373729 from a black-footed pygmy rice rat) and Brazil (Hu256 and Hu063 from HPS case-patients; EF446280 from a black-footed pygmy rice rat), as well as by Araucaria viruses (from HPS case-patients from Brazil) and Itapúa viruses from southern Paraguay, a viral lineage also detected in black-footed pygmy rice rats (DQ345765-66). (Sequences from Brazil JUQ hantaviruses, isolated from *O. nigripes* and HPS case-patients, were kindly provided

by S. Levis, Instituto Nacional de Enfermedades Virales Humanas, Argentina.)

Remarkably, JUQ and Araucaria viruses do not form monophyletic groups. The JUQ-like clade is sister to a clade formed by Hu39694, Ñeembucu, Bermejo, Andes Central Plata, Lechiguanas, and Oran; however, this relationship is weakly supported. Maximum-parsimony analysis (not shown) also recovers a strongly supported JUQ-like clade (bootstrap support 100%).

Sequence comparison for M and S segments showed that hantaviruses that cluster in the JUQ-like clade, including the Uruguay samples, form a homogeneous group that shows a high identity percentage at the nucleotide and amino acid levels. Under the general time reversible + gamma + proportion invariant model, the most similar lineages to the JUQ-like viruses are Hu39694 for the M segment and Oran for the S segment (Table 2).

Conclusions

Genetic and phylogenetic analyses showed that in Maldonado, *O. nigripes* and *O. nasutus* carried the same type of hantavirus. Moreover, the viruses from Uruguay form a well-supported clade with JUQ, Araucaria, and Itapúa viruses from HPS case-patients and black-footed pigmy rice rats (*O. nigripes*) from Argentina, Brazil, and Paraguay. In view of the high sequence similarity and the well-supported phylogenetic relationship, we propose that all of these viruses should be considered as JUQ-like hantaviruses. Additional studies will clarify whether all the viruses in the JUQ-like clade represent 1 viral type.

Table 2. Sequence comparison for medium and small segments of JUQ-like clade*

Identity or distance	Segment size	
	Medium	Small
Percentage identity†		
Nucleotide	92.6	95.4
Amino acid	98.9	99.7
Mean distance‡		
HU39694	0.451	0.475
Maporal	0.567	NA
Oran	0.631	0.326
Maciel	0.662	0.704
Pergamino	0.707	0.364
Castelo dos Sonhos	0.782	NA
Andes	0.820	0.434
Lechiguan	0.840	0.449
Bermejo	0.886	0.404
Araraquara	NA	0.437
JUQ-like mean distance§	0.093	0.039

*Parameters used in the analyses were medium segment, gamma shape 0.29, proportion of invariable sites 0.18; small segment, gamma shape 0.59, proportion of invariable sites 0.38. Sequence comparisons were conducted by using MEGA version 4.0 (www.megasoftware.net) and Modelgenerator (http://bioinf.may.ie/software/modelgenerator). JUQ, Juquitiba; NA, not available.
†Identity for the JUQ-like clade.
‡Distance between the JUQ-like clade and several South American hantavirus lineages, under the general time reversible + gamma + proportion invariant model.
§Intragenotypic.

No *Oxymycterus* spp. has been previously reported as being a hantavirus reservoir host. Antibodies to hantavirus in Uruguay *Oxymycterus* spp. may be interpreted as a secondary infection (spillover) (*10*). Interestingly, in the trapping area, the long-nosed mice are the most abundant rodent species, and the seroprevalence of hantavirus among them (6%) is higher than that previously reported in Uruguay (*6*). Black-footed pygmy rice rats are the second most abundant rodent captured; however, the seroprevalence of hantavirus among them (3.2%) is lower.

The presence of the same or similar hantavirus in 2 different rodent species may represent an event of host switching (*5*,*11*–*14*). Hantaviruses detected in Maldonado are similar, although not identical, and are carried by 2 distantly related rodent species that belong to different tribes, Akodontini (*Oxymycterus*) and Oryzomyini (*Oligoryzomys*). Further investigation of the *O. nasutus*–derived hantaviruses is needed to elucidate whether long-nosed mice are true reservoirs or only incidental hosts. Clarification may come from complete sequencing of M and S segments, viral isolation attempts, and new capture expeditions to look for more seropositive long-nosed mice in different areas of Uruguay.

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Rabies Postexposure Prophylaxis, Marseille, France, 1994–2005

Philippe Gautret, Georges Soula, Hamadou Adamou, Marie-José Soavi, Jean Delmont, Yolande Rotivel, Philippe Parola, and Philippe Brouqui

The administration of human rabies postexposure prophylaxis near Marseille (southern France) has changed since the eradication of terrestrial mammal rabies in 2001. Most injuries were associated with indigenous dogs; rabies vaccine was overprescribed. We suggest that the World Health Organization guidelines be adapted for countries free of terrestrial mammal rabies.

The last case of human rabies acquired in France was reported in 1924, and rabies was officially declared eliminated in terrestrial mammals in 2001 (*1*). However, confirmed rabid dogs from North Africa have been imported into France (*2*,*3*), and indigenous bats have been regularly found to be infected by rabies-related viruses (*4*). Marseille is the main international seaport in southern France; it handles heavy daily maritime traffic from North Africa, where numerous human cases are reported in relation with rabid dog bites. Management of patients exposed to these potentially rabid animals poses specific problems, and the decision to prescribe rabies vaccine and/or rabies immunoglobulin depends on the origin of the animal, as it does in the United Kingdom (*5*).

The Study

From 1994 through 2005, epidemiologic data on animal-related injuries and associated postexposure prophylaxis (PEP) treatment were prospectively collected for Marseille Rabies Treatment Centre patients. Only patients who had been injured in France were selected; rabies PEP for travelers who were injured abroad is detailed elsewhere (*6*). Of the 4,965 eligible patients, 4,367 were outpatients or inpatients (192–488/year), and from 2001 through 2005, a total of 598 were managed by teleconsultation only because their exposure risk was considered to be zero.

The number of inpatients and outpatients decreased markedly from 1999 to 2001 (Figure 1), which is consistent with the general decrease in the number of PEP treatments in France after the elimination of terrestrial mammal rabies (*7*). Furthermore, prescreening of persons by telephone also contributed to this decrease. The increase observed during 2004–2005 is likely an effect of the international alert in relation to the cases of rabid dogs imported from Morocco; these cases were intensively reported by the French media. The proportion of animal-related injuries tended to increase in late spring/early summer (Figure 2), probably as a result of increased outdoor activities in southern France, which makes contact with animals more likely.

The overall annual incidence of injured patients seeking care for rabies PEP was 16/100,000, which is consistent with incidence recently reported in United States (*8*,*9*) (where rabies is enzootic in bats and raccoons) but far less than that reported in recently available studies from the canine rabies–endemic countries of Turkey (467/100,000) (*10*) and India (1,700/100,000) (*11*). The overall mean annual incidence in our study was 20/100,000 before 2001 and 11/100,000 after 2001.

Dogs accounted for 81.2% of all injuries. By contrast, a recent study on pet demographics in France indicated that dog and cat populations are nearly similar at 8.51 million and 9.94 million, respectively (*12*). This finding suggests that dogs, more often than cats, are responsible for severe injuries that lead persons to seek care for rabies PEP. The mean annual incidence of animal-related injuries was lower in rural than in urban communities (online Technical Appendix, available from www.cdc.gov/EID/

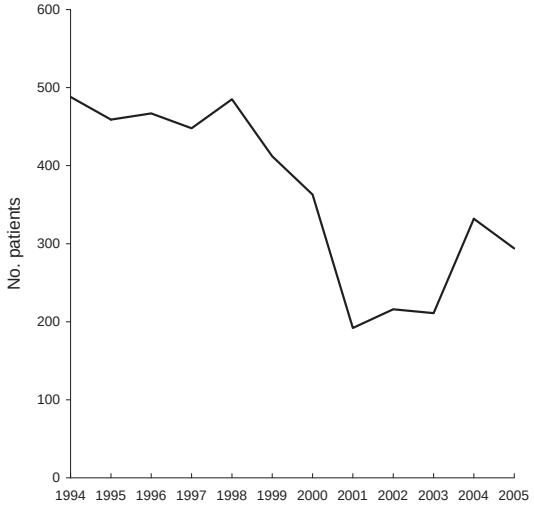

Figure 1. Number of injured patients per year seeking care for rabies postexposure prophylaxis, Marseille Centre, Marseille, France, 1994–2005.

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DOI: 10.3201/eid1409.071322

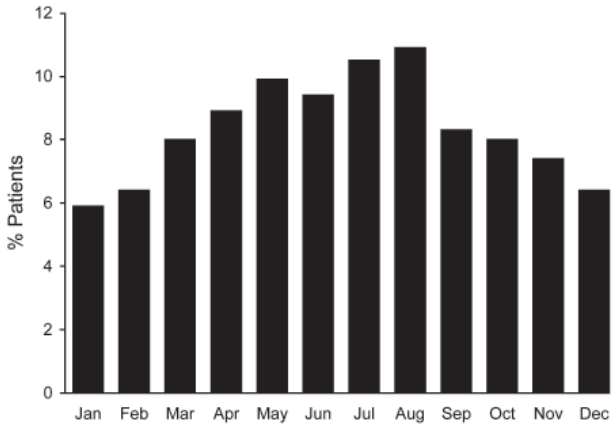

Figure 2. Average proportions of injured patients seeking care for rabies postexposure prophylaxis, by month, Marseille Centre, Marseille, France, 1994–2005.

content/14/9/1452-Techapp.pdf). Because an estimation of the dog population in France indicated that 41% live in urban areas (*12*), our results suggest that a high human population density increases the probability of human–dog interactions and risk for injuries.

Among patients seeking care for rabies PEP, most were male (male:female ratio 1.49) and mean age was 31.5 (median 29, range 0–96) years. Patients <15 years of age represented 26% of the cohort. The likelihood for animal-related injuries among male patients was also dependant on the animal species involved; dogs, bats, and monkeys accounted for most injuries (online Technical Appendix). In contrast, female patients were more likely to be injured by cats, a finding consistent with previous reports (*13*).

The mean time between injury and consultation was 2.6 days (range 0–365 days) and did not statistically vary by sex or age group. Time was longer in patients who were injured by bats ($p<10^{-6}$, online Technical Appendix), probably because most bat bites are nonpainful and considered benign by patients who ignore the risk for rabies after bat contact.

Most injured persons experienced severe contact with animals (95.1%), categorized by the World Health Organization (WHO) as category III (*14*). Most injuries were on the limbs (online Technical Appendix).

Animals were available for observation by a veterinarian in 1,441 cases (33%). Rabies testing of animal is not available in southern France, and animals from this region should be sent to the Rabies Laboratory at the Pasteur Institute in Paris, which was done for 89 cases, of which 20 cases were related to a confirmed rabid source from Africa or the Middle East (Table 1).

The proportion of patients who received treatment increased from 42% during 1994–2000 to 84.3% during 2001–2005 ($p<10^{-6}$) as a result of prescreening by telephone (Table 2). Since 2001, when the animal was not available for surveillance by a veterinarian (which includes numerous cases in which the animal was available for observation by its owner), complete treatment was given to most (89%) patients. Rabies immunoglobulin was provided to 3.2% of these patients, most of whom were injured by bats or severely injured by domestic animals when the owner was not identified or when surveillance of the responsible animal was not possible. No cases of rabies infection were identified in treated persons.

Conclusions

Our rabies PEP data are consistent with data from the national French Referral Center (*7*). The therapeutic approach in France is partly in accordance with WHO general recommendations that in rabies-free areas where adequate rabies surveillance is in effect, rabies PEP may not be required, depending on the outcome of a risk assessment conducted by a medical expert (*14*). Systematic rabies PEP is cost-effective and safe but should not be used if the biting animal is unlikely to be rabid. Furthermore, treating a patient with only vaccine when the animal is under observation could reduce the benefit of further administration of rabies immunoglobulin if the time between vaccination and rabies immunoglobulin injection is >7 days (*15*). If the treatment cannot be delayed, it should include both vaccination and rabies immunoglobulin in cases of category III injury. From 2001 through 2005, not vaccinating the patient when the animal was under observation by its owner or a veterinarian would have represented an overall savings of 177,600 Euros.

To minimize overprescription of vaccination for rabies PEP when treatment may be unjustified, we recommend delaying the initiation of rabies treatment in injuries involving an apparently healthy indigenous dog or cat that can be kept under veterinary or animal-owner observation for 2 weeks, which is the maximum rabies incubation time in these animals. Doing so would result in no rabies treatment for almost all such patients. However, when animals are not

Table 1. Characteristics of postexposure prophylaxis for patients exposed to confirmed rabies source, Marseille, France, 1994–2005

Date of exposure	No. treatments	Confirmed source	Location of exposure, France
1994 Jul	1	Fox	Northeast
1995 Nov	14	Dog*	Southeast
1998 May/Jun	2	Dog†	Southeast
2004 Aug	3	Dog‡	Southwest

*Imported from Burkina Faso.
†Unknown origin; rabid strain close to Egyptian isolates.
‡Imported from Morocco (187 treatments were given in France; most in Bordeaux Centre).

Table 2. Treatment for injured patients seeking care for rabies postexposure prophylaxis, by animal rabies status, Marseille, France, 1994–2005*

	Animal status					
	1994–2000, no. (%)			2001–2005, no. (%)		
Patient receipt of PEP*	Unknown†	Rabid‡	Not rabid§	Unknown†	Rabid‡	Not rabid§
Total	1,916 (61.5)	21 (0.6)	1,185 (37.9)	911 (73.2)	5 (0.4)	329 (26.4)
Unknown	0	0	0	4 (0.5)	0	0
None	761 (39.7)	1 (4.8)	1,048 (88.4)	34 (3.7)	0	158 (48.0)
Treatment completed	1,000 (52.2)	20 (95.2)	19 (1.6)	811 (89.0)	5 (100)	45 (13.7)
Treatment stopped	42 (2.2)	0	117 (9.9)	3 (0.3)	0	126 (38.3)
Lost to follow-up	113 (5.9)	0	1 (0.1)	59 (6.5)	0	0
RIG	2 (0.2)	20 (95.2)	1 (0.1)	29 (3.2)	0	14 (4.3)

*PEP, postexposure prophylaxis; RIG, rabies immunoglobulin (% as proportion of treatments including rabies PEP). 1994–2000, n = 3,122; 2001–2005, n = 1,245.
†Animal not available for observation by a veterinarian (including cases where animal was available for observation by its owner).
‡Animal proven to be rabid by laboratory testing or considered rabid upon clinical criteria.
§Animal proven to be not rabid by laboratory testing or after 2 weeks of observation by a veterinarian.

available for observation, complete rabies PEP treatment should be initiated. Given the risk for importation of rabid animals from nearby rabies-endemic countries, immediate rabies PEP treatment according to WHO guidelines should be given when the following are involved: indigenous bats; animals illegally imported from rabies-endemic countries; or animals found in railway stations, trains, or other ports of entry. If the animal is suspected of being rabid at the time of exposure, confirmatory testing should be conducted (online Technical Appendix). All travelers visiting countries where rabies is enzootic should be informed about the risks of bringing animals back to their home country and about the WHO recommendations regarding rabies vaccination of imported animals (*14*).

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Host Range and Genetic Diversity of Arenaviruses in Rodents, United Kingdom

Kim R. Blasdell, Stuart D. Becker, Jane Hurst, Mike Begon, and Malcolm Bennett

During a study to extend our knowledge of the host range and genetic diversity of arenaviruses in Great Britain, 66 of 1,147 rodent blood samples tested for antibody, and 127 of 482 tested by PCR, were found positive. All sequences most closely resembled those of previously identified lymphocytic choriomeningitis virus.

Viruses in the family *Arenaviridae* are separated into 2 distinct serocomplexes, the New World serocomplex and the Old World serocomplex (*1*). Several arenavirus species are known to cause human disease, including lymphocytic choriomeningitis virus (LCMV), which causes influenza-like clinical signs, occasionally with neurologic complications. Infection may be asymptomatic in up to one third of patients (*2*), and serious complications often occur in intrauterine infection (*3*). Less severe cases of adult human infection are likely underreported and often misdiagnosed (*4*).

LCMV is found worldwide, probably because of its association with its natural Old World host, the house mouse, *Mus musculus* (*5*). Although antibodies have also been detected in other rodent species (*6*,*7*), arenaviruses are known to be serologically cross-reactive. Few isolates of LCMV have been obtained from wild rodents so little is known about its genetic diversity. Recent studies on American arenaviruses found that diverse arenaviruses co-evolved with their rodent hosts (*8*), a finding that suggests that a more thorough study of European rodents might also identify novel arenaviruses. The purpose of this study was therefore to extend our knowledge of LCMV and LCMV-like arenaviruses in rodents in Great Britain.

The Study

In total 1,147 blood samples were collected from rodents: 1,060 were live-trapped, wild animals from <20 sites (Table 1), and 87 blood samples were collected from a captive colony of wild house mice (*9*) and tested serologically. All animal research was conducted under license, according to UK regulations.

Serum samples were separated by centrifugation (10,000 rpm, 10 min) and tested for LCMV antibody by using the manufacturer's protocol for commercial indirect fluorescent antibody assay slides (Charles River Laboratories, Wilmington, MA, USA). A 1:40 dilution of anti-rat or anti-mouse immunoglobulin G fluorescein isothiocyanate (Sigma-Aldrich, Gillingham, UK) or a combination of both were used as secondary antibody. Ninety-three serum samples (from the original serum samples tested for antibody) that were either antibody positive or from sites with high seroprevalence were tested for arenavirus RNA by PCR. Another 379 blood samples from the captive colony of house mice, which had not been previously tested for antibody, were also tested. The PCR targeted a fragment of the glycoprotein precursor gene (GPC) (*10*). A selection of samples found negative by the GPC PCR were subsequently retested by PCR targeted at a fragment of the nucleoprotein (N) gene (*8*), by using primers to sequences common to the Old World arenaviruses. Total RNA was extracted by using QIAamp viral RNA mini-kit (QIAGEN, Crawley, UK), converted to cDNA, and amplified by using a single-step kit (Superscript III one-step RT-PCR with Platinum Taq polymerase system; Invitrogen, Paisley, UK) in conjunction with oligonucleotides arena1^{+} and LCMV322^{-} (*10*) or 1010C and either OW1696R or NW1696R (*8*). Products were separated and visualized by agarose gel electrophoresis, and amplicons were purified with the QIAquick PCR purification kit (QIAGEN). Bidirectional sequencing was performed off-site (MWG Biotech AG, Ebersberg, Germany). The 97-nt sequences generated here were deposited with GenBank (accession nos. DQ275199–DQ275295).

The software package MEGA version 4.0 (*11*) was used to construct an alignment of a 283-nt fragment of the GPC gene nucleotide sequences and predicted amino acid sequences, and for phylogenetic analysis with the neighbor-joining method (p distance model), with bootstrap support based on 1,000 pseudoreplicates. Other GenBank sequences included for comparison are listed in Table 2. Pairwise genetic distances were calculated by using the p distance model; percentage sequence identities were calculated by subtracting the genetic distances from 1.0 and multiplying by 100.

Overall, 66 of 1,147 serum samples and 7 of 9 rodent species had antibodies to arenaviruses. *Sciurus vulgaris* had the highest prevalence, 26%, although only 15 squirrels were tested. *M. musculus* had the second highest prevalence, 17.5%. Antibodies were also detected in *Apodemus sylvaticus*, *Microtus agrestis*, *Micromys minutis*, captive-housed *Cynomys ludovicianus*, and *Rattus norvegicus*. Seroprevalence varied between species (1.4%–26%) and between sites (0%–50%) (Table 1).

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Table 1. Rodent species, numbers tested, seroprevalence, and viral RNA prevalence to LCMV at each UK and Republic of Ireland site*

Site code and year	Geographic location	Species tested for antibody	Species tested for viral RNA
PHF 2004	Cheshire	MMu (0/4), RN (0/2); SP = 0.0	
PHF 2005	Cheshire	***MMu (8/26)***; SP = 30.8	
BHF 2004	Cheshire	MMu (4/10); SP = 40.0	***MMu (2/9),*** RN (0/2) – 2 sequences
BHF 2005	Cheshire	***MMu (2/2)***, RN (0/2); SP = 50.0	
BGF	Cheshire	MMu (0/7), RN (0/2); SP = 0.0	
CLF 2004	Cheshire	MMu (0/6), RN (0/2); SP = 0.0	
CLF 2005	Cheshire	MMu (0/12), RN (0/4); SP = 0.0	
MF 2002	Cheshire	AS (0/10), MG (0/9), MA (0/2), MMu (0/30); SP = 0.0	***AS (1/10)***, MG (0/1), MA (0/1),
MF 2004	Cheshire	***AS (4/10)***, MG (0/1), MA (0/1), ***MMu (2/4)***; SP = 37.5	***MMu (2/4)*** – 3 sequences
CZ 2002	Cheshire	AS (0/4), MG (0/4), ***CL (4/61)***, MMi (0/22), RN (0/2); SP = 4.3	***AS (1/1)***, CL (0/4), MMi (0/1), MMu (0/1) – 0 sequences
CZ spring 2003	Cheshire	AS (0/9), MG (0/3), MA (0/4), ***MMi (1/3)***, SP = 5.3	
CZ autumn 2003	Cheshire	*AS (1/18)*, MG (0/19), MA (0/4), MMu (0/1), SP = 2.9	
CZ 2004	Cheshire	***MMu (1/19)***, RN (1/12), SP= 3.2	
DF	Cheshire	***MMu (1/69)***, SP = 1.4	
MW	Cheshire	MG (0/105), AS (0/45), SP = 0.0	
RH	Cheshire	MG (0/19), AS (0/49), SP = 0.0	
LVFS	Cheshire	RN (0/2, SC (0/4), SP = 0.0	
FA	Merseyside	MA (0/2), AS (0/24), SP = 0.0	
KF	Northumberland	***MA (2/104)***, SP = 1.9	
LI	North Devon	RN (0/40), SP = 0.0	
IOW	Isle of Wight	***SV (1/18)***, SP = 5.6	
TF	Thetford	***SV (1/21)***, SP = 4.8	
CF	Cumbria	SC (0/10), SV, (0/4), SP = 0.0	
NI	Northern Ireland	***AS (1/149)***, SP = 0.7	
CA	Republic of Ireland	MG (0/15), SP = 0.0	
CC	Republic of Ireland	AS (0/7), SP = 0.0	
TW	Republic of Ireland	AS (0/10), SP = 0.0	
Other	Various locations	***RN (1/6)***, SC (0/1), ***SV (2/26)***, SP = NA	AS (0/1), MA (0/2), MMu (0/31), RN (0/5), ***SV (1/4)*** – 0 sequences
Captive colony	Captive colony, Cheshire	***MMu (30/87)***, SP = 34.5	***MMu* (122/403)** – 92 sequences

*LCMV, lymphocytic choriomeningitis virus; SP, site prevalence (%). Species key: MMu, *Mus musculus* (house mouse); RN, *Rattus norvegicus* (brown or Norway rat); AS, *Apodemus sylvaticus* (wood mouse); MG, *Myodes glareolus* (bank vole); MA, *Microtus agrestis* (field vole); MMi, *Micromys minutis* (harvest mouse); CL, *Cynomys ludovicianus* (black-tailed prairie dog); SV, *Sciurus vulgaris* (red squirrel); SC, *Sciurus carolensis* (gray squirrel); NA, not available. Species containing positive animals are in ***boldface italics,*** with number positive and number tested in parentheses.

GPC PCR amplicons were obtained from 127 of 472 tested samples, and sequences were determined for 97 samples (Table 1). All positive samples were from *Mus musculus* except 1 from *A. sylvaticus*. Twenty samples negative in the GPC PCR, but seropositive or from high prevalence sites, were tested by N gene PCR, and 2 were weakly positive: 1 *S. vulgaris* and 1 *A. sylvaticus*. In neither case, however, could a sequence be obtained from the amplicon.

Table 2. Percentage nucleotide identities between the study sample sequences (all and from the captive colony only) and previously isolated LCMV and Lassa virus sequences*

Sequence	All study sequences, %	Captive colony sequences only, %
All study sequences	93.6–100	NS
Captive colony sequences only	NS	97.4–100
LCMV CIPV76001 Pasteur (AF095783; France)	78.7–80.5	78.7–80.5
LCMV CIP97001 (AF079517; France)	79.4– 83.1	80.9–83.1
LCMV Marseille (DQ286931; France)	82.8– 83.9	82.8–83.5
LCMV CH5871 (AF325215; Germany)	81.6–83.1	81.6–83.1
LCMV CH5692 (AF325214; Germany)	81.3–82.8	81.3–82.8
LCMV MX (EU195888; Slovakia)	78.7–80.5	78.7–80.5
LCMV Armstrong (M20869; USA)	82.8– 85.8	83.9–85.8
LCMV WE (M22138)	82.0–84.3	82.0–84.3
Lassa LP (AF181853)	58.1–59.9	58.8–59.9

*LCMV, lymphocytic choriomeningitis virus; NS, not shown.

Nucleotide and amino acid GPC sequence identities for all the samples in this study ranged from 93.6%–100% (Table 2), and 94.5%–100%, respectively (data not shown). When compared with other arenaviruses, the nucleotide sequences exhibited 78.7%–85.8% identity with LCMV reference sequences and only 58.1%–59.9% identity with Lassa virus (online Appendix Figure, available from www.cdc.gov/EID/content/14/9/1455-appF.htm).

Although antibodies to arenaviruses have been reported in a range of European rodent species, our study provided evidence of arenaviruses infecting red squirrels (*S. vulgaris*) and European harvest mice (*M. minutis*). Antibodies to arenaviruses have been reported in introduced *S. carolensis* in Great Britain (*12*) but were not detected in this study. We also reported antibodies to arenaviruses in black-tailed prairie dogs (*Cynomys ludovicianus*): those tested in this study were part of a colony in a zoo, however, and had contact with wild mice, some of which were seropositive. As found in previous studies, *Mus musculus* was more likely to be infected with LCMV than other rodent species.

The nucleotide sequences of most PCR amplicons clearly identified LCMV as the most frequent cause of the antibody detected. However, the detection of arenaviral RNA in 2 animals by the N gene PCR, but not by the LCMV-specific GPC PCR, may suggest the presence of another species of arenavirus. Further studies are needed to determine if other arenaviruses species are present in European rodent populations (*8*).

Genetic heterogeneity was present within and between sites (Figure), as seen in previous studies of arenaviruses (*13*,*14*). Sequences from animals in the captive colony and a nearby farm (MF) clustered and were different from those from a more distant farm (BHF). Furthermore, all of the British sequences clearly clustered separately from the reference strain sequences (from the United States, France, Germany, or Slovakia). These findings suggest spatial heterogeneity in sequence may be reflected in host range and pathogenicity. Sequencing might be useful in tracing sources of future human outbreaks.

Conclusions

This study has increased the list of European (and North American) rodents that may be infected with LCMV and that might therefore pose a risk to humans. The genetic variation observed and potential variations in pathogenicity may indicate that some wildlife populations pose more of a public health risk than others. Further studies are needed to assess which mutations cause increased pathogenicity and to establish whether or not LCMV represents the only arenavirus present in European rodent populations.

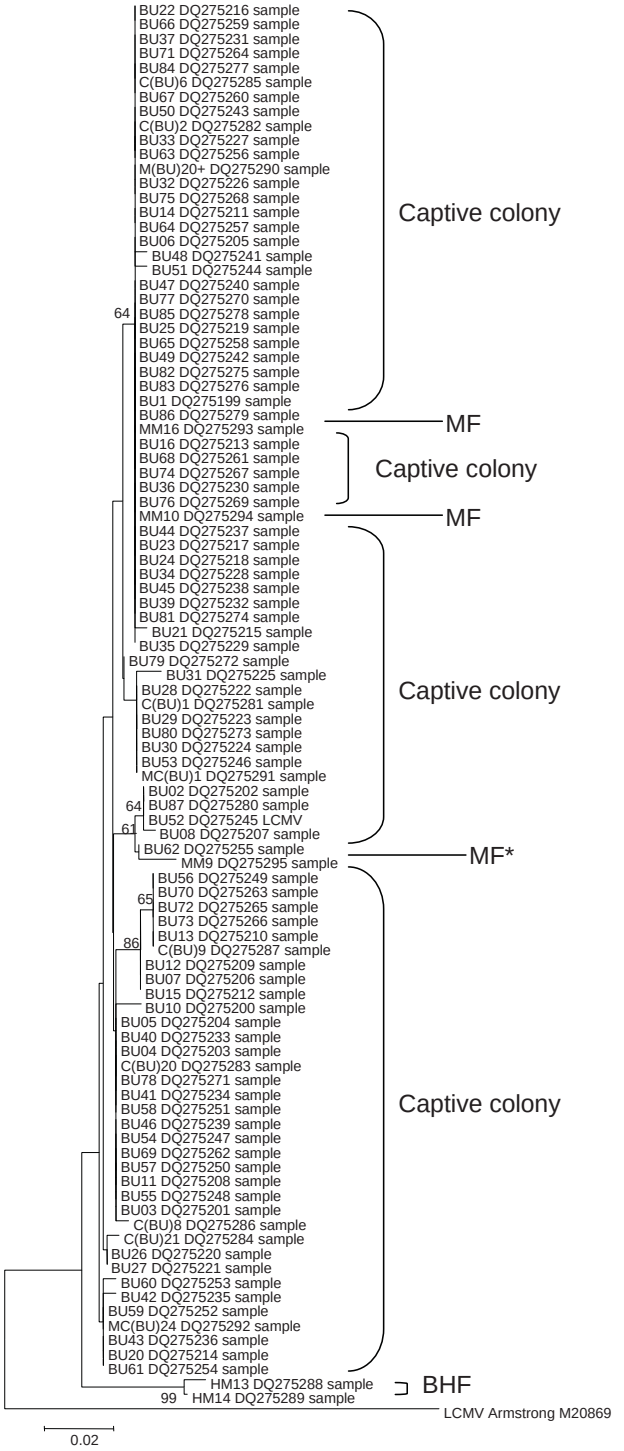

Figure. Unrooted neighbor-joining tree using the p-distance model (1,000 replicates) for a section of the glycoprotein precursor gene gene, showing bootstrap values of >60 for all sequences identified in this study (283 bp) and indicating site of origin. Captive colony, MF 2004, and BHF 2005 as in Table 1. MF* is from *Apodemus sylvaticus*, and all other sequences are from *Mus musculus*. Scale bar indicates number of substitutions per site.

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Knowledge, Attitudes, and Practices regarding Avian Influenza (H5N1), Afghanistan

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From February through April 2007, avian influenza (H5N1) was confirmed in poultry in 4 of 34 Afghan provinces. A survey conducted in 2 affected and 3 unaffected provinces found that greater knowledge about reducing exposure was associated with higher socioeconomic status, residence in affected provinces, and not owning backyard poultry.

Avian influenza (H5N1) has been reported in southern Asia (*1*). In Afghanistan, avian cases were confirmed from February through April 2007 in 4 of 34 provinces (*1*). No human cases have been detected, although limited human-to-human transmission has been reported from Pakistan (*2*). Backyard poultry (chickens) were affected in 20 of 22 outbreak sites in 4 eastern provinces. No outbreaks have been reported from commercial facilities. The response in Afghanistan was to cull all poultry within a 3-km radius, restrict poultry movement and importation, and conduct intensive influenza-like illness surveillance and information, education, and communication (IEC) campaigns within affected provinces. IEC campaigns included leaflets distributed in affected areas and broadcast media coverage on local television and radio. The campaign was designed to inform the public through messages aimed at reducing exposure to disease, preventing spread in poultry, and encouraging reporting. Additional IEC messages were aired nationally and outbreaks were widely reported by local news media. We conducted a survey of knowledge, attitudes, and practices (KAPs) regarding avian influenza in Afghanistan. The aim was to assess factors associated with KAPs.

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The Study

Five provinces in Afghanistan were selected as a convenience sample (accessibility) that included both affected and unaffected areas. Two accessible districts in each province were randomly selected by using a random number generator. Random transects were used to select 10 households per village. To give an approximately equal male:female ratio, either the head of household, spouse (woman), or the oldest person available at the time was selected. Participants provided informed consent. Ethical approval was provided by the Institutional Review Board, Ministry of Public Health, Afghanistan.

A standardized, structured questionnaire collected information on demographic and socioeconomic measures, avian influenza information sources and knowledge of appropriate preventive measures, poultry and animal handling, food and generic hygiene, and human influenza knowledge and treatment seeking. Questions related to KAPs were scored by a panel of experts in related disciplines. The questions were ranked for importance in preventing avian influenza transmission in poultry or reducing human exposure and awarded 5 points, 3 points, or 1 point for correct answers. For each respondent, the sum of scores for correct answers divided by the sum of available points generated a percentage score. Blank responses to questions were counted as such and not included in individual denominators. The questionnaire was back-translated and pilot-tested. The survey was conducted in May 2007, by trained Afghan surveyors. Data were double-entered by using Microsoft Access (Microsoft, Redmond, WA, USA) and analyzed by using Stata 8 software (Stata Corporation, College Station, TX, USA).

KAP scores provided a weighted measure of KAPs related to prevention of avian influenza. Percentage scores for each respondent were ranked and classified as above or below the median. The primary analysis was conducted to compare factors (age, sex, socioeconomic status, provincial exposure to avian influenza IEC campaigns, and poultry ownership) associated with knowledge above the median. Socioeconomic quintiles (SEQs) were defined by principle components analysis using employment, education, and household assets as indicators (*3*). Factors independently associated by univariate regression at the 95% confidence level were included in a stepwise multivariate logistic regression model. To numerically evaluate KAP levels, a secondary analysis assessed differences between mean percentage scores, stratified by factors identified by logistic regression analysis.

Data for 304 respondents were included in the analysis. Of the 5 provinces, Kabul and Nangahar had had influenza outbreaks in poultry in 2007. Enrollment characteristics are shown in the Table. Median age of respondents (38 years) was high, but it reflected the age of heads of households

Table. Enrollment data for avian influenza knowledge, attitudes, and practices survey, Afghanistan, May 2007

Characteristic	Value
No. respondents	304
% Male	46.8
Median age, y (interquartile range)	38 (27–50)
Age range, y, no. (%)*	
15–20	30 (10.0)
21–30	85 (28.2)
31–40	64 (21.3)
>40	122 (40.5)
No. (%) in each province	
Herat†	32 (10.5)
Kabul‡	64 (21.0)
Kandahar	79 (26.0)
Nangahar‡	64 (21.0)
Samangan	65 (21.0)
No. (%) with no formal education	
Male	36 (26.1)
Female	117 (75.0)

*Age data missing for 3 respondents.
†Only 1 district reported results because of security concerns.
‡Provinces exposed to avian influenza and intensive information, education, and communication campaigns (Kabul, March 2007, and Nangahar, February 2007).

and spouses. Poultry ownership was reported by 65.2% of households (>95% backyard ownership) and differed significantly between SEQs (poorest 53/62 [85.5%] vs. least poor 20/55 [36.4%]; χ^2 30.0, p<0.001).

SEQ was positively associated with KAP score above the median (lowest vs. highest: adjusted odds ratio [AOR] 14.3, 95% confidence interval [CI] 5.2–39.9), as was provincial exposure to avian influenza IEC campaigns (AOR 9.5, 95% CI 4.9–18.6). Backyard poultry ownership (nonowners vs. owners: AOR 0.3, 95% CI 0.2–0.7) and older age group (15–20 years vs. >40 years: AOR 0.3, 95% CI 0.1–0.8) were both negatively associated.

For secondary analysis, overall mean KAP score was 44.4%. Mean KAP score differed between SEQ (p<0.001, by analysis of variance) and was higher in provinces previously exposed to IEC campaigns (50.2% vs. 40.1%; p<0.001, by *t* test).

Specific, self-reported practices also differed by SEQ. Reporting of sick or dead poultry to authorities was less frequent among lowest SEQ (8/47 [13%]) than highest SEQ (20/49 [37%]; χ^2 6.6, p = 0.02) where selling poultry in the event of a local outbreak was more commonly reported (21/66 [66%] vs. 10/51 [18%]; χ^2 27.2, p<0.001). Presence of coops was less frequent in lowest SEQ (9/49 [18.4%]) than in highest SEQ (21/46 [45.6%]; χ^2 8.2, p = 0.004).

Conclusions

Human cases of avian influenza (H5N1) have resulted from contact between humans and infected backyard poultry (*4*). Risk to humans is also related to frequency of disease occurrence in the avian population (*5*). Recently, human-to-human transmission has been reported in the neighboring Northwest Frontier Province of Pakistan (*2*). Knowledge of disease is therefore a key factor in reducing exposure and enhancing reporting.

Overall knowledge was low, although in provinces exposed to intensive IEC campaigns, KAP scores of the population were higher. This finding indicated that campaigns had some success in increasing awareness. The level of concern generated by the campaign, government response, media reports, and proximity to the outbreak are all likely to contribute to this association. Despite this encouraging evidence, level of knowledge was far higher among persons with higher socioeconomic status. This finding contrasts with frequency of poultry ownership. Exposure risk is therefore likely to be considerably higher among lower socioeconomic groups.

Our results can be broadly generalized to the population, although we did not have access to unsafe districts (most of the districts in southern and eastern Afghanistan). This limitation may introduce selection bias, which would underestimate the effect of socioeconomic status because those living in inaccessible areas likely have a lower status than persons in accessible areas. Preintervention and postintervention surveys would provide a more robust measure of effectiveness. In the immediacy of an outbreak, this was unfeasible and would have been unethical. Although there are limitations to the study design in concluding intervention effectiveness, the results provide evidence to support further intensive campaigns as a response to influenza outbreaks in poultry.

Several reports have examined KAPs and behavior related to avian influenza (H5N1) (*6*–*9*). Similar to the finding in the Lao People's Democratic Republic (*6*), our study suggests that conventional education and behavior change messages have a limited effect in populations with highest exposure. Efforts to ensure that IEC messages are suitable for lower socioeconomic groups should be adopted, specifically by improving the knowledge of community leaders, designing messages in a suitable format for the poor and illiterate, and ensuring that the most accessible channels are used. Messages should carefully balance the risk for human disease against potential nutritional and economic consequences of high population concern (e.g., food scares).

Successfully promoting behavior change is a lengthy process and requires frequent reinforcement. The acuteness of avian influenza (H5N1) outbreaks requires a concerted effort to enhance knowledge and change behavior among those most at risk in low-income countries.

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Emergence and Spread of *Chlamydia trachomatis* Variant, Sweden

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A variant of *Chlamydia trachomatis* that had escaped detection by commonly used systems was discovered in Sweden in 2006. In a nationwide study, we found that it is now prevalent across Sweden, irrespective of the detection system used. Genetic analysis by multilocus sequence typing identified a predominant variant, suggesting recent emergence.

In 2006 a new variant of *Chlamydia trachomatis* (nvCT) was discovered in Sweden (*1*). Because of a 377-bp deletion in the target sequence for amplification, the variant had escaped detection by 2 widely used nucleic acid amplification tests, Abbott m2000 (Abbott Laboratories, Abbott Park, IL, USA) and Cobas Amplicor/TaqMan48 (Roche Diagnostics, Basel, Switzerland) (*1*,*2*). The other test commonly used in Sweden, ProbeTec ET (Becton Dickinson [BD], Franklin Lakes, NJ, USA), detects the new variant because it uses a different DNA target sequence in the cryptic plasmid (*3*). The nvCT has now been reported from several of Sweden's 21 counties (Figure 1). The aim of this study was to provide a national overview of the characteristics and extent of the new chlamydia variant through examination of surveillance trends, microbiologic laboratory data, and genetic analysis of new variant strains.

The Study

We examined national surveillance data reported to the Swedish Institute for Infectious Disease Control (Smittskyddsinstitutet) about chlamydia cases detected and the number of chlamydia tests performed. Trend analysis from 2004 to 2006 included only data for the first 6 months of each year to avoid any influence of changes in detection systems; some counties introduced different detection systems in late 2006 in response to the emergence of nvCT. The statistical methods are described in the online Technical Appendix (available from http://www.cdc.gov/EID/content/14/9/1462-Techapp.pdf). The total number of chlamydia cases detected in Sweden in the first 6 months of 2006 was lower than that in 2005, and the proportion of tests that were positive also fell (Table 1). In 2004, the proportion of positive chlamydia tests was similar whether laboratories used Abbott/Roche or BD test systems. From 2004 to 2005, there was a relative reduction of 3.4% (95% confidence interval [CI] 5.8–1.0) in chlamydia positivity in laboratories using the Abbott or Roche methods (p = 0.006) but no change in the proportion of positive samples in laboratories using the BD test system (–0.4%, 95% CI –4.2 to +3.5). During the first 6 months of 2005 and 2006, the positivity rates of samples tested by Abbott or Roche systems fell further; samples tested that used the BD system remained stable. The estimated difference in proportions of chlamydia-positive samples in counties that used Abbott or Roche tests compared with counties that used the BD method was –9.5 % (95% CI –14.1 to –4.7, p = 0.0005), after baseline differences and county differences in testing were controlled for.

We conducted microbiologic analyses on consecutive samples that were collected from 12 counties in late 2006 and early 2007. Cases of nvCT were identified by testing specimens with additional methods using alternative se-

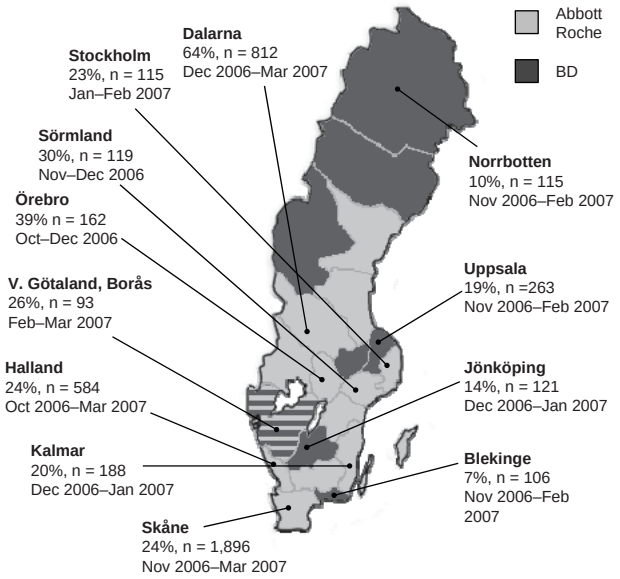

Figure 1. Map of Sweden showing proportions of the new variant of *Chlamydia trachomatis* in different counties. Light gray shading indicates counties that used Abbott or Roche test systems before the discovery of the new variant; dark gray shading indicates counties that used the Becton Dickinson (BD) system. The 1 county that used both Roche and BD assays is indicated with stripes. n, number of positive chlamydia cases analyzed. The period in which samples were collected is given for each county.

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Table 1. Numbers of chlamydia tests, positive results, and change in positivity rates, Sweden, 2004–2006

Diagnostic test*	No. counties	No. positive results/total no. tests (%) in first 6 mo		
		2004	2005	2006
Abbott/Roche	14	11,721/150,080 (7.8)	11,111/147,311 (7.5)	10,236/152,960 (6.7)
Becton Dickinson	8	4,262/54,260 (7.9)	5,220/66,728 (7.8)	3,363/43,189 (7.8)
All tests	21	15,983/204,340 (7.8)	16,331/214,039 (7.6)	13,599/196,149 (6.9)

*One county used both Roche and Becton Dickinson tests.

quence targets (online Technical Appendix). The proportion of nvCT ranged from 20% to 64% in the 8 counties that used Abbott or Roche detection systems, compared with 7% to 19%, respectively, for counties that used BD tests (Figure 1). Additional data about gender, age, and clinical setting of diagnoses were available for 600 chlamydia-positive patients in the 4 counties using the BD system (Table 2). The proportion of nvCT cases varied between clinics ($p = 0.020$) and was higher at youth and venereal disease clinics than at antenatal and gynecology clinics. This variance might be because persons seeking treatment in these settings have higher levels of risk taking and more frequent changes in partners (*4*). There was no evidence of differences in the proportion of cases by gender ($p = 0.103$) or age ($p = 0.558$) because of nvCT.

Genetic characterization with a new high-resolution genotyping system (*5*) was performed on 48 specimens of nvCT from 2 counties that used the BD test system (n = 21); 2 counties that used Roche and Abbott systems (n = 18); and 9 specimens from Norway, Ireland, and France (online Technical Appendix). The nvCT showed a new genetic variant in the chromosomal target region *hctB* compared with previous findings in wild-type strains and thus constitutes a separate clone with the designation 21 (*hctB*), 19 (CT058), 1 (CT144), 2 (CT172), and 1 (*pbpB*) in our system. All 48 specimens tested were of genotype E, and 46 were identical in the *ompA* gene to the reference strain E/Bour. The divergent specimens were from 2 persons known to be sexual partners and differed in a single nucleotide position. In the other 5 target genes, altogether comprising some 5,500 bp, all 48 specimens were identical.

Conclusions

Our study was a national systematic overview including surveillance, demographic, microbiologic, and genetic data about the emergence and spread of a mutant strain of *C. trachomatis* in Sweden. A fall in the proportion of positive chlamydia test results in counties using Abbott or Roche test systems began in 2005 and continued in 2006, whereas positivity rates in counties using BD tests did not change. The mutant strain has now spread throughout Sweden. Notably, the new variant has scarcely been found outside Sweden (*6*), indicating that we need to improve our understanding of the sexual networks through which chlamydiae spread (*7*).

Our analysis suggests that widespread transmission of nvCT is recent, even if the mutation itself occurred some time ago, because 46 of 48 specimens from different places had identical sequences when we used a highly discriminatory multilocus sequence typing system (*5*). This hypothesis is supported by the lack of diversification in the mutant strains compared with the high degree of sequence variation in other sample collections that we have analyzed (*5*; and unpub. data). We expect new nucleotide substitutions to occur over time.

The area in Sweden in which nvCT originated is not known, but the consistently high proportion of nvCT found in the county of Dalarna suggests that the mutant might have been present in this region for longer than in other counties studied. nvCT comprised 64% of chlamydia-positive specimens over the study period in Dalarna as a whole, and up to 78% in some localities (*7*); elsewhere in Sweden (*8–10*) and worldwide (*11*,*12*), genotype E strains of different subvariants comprise ≈40% of chlamydia-positive speciments in heterosexual populations. These data might indicate that the high proportion of nvCT is not only a result of accumulation of chlamydia cases when diagnostics failed and treatment and contact tracing were inadequate. Further studies will be needed to determine whether nvCT also has a selective advantage that might outcompete the wild-type bacterium over time.

The emergence of this mutant strain of a sexually transmitted pathogen has implications for public health practice. A recent study estimates that some 8,000 chlamydia cases

Table 2. Distribution of cases of nvCT by gender, age, and clinic category in 4 selected counties that used the Becton Dickinson system*

Factor	% nvCT (n/N)	p value†
Gender		
Male	16 (45/273)	0.103
Female	12 (39/327)	
Age, y		
15–19	14 (23/162)	0.558
20–24	16 (44/279)	
25–29	11 (11/98)	
≥30	10 (6/59)	
Clinic		
Venereal disease	15 (19/129)	0.020
Youth clinics	19 (39/210)	
Gynecology	9 (13/150)	
Antenatal/general practice	8 (6/77)	
Others	21 (7/34)	

*nvCT, new variant of *Chlamydia trachomatis*; n, number of nvCT cases detected; N, total number of *C. trachomatis* cases detected.
†Determined by χ^2 test.

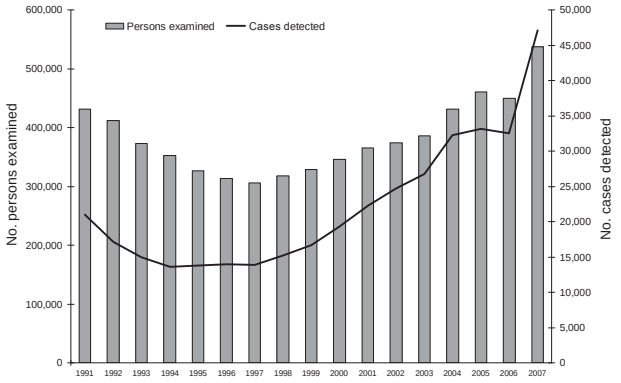

Figure 2. *Chlamydia trachomatis* reports, Sweden, 1991–2007. The number of persons examined and cases detected in 2007, when diagnostic tests for chlamydia had been changed, is in line with the increasing trend from 2004 and before. The figures for 2005 and 2006 reflect the failure to detect cases of the new chlamydia variant in some counties.

escaped detection in 2006 (*7*). This would have resulted in an ≈20% increase in reported chlamydia cases. Actual national figures for 2007 confirm such an increase, and the number of reported chlamydia cases has reached an all-time high in Sweden (Figure 2). *Chlamydia* infections, caused not only by nvCT, continue to rise (*13,14*), but the areas most heavily affected by the spread of nvCT have been in much the same situation as before chlamydia was first recognized as a pathogen. Failure to detect the nvCT over time have resulted in episodes of complicated infection all over the country, leading to ectopic pregnancies and infertility. Research is now needed to determine whether sequelae associated with chlamydia occur disproportionately in counties where test systems fail to diagnose the nvCT. The ability of this new variant to escape detection for so long shows that developers of future diagnostic tests need to take into account the structure and function of genomes when selecting appropriate target nucleic acid sequences in microorganisms. Currently, there are unique opportunities for research that could lead to insights into the immunobiology, transmission, and consequences of *C. trachomatis*.

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Crack Cocaine and Infectious Tuberculosis

Alistair Story, Graham Bothamley, and Andrew Hayward

We hypothesize that crack cocaine is independently associated with smear-positive tuberculosis (TB). In a case–control study of TB in London, 19 (86%) of 22 crack cocaine users with pulmonary TB were smear positive compared with 302 (36%) of 833 non–drug users. Respiratory damage caused by crack cocaine may predispose drug users to infectivity.

Tuberculosis (TB) has reemerged as a public health problem in London, and drug users are at high risk of contracting and spreading the disease (*1*). The United Kingdom has seen a substantial increase in the prevalence of drug use in the past decade, particularly crack cocaine use (*2*). Numbers of crack cocaine users assessed while in police custody in London increased 3-fold from 1993 through 2003 (*3*). There are an estimated 46,000 crack cocaine users in London; most also use opiates (*4*). Evidence to directly link risk for TB with crack cocaine use is lacking, although an association with tuberculin positivity has been shown. Increased exposure risk is considered largely attributable to social and lifestyle factors including homelessness, imprisonment, and drug and alcohol abuse (*5*). Drug users are commonly immunocompromised through HIV infection and malnutrition, resulting in increased risk for TB infection and rapid progression to active disease.

Habitually smoking crack cocaine causes pulmonary damage (crack lung) (Figure). Consequently, alveolar macrophage function and cytokine production is impaired, which may enhance susceptibility to infectious diseases (*6*). *Mycobacterium tuberculosis* is an intracellular pathogen that begins the disease process after a person inhales bacilli into the terminal bronchi and pulmonary alveoli (*7*). Alveolar epithelial cells likely resist invasion by *M. tuberculosis* bacilli, enabling resident alveolar macrophages and dendritic cells sufficient time to traverse the epithelium and phagocytose potential invading microbes (*8*). Several pulmonary complications are associated with the inhalation of crack cocaine (e.g., intensive cough, hemoptysis, shortness of breath, chest pain, acute bilateral pulmonary infiltrates, thermal airway injury, pneumothorax and noncardiogenic pulmonary edema, production of carbonaceous sputum, and exacerbation of asthma) (*9*). Collectively, these complications have been reported as crack syndrome (*10*). We hypothesize that crack cocaine use increases the risk for smear-positive pulmonary TB and that a component of this risk relates to lung damage caused by crack cocaine inhalation.

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The Study

Detailed clinical and social data were collected by case managers for all TB patients undergoing treatment in London on July 1, 2003. The study was approved by the Metropolitan Multicentre Research Ethics Committee–United Kingdom. Analyses were restricted to pulmonary patients 15–60 years of age (n = 970). We used univariate analyses to compare the characteristics of crack cocaine users, other hard-drug users (predominantly heroin users but excluding those who used only alcohol and marijuana), and those not known to use drugs. A separate category was included for hard-drug users not known to use crack cocaine to have a group with comparable levels of social deprivation, addiction related problems, and difficulty in accessing health services. To test the hypothesis that smear positivity at diagnosis was associated with crack cocaine use, we used a multivariate model with backwards elimination to exclude variables that did not make a significant contribution to the model. Variables initially included are shown in Table 1; the final model is shown in Table 2.

TB patients who used crack cocaine were predominantly 20–49 years of age. Crack cocaine users and other

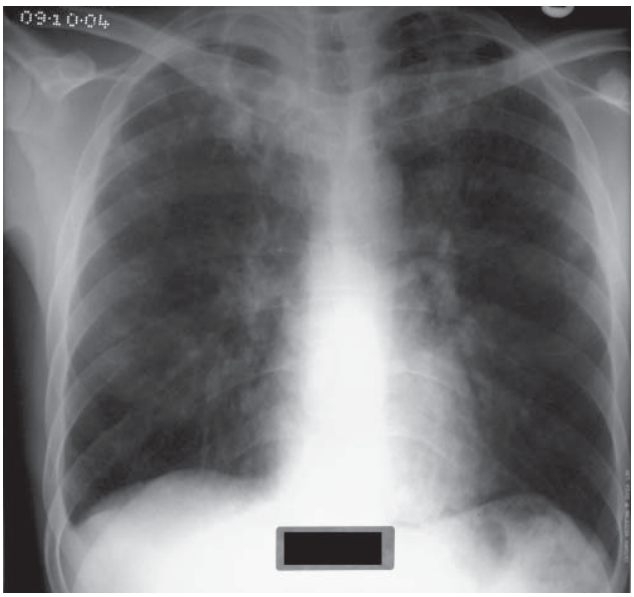

Figure. Chest radiograph of a tuberculosis patient addicted to crack cocaine.

Table 1. Univariate analysis of drug-using and non–drug-using patients with pulmonary TB in London, United Kingdom, 2003–2004*

Variable	No known drug use, n = 833, no. (%)	Hard-drug user (unconfirmed crack cocaine user), n = 115, no. (%)	Hard-drug user (confirmed crack cocaine user), n = 22, no. (%)	p value
Gender				<0.0001
Male	445 (54.1)	99 (86.8)	12 (54.6)	
Female	377 (45.9)	15 (13.2)	10 (45.5)	
Ethnicity				<0.0001
White	142 (17.1)	54 (47.0)	5 (22.7)	
Black African	344 (41.5)	25 (21.7)	5 (22.7)	
Black Caribbean	32 (3.9)	15 (13.0)	9 (40.9)	
South Asian	244 (29.4)	17 (14.8)	0	
Other	68 (8.2)	4 (3.5)	3 (13.6)	
Born in the United Kingdom	162 (19.6)	62 (54.9)	14 (63.7)	<0.0001
Previous TB	78 (9.4)	25 (21.7)	5 (22.7)	<0.0001
Previous TB past 2 years (relapsed)	36 (4.3)	19 (16.5)	4 (18.2)	<0.0001
Known HIV+	95 (11.4)	9 (7.8)	3 (13.6)	0.478
Delay in diagnosis ≥3 mo	109 (13.1)	19 (16.5)	2 (9.1)	0.499
Sought treatment at ED	126 (15.1)	32 (27.8)	10 (45.6)	<0.0001
Cough during initial examination	589 (70.7)	99 (86.1)	19 (86.40	0.001
Sputum smear positive at diagnosis	302 (36.3)	68 (59.1)	19 (86.4)	<0.0001
MDR	32 (3.8)	7 (6.1)	0	0.333
Linked to known INH resistance outbreak	9 (1.1)	10 (8.7)	11 (50.0)	<0.0001
INH resistance (not outbreak)	54 (6.5)	11 (9.6)	1 (4.5)	0.783
Treated with DOT from start	74 (9.0)	19 (16.5)	6 (27.3)	0.001
Nonadherent to treatment in first 2 mo	125 (15.0)	59 (51.3)	15 (68.2)	<0.0001
Lost to follow-up	19 (2.3)	12 (10.4)	6 (27.3)	<0.0001
Homeless	37 (4.4)	22 (19.1)	13 (59.1)	<0.0001
Mental health problems	28 (3.4)	27 (23.5)	9 (40.9)	<0.0001
Imprisoned during current episode of TB	9 (1.1)	22 (19.1)	14 (63.6)	<0.0001

*TB, tuberculosis; ED, emergency department; MDR, multidrug resistant; INH, isoniazid; DOT, directly observed therapy.

drug users were significantly more likely than non–drug users to have been born in the United Kingdom, of white or black Caribbean ethnic origin, homeless, alcohol abusers, or have a history of imprisonment. Non–crack drug users tended to have the longest delays between diagnosis and treatment and crack users the shortest, but this tendency did not reach significance (Table 1). Crack cocaine users were statistically significantly more likely to seek treatment at emergency departments, to adhere poorly to treatment regimen, or default from treatment altogether. Drug users were also more likely to have isoniazid-resistant disease. Among crack cocaine users this was primarily related to a large outbreak of isoniazid-resistant TB (*11*).

Among crack cocaine users, diagnosis showed that 86% were smear positive compared with 36% of patients not known to use drugs (relative risk [RR] 2.4, 95% confidence interval [CI] 2.0–2.9), p<0.001) and 59% of drug users not known to use crack cocaine (RR 1.6, 95% CI 1.4–2.0, p<0.001). Multivariate analysis showed that the risk for smear-positive disease was higher for drug users than for those not known to use drugs (odds ratio [OR] 1.9, 95% CI 1.2–3.0, p = 0.007) and highest in crack cocaine users (OR 6.6, 95% CI 1.8–24.3, p = 0.005). Other significant risk factors for smear positivity were being of black Caribbean ethnicity, having multidrug-resistant disease, and seeking treatment at an emergency department. When the multivariate model was restricted to include only hard-drug users, crack cocaine users were still significantly more likely than other drug users to be smear positive (p = 0.02).

Conclusions

Smear-positive disease is 2.4 times more likely to be diagnosed in crack cocaine users than in non–drug users, whereas hard-drug users not known to use crack cocaine are 1.6 times more likely to be diagnosed with smear-positive disease. Crack cocaine users were significantly more likely than other drug users to be smear positive on diagnosis.

The increased risk for smear-positive disease in crack cocaine users was not due to diagnostic delays. Hard-drug users who were not confirmed as crack cocaine users had the longest diagnostic delays. Crack cocaine users had the shortest diagnostic delays, potentially attributable to rapidly progressive, debilitating disease. Crack cocaine users were also more likely to seek treatment at an emergency department rather than primary care services. Again, the choice of healthcare service may be related to the severity of disease. Symptom duration before diagnosis is difficult to measure, especially among drug users. We included non–crack drug

Table 2. Multivariate analysis of risk factors for smear-positive disease on diagnosis among drug-using and non–drug-using patients with pulmonary TB in London, UK, 2003–2004*

Variable	OR	95% CI	p value
Not a hard-drug user	Baseline		
Hard-drug user (not known to use crack cocaine)	1.87	1.19–2.95	0.007
Crack cocaine user	6.59	1.78–24.31	0.005
Age, y			
0–14	0.10	0.08–0.56	0.002
15–29	1.10	0.81–1.48	0.55
30–59	Baseline		
≥60	0.69	0.45–1.14	0.14
Ethnicity			
South Asian	Baseline		
Black African	1.75	0.96–1.95	0.08
White	1.51	0.99–2.31	0.053
Black Caribbean	2.70	1.34–5.43	0.005
Other ethnicity	1.61	0.91–2.85	0.101
No drug resistance	Baseline		
INH (not outbreak strain)	1.23	0.72–2.11	0.441
INH (outbreak strain)	0.96	0.37–2.50	0.929
MDR	2.90	1.44–5.78	0.003
Sought treatment at ED	3.33	2.20–4.82	<0.001

*OR, odds ratio; CI, confidence interval; INH, isoniazid resistant; MDR, multidrug-resistant; ED, emergency department.

users as a comparison group because they have a similar social profile and similar access to healthcare. Therefore, we are confident that the extremely high levels of smear positivity on diagnosis in crack cocaine users are not due to a long duration of clinical illness preceding diagnosis.

In the multivariate model, crack cocaine use remains strongly associated with smear-positive disease after controlling for a wide variety of other potential confounders. Other risk factors include ethnicity (drug use was common among black Caribbean patients and may have been underreported); treatment at an emergency department (possibly a marker of disease severity); and multidrug-resistant disease. We are uncertain why multidrug-resistant cases were more likely to be smear-positive on diagnosis; however, previous studies have found that cavitary disease is a risk factor for drug resistance (*12*).

The fact that smear positivity was significantly more prevalent in patients known to use crack cocaine when compared with other hard-drug users suggests that this additional risk may be attributable to a biological component. Plausible biological mechanisms to explain the increased risk of smear-positive disease include poor alveolar macrophage antimicrobial activity in crack cocaine users due to decreased inducible nitric oxide synthase activity (*13*) and direct effects on the lung (*10*).

It is likely that a proportion of hard-drug users were incorrectly classified as not using crack cocaine due to nondisclosure. This would reduce the apparent differences in levels of smear positivity between the groups. Nevertheless, despite relatively small numbers of known crack cocaine users, there is a significantly ($p = 0.02$) higher proportion of smear-positive disease in these patients compared with other hard-drug users.

Previous studies have shown TB transmission associated with crack cocaine use (*14*). Persons frequenting crack houses are likely to have multiple risk factors for active pulmonary TB. Prolonged sharing of closed and confined airspace, intensive coughing, and other acute pulmonary complications of crack cocaine inhalation promote transmission. Drug users are more likely than non–drug users to default treatment, to remain infectious for prolonged periods after diagnosis, and to acquire drug-resistant TB (*15*). We studied smear status at diagnosis to exclude the effect of poor treatment adherence.

Our study suggests a dangerous synergy between TB and crack cocaine. Users may experience addiction-related problems that complicate access to healthcare and aggravate transmission, possibly aggravated by a biological driver that may increase susceptibility to infection and progression to infectious disease. Additional studies are needed to investigate the possible biological role of crack cocaine in the development of infectious forms of TB.

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Mr Story is a consultant nurse and epidemiologist at the Health Protection Agency Centre for Infections, London. His main area of interest is the control and public health effect of infections among hard-to-reach groups, particularly drug users, prisoners, and the homeless.

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Human Case of Swine Influenza A (H1N1) Triple Reassortant Virus Infection, Wisconsin

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Zoonotic infections with swine influenza A viruses are reported sporadically. Triple reassortant swine influenza viruses have been isolated from pigs in the United States since 1998. We report a human case of upper respiratory illness associated with swine influenza A (H1N1) triple reassortant virus infection that occurred during 2005 following exposure to freshly killed pigs.

Human infections with swine influenza A viruses occur sporadically in the United States and Canada (*1–8*). Triple reassortant swine influenza viruses (containing genes derived from human, swine, and avian influenza A viruses) have been isolated from swine in the United States since 1998 (*9*,*10*), and human infections with swine reassortant viruses have been documented (*11–13*). We report a case of respiratory illness in an adolescent boy associated with swine influenza A (H1N1) triple reassortant virus infection.

The Study

On December 7, 2005, a previously healthy 17-year-old boy with no history of recent travel became ill; symptoms were headache, rhinorrhea, low back pain, and cough without fever. He had received inactivated influenza vaccine administered intramuscularly on November 11, 2005. During an outpatient clinic visit on December 8, 2005, a nasal wash specimen was obtained and tested positive for influenza A by rapid influenza diagnostic test (BinaxNow A&B, Binax, Inc., Scarborough, ME, USA). Results of a chest radiograph were normal. The patient's symptoms resolved on December 10, 2005. The specimen was sent to the Wisconsin State Laboratory of Hygiene (WSLH), and an influenza A virus was isolated by shell vial tissue cell culture (MDCK cells, WSLH, Madison, WI, USA). Real-time reverse transcription–PCR (rRT-PCR) was positive for influenza A virus but negative for human subtypes H1, H3, and Asian avian H5. At the Centers for Disease Control and Prevention (CDC), rRT-PCR testing of the shell vial viral culture material was positive for influenza A virus, but negative for human subtypes H1 and H3, as well as avian subtypes H5, H7, and H9. Complete genomic sequencing of the virus at CDC identified it as a swine influenza A (H1N1) triple reassortant virus, A/Wisconsin/87/2005 H1N1.

Investigation by the Wisconsin Division of Public Health and the Sheboygan County Division of Public Health showed that the patient had assisted his brother-in-law in butchering pigs at a custom slaughterhouse 3 days before illness onset. Thirty-one swine were delivered to the facility that morning by a distributor who had acquired the animals from multiple sources. None of the pigs appeared ill. The patient helped hold and abduct the forelimbs of 1 freshly killed pig while his brother-in-law eviscerated it. No facial or respiratory protection was worn during this procedure. A few chickens were housed at the slaughterhouse premises, but no poultry were slaughtered on site.

The patient denied any other contact with swine, poultry, or other animals during the 7 days before becoming ill. Eight days before illness onset, the patient's father obtained a live chicken that was kept in the home for 1 day before it was sacrificed during a ritual ceremony. The patient was never within 10 feet of the chicken and did not attend the ceremony. None of the patient's household members or any slaughterhouse employees reported illness during the 2 weeks before or after the patient became ill.

Paired serum specimens were obtained from the patient and 4 family members on December 13, 2005, and January 9, 2006. A single serum specimen was obtained from the patient's brother-in-law on December 19, 2005. Serologic testing was conducted at CDC by a standard hemagglutinin inhibition (HI) antibody assay and reference antisera against influenza A (H1), A (H3), B, and swine A (H1N1) viruses A/swine/Wisconsin/238/97, A/swine/Wisconsin/NJ56371/99, A/swine/Minnesota/593/99, and A/Wisconsin/87/2005 (isolated from the patient). HI antibody testing was negative for influenza A (H1), A (H3), and swine subtype H1N1 on all serum specimens, but 1 family member had evidence of a 4-fold rise in HI antibody titer to influenza B/Hong Kong/330/2001 (B/Victoria/2/87

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lineage) virus, which suggested an acute influenza B virus infection. All serum samples were also tested by microneutralization assay at CDC using the patient's swine influenza A (H1N1) virus isolate and a human influenza A virus (A/New Caledonia/20/99 H1N1). The patient's serum specimens demonstrated a 2-fold increase in neutralizing antibody titer against swine influenza A/Wisconsin/87/2005 subtype H1N1 virus, but the level of neutralizing antibodies to A/New Caledonia/20/99 (H1N1) virus was unchanged in acute- and convalescent-phase serum specimens, which is consistent with his history of influenza vaccination in mid-November 2005.

Conclusions

We report a human case of swine influenza A (H1N1) triple reassortant virus infection in the United States. The case-patient experienced a mild and acute respiratory illness and recovered fully. Swine influenza A/Wisconsin/87/2005 (H1N1) virus was isolated from an upper respiratory specimen obtained from the patient, and serologic testing suggested, but was not diagnostic of, an immune response to acute infection. Epidemiologic investigation showed the patient had direct and close exposure to freshly killed pigs and their organs while assisting his brother-in-law in butchering them. Although the pigs did not appear ill, the most plausible source of the patient's swine influenza A virus infection was respiratory secretions of freshly killed pigs.

Surveillance data suggest that triple reassortant subtype H1N1 viruses are the predominant genotype of subtype H1N1 viruses in North American pigs (*14*). Persons having direct contact with swine are at greatest risk of infection with swine influenza viruses (*4–7,13*), but such contact is not documented in all cases (*7*). Human-to-human transmission of swine influenza virus is rare, but evidence suggests that it has occurred (*1–3,7,8,13*). Human illness caused by infection with swine influenza viruses is often indistinguishable clinically from infections caused by other influenza viruses (*1,7,12,13*); complications, including pneumonia and death, have been documented (*3,4,7*). Asymptomatic infections in humans caused by swine influenza viruses may occur (*5,8,13*) and therefore, the true frequency of swine-to-human influenza virus transmission is unknown (*3,5–7*).

We were limited in assessing other possible swine influenza A (H1N1) virus infections and in confirming swine influenza in the pigs. Pigs delivered to the slaughterhouse the day of the patient's exposure originated from multiple farms, but specimens were unavailable for testing due to delays during animal traceback. Our findings suggest that microneutralization assay may be more sensitive than a standard HI assay in detecting human antibodies to swine influenza A viruses. Primers and probes for detection of human influenza A viral RNA by rRT-PCR identified a nonhuman influenza A virus, triggering further analyses that specifically identified the virus. We could not confirm whether the patient's influenza vaccination and high levels of vaccine-derived subtype H1N1 neutralizing antibody influenced his relatively mild clinical course of illness. The patient did not have a 4-fold increase in neutralizing antibody titer to the swine influenza A (H1N1) virus isolated from his respiratory specimens, which would be more suggestive of acute infection. Further studies are needed to understand the human immune response to infection caused by swine influenza A viruses and to interpret serologic test results.

Human infections with novel influenza A subtype viruses are now nationally notifiable in the United States. Clinicians should inquire about exposure to animals (including pigs) and visits to petting zoos and county fairs when evaluating patients with unexplained influenza-like illnesses (*15*). Ideally, joint animal health and public health investigations should be conducted promptly to identify and control the source of swine influenza. Investigations should attempt to specifically identify the virus in animals and persons; define the scope and clinical spectrum of human illnesses, including appropriately timed collection of serum specimens from ill persons and exposed individuals; determine risk factors for human infection; and assess the potential for human-to-human transmission of swine influenza A viruses.

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Diagnostic Challenges of Central Nervous System Tuberculosis

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Central nervous system tuberculosis (TB) was identified in 20 cases of unexplained encephalitis referred to the California Encephalitis Project. Atypical features (encephalitic symptoms, rapid onset, age) and diagnostic challenges (insensitive cerebrospinal fluid [CSF] TB PCR result, elevated CSF glucose levels in patients with diabetes, negative result for tuberculin skin test) complicated diagnosis.

Tuberculosis (TB) of the central nervous system (CNS) is classically described as meningitis. However, altered mental status, including encephalitis, is within the spectrum of clinical manifestations. Because early treatment can dramatically improve outcomes, consideration of TB as a potential pathogen in CNS infections, including encephalitis, is vital. The California Encephalitis Project (CEP), initiated in 1998 to study the causative agents, epidemiology, and clinical features of encephalitis, has identified 20 cases of culture-confirmed tuberculous encephalitis. In most instances, TB was not initially considered to be a likely cause.

The Study

Referrals are received by the CEP statewide from clinicians seeking diagnostic testing for immunocompetent patients, including TB PCR testing when appropriate, who meet the CEP case definition of encephalitis (*1*). Mycobacterial testing was often also conducted by the referring hospital. Inclusion criteria for this report were a positive cerebrospinal fluid (CSF) culture for *Mycobacterium tuberculosis* complex or a positive CSF TB PCR result. Clinical data were compiled from case history forms and other medical records when available. To evaluate differences among causes of encephalitis, TB patients were compared with CEP patients with cases of enterovirus and herpes simplex virus 1 (HSV-1) encephalitis. Demographic, clinical, and laboratory data were compared by using the Fisher exact test, χ^2 test, or Kruskal-Wallis test as appropriate (statistical significance was set at $\alpha = 0.05$).

From June 1998 through October 2005, a total of 1,587 patients were enrolled in the CEP; 20 patients fulfilled criteria as TB cases. Demographic and clinical information for the study population are detailed in the online Appendix Table (available from www.cdc.gov/EID/content/14/9/1473-appT.htm). Median age was 41 years (range 8 months to 77 years). The median time from symptom onset to first lumbar puncture was 5 days (range 0–62 days). Seventeen patients (85%) had a second lumber puncture.

In general, CSF values became more abnormal over time, with increasing leukocyte counts and protein levels and decreasing glucose levels (Appendix Table). Most patients had a CSF mononuclear cell predominance, although 4 patients (21%) had a neutrophil predominance. All patients had cranial neuroimaging, magnetic resonance imaging (18 of 20), and computed tomography (20 of 20) (Appendix Table; Table). Results of computed tomography scans were often normal (50%).

Of patients in whom the results of a recent tuberculin skin test (TST) were known, 59% (10 of 17) had a negative result (Appendix Table). Many chest radiographs (9 of 18, 50%) showed no abnormalities. Concurrent culture positive pulmonary disease was found in 4 (50%) of 8 patients tested. A history of foreign birth (53%) or foreign travel (80%) was common. When these factors were reported, 5 patients (25%) had a history of treatment for TB and 5 patients (63%) had contact with a known case of TB. Only 2 patients did not have at least 1 of these risk factors.

Of the 20 cases identified, all had a positive CSF culture for *M. tuberculosis* complex. Only 4 (24%) of 17 were CSF TB PCR positive and none had a positive CSF acid-fast bacilli smear (Appendix Table). All but 3 patients had pan-susceptible *M. tuberculosis* isolates; 2 patients had *M. bovis* isolates (resistant to pyrazinamide) and 1 patient had an isoniazid-resistant isolate.

When patients with TB were compared with patients with viral causes of encephalitis (Table), those with enterovirus encephalitis were significantly younger, were less likely to require intensive care, had shorter hospitalizations, had fewer abnormal results for CSF and neuroimaging, and were less likely to die (all $p<0.05$). Patients with HSV-1

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Table. Comparison of TB encephalitis with viral encephalitis, California Encephalitis Project*

Characteristic	CNS TB	HSV-1	Enterovirus
Total no. cases	20	39	44
Patient demographics			
Male, no. (%)	12 (60)	14 (36)	25 (57)
Median age, y (range)	41 (8 mo–77y)	55 (8 mo–89 y)	13 (6 mo–74 y)†
Race white, non-Hispanic, no. (%)	3 (15)	22 (69)‡	14 (33)
Clinical data			
Interval from CNS onset to admission, d, median (range)	5 (0–62)	2 (0–28)	2 (0–18)†
ICU care, no (%)	15 (75)	19 (56)	20 (53)
Fever, no. (%)	15 (75)	35 (92)	35 (80)
Seizures, no. (%)	7 (35)	22 (58)	12 (27)
Altered consciousness, no. (%)	13 (65)	30 (79)	20 (50)
Personality change, no. (%)	9 (45)	17 (45)	7 (17)†
Hallucinations, no. (%)	3 (16)	7 (21)	3 (7)
Stiff neck, no. (%)	14 (70)	12 (32)§	19 (44)
Ataxia, no. (%)	7 (37)	6 (23)	11 (28)
Length of hospital stay, d, median (range)	30 (8–753)	15 (0–738)§	6 (0–1,124)¶
Laboratory results			
CSF leukocytes, per mL, median (range)	201 (42–2,845)	47 (0–975)‡	85 (0–1,080)†
CSF protein, mg/dL, median (range)	174 (66–357)	71 (15–297)‡	60 (19–881)¶
CSF glucose, mg/dL, median (range)	35 (9–132)	69 (39–112)‡	67 (38–159)¶
MRI/CT (abnormal, initial study), no. (%)	17 (85)	36 (95)	12 (39)¶
Inpatient deaths, no. (%)	6 (30)	8 (21)	4 (9)†

*CNS, central nervous system; TB, tuberculosis (*Mycobacterium tuberculosis*); HSV-1, herpes simplex virus 1; ICU, intensive care unit; CSF, cerebrospinal fluid; MRI, magnetic resonance imaging; CT, computed tomography. Denominators may vary slightly depending on available data. Data were analyzed (2-way analysis) by using Fisher exact test, χ^2 test, or Kruskal-Wallis test as appropriate, with statistical significance set at $\alpha = 0.05$. Comparisons without a symbol did not reach statistical significance.
†CNS TB vs. enterovirus, $p<0.05$.
‡CNS TB vs. HSV-1, $p\leq0.001$.
§CNS TB vs. HSV-1, $p<0.05$.
¶CNS TB vs. enterovirus, $p\leq0.001$.

encephalitis were more likely than those with CNS TB to be white and non-Hispanic and to have shorter hospital stays, lower CSF leukocyte and protein levels, and higher CSF glucose levels.

Conclusions

Although tuberculous meningitis is well described, prominent encephalitic features are less commonly reported. Illness and death associated with neurotuberculosis are highly dependent on the stage of disease at diagnosis; early diagnosis and treatment correlates with better outcomes (*2*). Although the TB cases reported here represent only a small percentage of CEP cases (<1%), CNS TB with an encephalitic picture warrants further discussion because of high morbidity and mortality rates and need for early diagnosis and appropriate treatment.

This study found atypical features of CNS disease that may have confounded early diagnosis. Tuberculous CNS disease is typically described as a chronic meningitis with insidious onset in children <5 years of age or in older adults with relatively few cases during school age years or adolescence (*3*). In contrast, CEP TB patients came to a hospital within 2 weeks of symptom onset and the greatest percentage of CEP TB patients was found in persons 10–19 years of age (22%).

Although typical CSF studies (mononuclear cell pleocytosis, low glucose levels, and elevated protein levels) (*4*) were often found in CEP CNS TB patients, atypical findings were noted. CSF glucose levels were often normal in patients with diabetes, although the ratio of CSF to serum glucose was invariably low. Additionally, a CSF neutrophil predominance was found in 4 patients, erroneously suggesting pyogenic meningitis. Although clinicians may be tempted to ascribe abnormal CSF values to viral meningitis or encephalitis based on abnormal CSF values, CEP patients with enterovirus and HSV-1 encephalitis rarely had glucose levels <40 mg/dL. Median protein levels were significantly higher in patients with CNS tuberculosis (174 mg/dL in TB) than in patients infected with HSV-1 (71 mg/dL) or enterovirus (60 mg/dL) ($p<0.001$).

Diagnostically, the low sensitivity of CSF TB PCR is problematic. Potential explanations for the lack of sensitivity in CSF specimens include low bacillary load in CSF, small sample volumes, and PCR inhibitors in the sample (*5*). Given that all of our patients had positive CSF cultures, we would have expected a higher PCR yield. Most concerning was the finding that many providers caring for these patients were dissuaded from pursuing TB as a diagnostic possibility when the PCR result was negative.

Given the difficulties in obtaining a rapid diagnosis, therapy must often be initiated empirically. Unfortunately, a history of TB and TST or chest radiograph results were not reliable indicators of active disease and might be difficult to obtain. Further complicating therapy, lengthy cultures, and isolate sensitivities are necessary to optimize choices of antimicrobial drugs. Ten percent (2) of our patients were infected with *M. bovis*, a relatively higher percentage than in other reports (*6*–*8*). Intrinsic pyrazinamide resistance (*9*) of *M. bovis* required modification of use of empiric antimicrobial drugs.

A limitation of this series is inclusion of only patients with a positive CSF culture. Because historical data suggest that only 25%–70% of patients with a diagnosis of CNS TB have a diagnosis confirmed by microbiologic testing (*2*), there were likely additional CEP patients with CNS tuberculous disease without a positive acid-fast bacilli culture who were not included in this series. Additionally, the series was limited by the referral bias inherent in the project; CEP patients are typically sicker and present greater diagnostic challenges. Thus, those with obvious or mild CNS tuberculous disease would be underrepresented. Despite this potential bias, outcomes were similar (mortality rate 30%) compared with reported mortality rates (18%–72%) and morbidity rates (16%–48%) in previous studies (*10*).

This report emphasizes some atypical features of CNS TB manifested as encephalitis. Encephalopathic changes, a relatively rapid course, nonclassic age distribution, and negative TB PCR and TST results should not dissuade a clinician from considering TB, particularly when CSF:serum glucose ratio (*11*) is <0.5 and CSF protein level is >100 mg/dL. The CEP TB patients reported here may represent a severe part of the continuum of TB meningitis or may represent a distinct encephalitic subset with atypical features.

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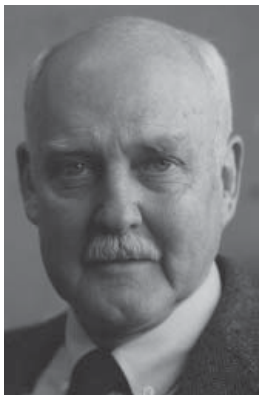
In Memoriam: Michael B. Gregg (1930–2008)

David M. Morens



Dr Gregg

"He has in his make-up two essential elements—common sense and the will to work ... in collecting [facts] he is diligent, patient, careful, thorough and unbiased ... he avoids needless or obfuscating high mathematics and formulae ... he does not twist the facts... He remains broad-minded and open-minded ... He is humble in his ignorance but bold in his search for truth" (*1*). So wrote legendary American epidemiologist Leslie Lumsden (1875–1946). He was responding to the question, posed by the American Journal of Public Health in 1942, "What and who is an epidemiologist?" (*1*).

Michael B. Gregg, MD, who died on July 9 in Brattleboro, Vermont, was a 12-year-old boy in 1942 when Lumsden described the quintessential epidemiologist. But Gregg (or Mike, as just about everyone at the Centers for Disease Control and Prevention [CDC] called him) grew up to become not only an epidemiologist but also a teacher, a mentor, a friend, and for many the embodiment of Lumsden's ideal. Among the many hundreds of students Gregg taught and influenced at the National Institutes of Health, and most notably at CDC, are countless leaders in epidemiology, public health, and the biomedical sciences. They practice all over the country, in health departments, in universities, in federal agencies, and indeed around the world. Roger Bernier, an epidemiologist who trained under Gregg at CDC, has compared Gregg's professional approach to one of his hobbies: gardening. He planted, he nurtured, and he took care of and kept an eye on things; as the garden flourished, he kept it in bloom by careful but unobtrusive attention, preventive maintenance, and teaching and inspiring others to pitch in and help.

Mike's textbook, Field Epidemiology (*2*), remains the authoritative work on outbreak investigations and the "shoe leather" approach to public health problem solving. It reflects not only his style but also his unparalleled experiences in national and international epidemiology. Between the mid-1960s and 1990, when he retired from CDC, Mike was often in the eye of the hurricane, presiding as deputy director over CDC's Bureau of Epidemiology during many of the most memorable outbreak investigations of the past century. These included Pontiac fever/Legionnaires' disease (1968/1976), swine flu (1975–1976), Guillain-Barré syndrome (1977), Ebola fever in Sudan and Zaire (1976), and AIDS (1981). He also helped put on the epidemiology map such new diseases as Reye syndrome (1973–1977), Kawasaki disease (1977), and toxic-shock syndrome (1980). And he helped steer us into an era of control (and future eradication) of such vaccine-preventable diseases as measles and poliomyelitis.

Less appreciated, his methods and perspectives led unself-consciously to the conceptualization and the development of approaches to understanding what would later be called emerging infectious diseases. He believed that such emergences were an inevitable consequence of microbial genetic dynamism let loose in ecosystems shared by humans and environmental determinants. If this were not enough, Mike described it all in the Morbidity and Mortality Weekly Report (MMWR), put out in the pithy, painfully unambiguous, "just-the-facts-ma'am" style he perfected. As an unintentional by-product of his editorial skills, he turned hundreds—perhaps thousands—of epidemiologists into decent medical writers and editors, instilling in them the idea that good writing is not only a result of, but also a path to, the kind of clear thinking needed in epidemiologic practice. Anne Mather, managing editor of the MMWR for 7 years under Mike's leadership in the 1970s and 1980s, notes that his mantra for editing, "This is getting into the thick of thin things," is advice she still follows today.

Even in full retirement, Mike was there for us to consult. When the outbreak of severe acute respiratory syndrome (SARS) erupted in 2003, Emerging Infectious Diseases called him into service to review a major report of a SARS investigation. Although he strongly recommended publication of the article, Mike's 5-page review nonetheless respectfully took it apart line by line, then put it back together in the way it should have been done in the first place. The authors may have wished they had had this anonymous reviewer on their team during the actual investigation.

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At CDC Mike avoided the limelight but devoted tremendous energy to supporting others, especially young epidemiology trainees (Epidemic Intelligence Service, or EIS, officers). When I first met him in 1975, he wore three tall hats: MMWR editor, Viral Diseases Division director, and deputy director of the Bureau of Epidemiology (this was 7 years before the "Center" became the "Centers," a time when the "Bureau of Epi" was still CDC's crown jewel). He was my first boss and already something of a legend. He taught us by making us apprentices in everything he did, including participation in countless "curbside" discussions of epidemics, methods, publications, theories, and epidemic history. The discussions were often conducted on the run, carried on from office to office like a movable feast, spilling into hallways, migrating to the cafeteria, or even the library. It always amazed me that such a quiet man was at the center of so much bustling activity.

Mike was also a historian of sorts, retaining much of the institutional memory of CDC. He had encyclopedic recall of past CDC outbreak investigations, perhaps because he had been involved in supervising many of them, reading and editing the "Epi-2" reports, and then publishing write-ups in the MMWR. The day we learned of an outbreak of respiratory disease among American Legionnaires in Philadelphia, Mike was having lunch in the CDC cafeteria with 3 young EIS officers (me among them), each of us less than a month on the job. Everyone was excited: obviously, something really big was happening. But, as is usually the case, information was dribbling in sporadically, incompletely, and with maddening imprecision. After listening to his young colleagues speculate a bit, Mike calmly reflected on whether this could be "the big one," return of a 1918-like influenza pandemic presaged by the swine flu outbreak earlier that year. He ran through the old and the recent history of influenza and other epidemic respiratory diseases, calling up all sorts of experiences completely new to us, commenting on what we (Pennsylvania health officials and CDC) needed to look for, what facts would be for and against influenza, what we needed to do next, and so on. I was thrilled. On ward rounds only a few weeks beforehand, I was now suddenly in the middle of a national epidemic, having lunch with one of the nation's top epidemiologists, learning about fascinating things I had not imagined last month. "Yes!" I thought, "This epidemiology thing is what I want to do from now on." Many others had similar epiphanies.

Within a day or two of that lunch discussion, accumulating information made it clear that the Philadelphia outbreak could not be influenza. Remembering a mysterious unsolved epidemic 8 years beforehand, and having himself caught that mystery disease in the line of duty, Mike was then perhaps the first to say "This is beginning to look like Pontiac fever." And so it turned out to be.

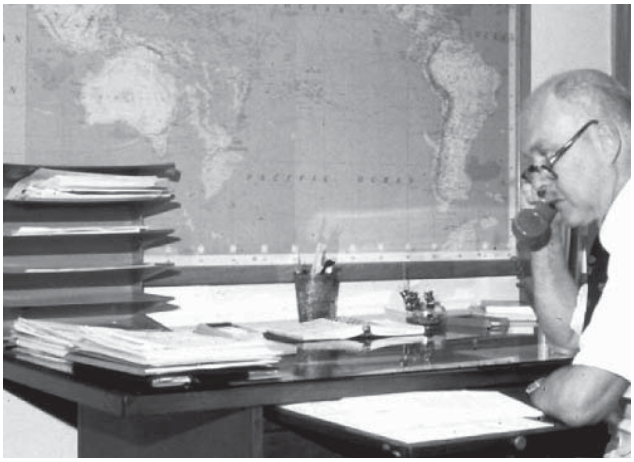

Figure. Mike Gregg at work on the Morbidity and Mortality Weekly Report. Source: Steve Thacker.

Each July, Mike addressed the incoming class of EIS officers, typically fresh from the wards of Ivy League medical schools, in the only epidemiology course most would ever attend. His first subject would be "how to investigate an epidemic," one of the few medical subjects most of his audience knew absolutely nothing about. Peering up shyly over his glasses, held midway down the bridge of his nose, he would look down at the floor for a minute and then begin so softly that those in the back would have to strain to hear. "First," he would say, "you need to find a good map..." Then, in a slow build-up of pragmatic and mundane steps, he would walk us through an outbreak, taking care to omit none of those boringly essential details that seemed too obvious to mention. His unstated message was clear and powerful. This isn't medical school. Memorizing the Krebs cycle, the cranial nerves, and the entire Washington Manual won't get you through an outbreak investigation. You are now a detective, and you have to go about investigating the scene of the "accident" in "real time," with little opportunity to research or look things up, keeping an open and inquisitive mind, remaining flexible and creative, always rethinking things, backtracking and verifying when necessary, being thorough but also moving quickly to assemble all of the puzzle pieces that seek to become a coherent picture.

"Quick and dirty," one of his favorite terms, was how he described much of what we epidemiologists did, and not in a pejorative sense of the word but with genuine affection for the game of hide-and-seek he never tired of playing. In epidemiologic investigations, there was no time for elegance and perfection. Epidemiology helped real people with real problems. As Michael O'Leary, another of Mike's former epidemiology students, paraphrased him: "It's better to be approximately right today than exactly right tomorrow." Were Mike Gregg standing in Golden Square in 1854, he would have been impressed less by John Snow's brilliant reasoning

than by Snow's ability to get the Broad Street pump handle removed quickly, thereby saving more lives.

Avuncular, almost fatherly at times, Mike clearly enjoyed mentoring young colleagues, and in doing so he revealed to us a broader view of a bigger, more complicated world into which we might one day be admitted. His door was always open, no matter how low on the totem pole you might be; but if he needed to see you, he popped into your office rather than asking you to come to his. In conversation he valued and respected the ideas of even the newest of neophytes. He listened more than he spoke; and when he did speak, it was softly and courteously, tentatively offering up his own thoughts on the subject, as if this were a democratic effort, rather than the boss telling you how things really were. He embraced teamwork as the professional norm and ignored, without discounting or disparaging it, individual accomplishment. His modesty was genuine; his gentleness, memorable. He walked the halls with his head aimed shyly down, moving so close to the cinderblock wall that you expected him to bump into it at any moment, occasionally lifting his shoulder to avoid just such an accident, dressed casually and in a manner that never telegraphed his stature, his slightly battered Hush Puppies pressing silently on linoleum floors. Had you passed Mike in the cafeteria, or even sat down next to him at a meeting, you might have mistaken him for a visitor who had accidentally wandered into Building 1.

Mike's conversation was sometimes reflective and philosophical but at other times spare, to the point, and without adornment. Frequently, before speaking he softly cleared his throat, as if to test that he had enough voice to be heard, or perhaps as a preventive warning against interrupting someone else. If he became irritated or impatient, which happened only rarely, there might appear a quick gleam in his eyes, but any anger he felt dissipated quickly. In more than 30 years, I never heard him raise his voice above a moderate level. When others drowned him out, he let them do so, dropping his own words to listen with interest and respect to theirs.

His sense of humor was expressed not in guffaws but in short chuckles, sometimes accompanied by the split-second flash of an almost-mischievousness grin. Like everything else about him, his humor was understated. One example comes to mind. Gracious hosts, he and his wife, Lila, sometimes opened their home to the annual picnics for incoming EIS officers and their families. On the first of these I attended, in 1976, I remember him relating, with a seriousness that may well have been intentionally hilarious (I could never be sure), the story of a scandalous, and possibly apocryphal, EIS-picnic-from-hell of some years past. In Mike's telling, someone on the CDC staff had gotten a not-so-bright idea for a training exercise: put methylene blue dye in one of the foods at the EIS picnic and, when the results were discovered, send the EIS officers off on an outbreak investigation to identify the food source. Apparently, however, when spouses and children began urinating blue liquid into their home toilets, a few among them were more alarmed than amused, and the exercise had to be abandoned. Mike told this story dryly but with a twinkle in his eye, as if to say "What a dumb thing to do (but wasn't it a great idea for an epidemic—more than 'eleven blue men' [*3*] but women and children too?").

One of my most vivid memories of Mike concerns a brief discussion we had in about 1978. I was sitting outside the office of Phil Brachman, then the director of the Bureau of Epidemiology, when Mike walked up. Unexpectedly, he greeted me in French, "Ça va"?. Remembering his Parisian birth, I replied in kind, then asked how he had come to be born in France. His father had worked there at the Rockefeller's European office, he said, continuing on with a few remembrances about the man. I had vaguely remembered hearing about his famous father, but Mike's recollections were about a beloved "Dad," not a famous man. It was some years later that I learned who Alan Gregg was—a legendary Rockefeller Foundation official who made a tremendous and lasting impact on biomedical research—but I never forgot the touching sense of fondness and reverence with which Mike spoke about his dad that day.

At age 78, Mike Gregg died too young and too quickly for many to grasp the meaning and consequences of his passing. His touch was so light that it is difficult to comprehend the breadth and depth of his legacy or to measure his influence on epidemiology and American public health. He would surely take quiet pride in being warmly remembered as the quintessential epidemiologist by friends and colleagues around the world. But I imagine he would be prouder still to know that his gardening was a success, that the garden still flourishes, and that it is now tended by those who learned from him and will one day share his secrets with others.

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Photo Quiz

Who is this man and what did he accomplish?

Here is a clue. He said: "For if medicine is really to accomplish its great task, it must intervene in political and social life. It must point out the hindrances that impede the normal social functioning of vital processes, and effect their removal."

Is he:
A) Robert Koch
B) Charles Nicolle
C) Louis Pasteur
D) Rudolf Virchow
E) Max von Pettenkofer

Decide first. Then turn the page.

Rudolf Virchow

Myron Schultz

This is a photograph of Rudolph Virchow (1821–1902). Virchow was one of the 19th century's foremost leaders in medicine and pathology. He was also a public health activist, social reformer, politician, and anthropologist.

Virchow was the only child of a farmer and city treasurer in Schivelbein, Germany. He had a strong interest in natural science. In 1839, he received a scholarship from the Prussian Military Academy, where he was given the opportunity to study medicine in preparation for a career as an army physician. He studied medicine in Berlin and then taught there for the most of his life, with interludes in Silesia and Würzberg. In 1847, he and a colleague, Benino Reinhardt, founded the Archiv für Pathologische Anatomie und Physiologie (now known as "Virchow's Archives"), which still survives as a leading journal of pathology. He encouraged his students to use microscopes and "think microscopically." Virchow had a major impact on medical education in Germany. He taught several persons who became famous scientists in Germany, including Edwin Klebs, Ernst Haekel, and Adolf Kussmaul. He also taught William Welch and William Osler, 2 of the 4 famous physicians who founded Johns Hopkins Hospital.

Virchow's greatest accomplishment was his observation that a whole organism does not get sick—only certain cells or groups of cells. In 1855, at the age of 34, he published his now famous aphorism "*omnis cellula e cellula*" ("every cell stems from another cell"). With this approach Virchow launched the field of cellular pathology. He stated that all diseases involve changes in normal cells, that is, all pathology ultimately is cellular pathology. This insight led to major progress in the practice of medicine. It meant that disease entities could be defined much more sharply. Diseases could be characterized not merely by a group of clinical symptoms but by typical anatomic changes. Pathologic anatomy, in addition to its great scientific merit, had tremendous practical consequences. If the physician was able to find out what anatomic changes had occurred in a patient, he could make a much more accurate diagnosis of the disease than he could in the past. This also empowered physicians to give more precise treatment and prognosis. In many of his speeches Virchow stated that the practice of medicine in Germany should shift away from being a largely theoretical activity. He advocated for the study of microscopic pathological anatomy, for research to be performed by physicians, the importance of making systematic clinical observations, and the performance of animal experimentations.

Virchow's many discoveries include finding cells in bone and connective tissue and describing substances such as myelin. He was the first person to recognize leukemia. He was also the first person to explain the mechanism of pulmonary thromboembolism. He documented that blood clots in the pulmonary artery can originate from venous thrombi. While Virchow, in Germany, was developing the new science of cellular pathology, Louis Pasteur, in France, was developing the new science of bacteriology. Virchow fought the germ theory of Pasteur. He believed that a diseased tissue was caused by a breakdown of order within cells and not from an invasion of a foreign organism. We know today that Virchow and Pasteur were both correct in their theories on the causality of disease.

Virchow noted the link between diseases of humans and animals and coined the term "zoonosis" to indicate the infectious diseases links between animal and human health. In addition to his groundbreaking work in cellular pathology he created the field of comparative pathology. Yet, Virchow's concept of "One Medicine," was not uniformly appreciated during his lifetime.

In 1848, Virchow served on a commission to investigate an epidemic of typhus, for which he wrote a penetrating report that criticized the social conditions that fostered the spread of the disease. He had already established a reputation as a crusading social reformer, and this report consolidated that reputation. He has since been identified as much with what came to be called "social medicine" as with his primary specialty of pathology.

Virchow was an outspoken advocate for public health. His writings and teachings are full of observations and rec-

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ommendations about ways to improve people's health by improving their economic and social conditions. He entered politics, serving in the German Reichstag (1880–1893), while also directing the Pathological Institute in Berlin. He helped to shape the healthcare reforms introduced in Germany during the administration of Otto von Bismarck. His prolific writings, while mainly on topics of pathology, included many essays and addresses on social medicine and public health.

Among Virchow's many interests was helminthology. He described the life cycle of the roundworm *Trichinella spiralis* in swine and its zoonotic consequences. He was opposed to Bismarck's excessive military budget, which angered Bismarck sufficiently to challenge Virchow to a duel. Virchow, being entitled to choose the weapons, chose 2 pork sausages: a cooked sausage for himself and an uncooked one, loaded with *Trichinella* larvae, for Bismark. Bismarck, the Iron Chancellor, declined the proposition as too risky.

Virchow also contributed substantially to the fields of anthropology, paleontology, and archeology. It should be noted that even men of great accomplishment, like Virchow, are fallible. Virchow believed that the Neanderthal man was a member of the modern species *Homo sapiens*, whose deformations were caused by rickets in childhood and arthritis later in life, with the flattened skull due to powerful blows to the head. Subsequent discoveries and research showed that the Neanderthals are, indeed, ancient.

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Tularemia in a Park, Philadelphia, Pennsylvania

To the Editor: Tularemia is a bacterial zoonosis caused by the gram-negative, nonmotile coccobacillus *Francisella tularensis,* which is endemic in lagomorphs in North America (*1*,*2*). Tularemia is considered a possible biological weapon of terrorism (Centers for Disease Control and Prevention [CDC] category A) because of its high infectivity, ease of dissemination, and considerable ability to cause illness and death in humans (*3*). The BioWatch Program monitors the environment in urban areas throughout the United States for *F. tularensis* and other potential bioterrorism agents. The epidemiology of many of these pathogens in urban ecosystems is not well understood; reservoirs may not be known or suspected, which leads to an inability to differentiate natural infection from a bioterrorism event. We describe a cluster of tularemia infections (in the absence of identified human illness or environmental detection) in feral rabbits found in a 0.5-km^2 area of a large city park in Philadelphia, Pennsylvania, USA.

During the spring and summer of 2006, a total of 14 eastern cottontail rabbits (*Sylvilagus floridanus*) and 2 woodchucks (*Marmota monax*) were found dead or trapped and euthanized (2 rabbits only) at a zoological park. The animals were necropsied, and specimens of liver and spleen were sent to the Pennsylvania Bureau of Laboratories (BOL) for *F. tularensis* culture and PCR. Two years earlier, in the spring of 2004, a single rabbit found dead at this same location had tested positive for *F. tularensis*; PCR and culture identified the organism in liver and spleen. Of the 14 rabbits submitted in 2006 for *F. tularensis* testing, 6 were positive (collection dates ranged from March through August). Five of these were positive by PCR and culture, and 1 was positive by PCR alone; *F. tularensis* was identified only in animals found dead. The 2 woodchucks tested negative by PCR and culture. The 2004 isolate and 2006 isolates were identified by CDC as type A *F. tularensis* and were found genetically identical by pulsed-field gel electrophoresis.

These additional 2006 positive findings triggered efforts to use available resources to identify other tularemia sources: the Philadelphia Department of Public Health (PDPH) heightened surveillance for tularemia by requesting that other city agencies and wildlife rehabilitation centers report and submit for testing any mammals found dead from unknown causes. (City agencies reported a few larger mammals, e.g., groundhogs and raccoons, dead from trauma; these animals were not tested.) The zoological park continued routine illness monitoring of collection animals, animals on grounds, and staff. In addition, during October 2006 and March 2007, the PDPH collected ticks on the outskirts of a heavily wooded area with frequent foot traffic ≈1.5 miles from the site where the rabbits were found dead. (The specific tick collection method involved dragging a white cotton bath towel along the edge of a wooded area; this activity took place during the hours of 10:00 AM–2:00 PM Other tick-dragging attempts during August 2007, on the outskirts of a heavily wooded area ≈0.5 miles away that was accessible to foot traffic but across the river from the zoological park, yielded no results.) A total of ≈30 deer ticks (*Ixodes scapularis*, which are not a known vector for tularemia) were collected each month; no other species were identified. These tick specimens were submitted to BOL for *F. tularensis* testing by PCR and culture. During November and December 2006, 5 crayfish (*Procambarus acutus acutus*, cited as a possible reservoir for type B tularemia by Anda et al.) (*4*), were trapped from a pond near the site where the rabbits were found dead and submitted to BOL for *F. tularensis* testing by PCR and culture. None of these readily available surveillance activities resulted in identifying tularemia except in the rabbits found dead in the zoological park. Additionally, no cases of human tularemia were reported to PDPH during this period, despite distribution of a health alert to medical providers to heighten clinical suspicion for the disease. Furthermore, the organism was not detected by routine environmental monitoring of air samples by the city's BioWatch sensors.

Even though this limited investigation failed to identify additional *F. tularensis* infections in humans and in any of the animals and ticks tested, the cluster of infections in rabbits in Philadelphia indicates that *F. tularensis* is present in the environment in sufficient numbers to cause a noteworthy die-off of animals (i.e., 6 rabbits in a 0.5-square-mile area over a 5-month period). Environmental biomonitors in other metropolitan areas have been triggered by reported detection of tularemia on at least 2 occasions in the past 5 years—Houston in 2003 and the Washington, DC, National Mall in 2005 (*5*).

This investigation underscores that *F. tularensis* identification in the environment requires a systematic approach beyond environmental biomonitoring, random convenience sampling, and increased passive surveillance for human cases. Standard methods such as serologic studies of wildlife may not be available to resource-limited urban institutions. Possible strategies such as the collection of ticks, specifically the American dog tick, *Dermacentor variabilis* (a known vector for tularemia), from animals upon entry into urban animal shelters and mapping of areas where the animals were found need to be considered if resources are limited. Additional research is necessary to understand the occurrence of disease caused by *F. tularensis* in humans and animals, especially in urban environments (*6*).

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Genotyping of *Orientia tsutsugamushi* from Humans with Scrub Typhus, Laos

To the Editor: Rickettsial diseases have been only recently identified as underrecognized but important causes of fever of unknown origin in Laos. In 2006, 63 (14.8%) of 427 adults with negative blood cultures admitted to Mahosot Hospital in Vientiane had scrub typhus, an infection caused by *Orientia tsutsugamushi* and transmitted by the bite of larval trombiculid mites (*1*). *O. tsutsugamushi* is characterized by a wide antigenic diversity, and isolates are conventionally classified on the basis of reactivity with hyperimmune serum against prototype strains (e.g., Karp, Kato, Gilliam, Kawasaki, Kuroki, or Shimogoshi). The 4 hypervariable regions within the 56-kDa type-specific antigen of *O. tsutsugamushi,* which is located on the outer membrane surface, are considered to play an essential role in type strain assignment (*2*).

In the Lao study (*1*), in addition to acute-phase serum samples, a 5-mL blood sample anticoagulated with EDTA was collected at admission from all patients. After centrifugation, buffy coat of the serum sample was removed and stored at –80°C (*1*). DNA was extracted from buffy coat samples of 63 patients whose conditions were diagnosed by imunofluorescence assay as scrub typhus (*3*). Two amplification reactions were performed, a real-time quantitative PCR with a probe targeting the *O. tsutsugamushi* 47-kDa outer membrane protein gene with appropriate primers and probes (*4*) and a standard PCR targeting a 372-nt fragment of the 56-kDa protein gene (*3*).

Buffy coat samples from 11 (17.5%) patients were positive for *O. tsutsugamushi* in the real-time quantitative PCR and 56-kDa antigen gene PCR (Table). All 11 patients were from Vientiane or Vientiane Province. PCR products for the 56-kDa gene fragments were purified and sequenced as described (*3*). Comparison (*3*,*5*) of amplicons for the 11 patients with each other and with GenBank sequences identified 6 genotypes. Percentages of nucleotide sequence similarity with other sequences available in GenBank ranged from 95.9% to 100% (Table). Interpretation of our results was also supported by recent phylogenetic studies that compared sequences of the entire 56-kDa type-specific antigen gene of isolates from Thailand (*6*). LaoUF238 and LaoUF220 genotypes clustered with those of strains related to the Karp serotype, and LaoUF136 and LaoUF187 clustered with genotypes of strains related to the Gilliam serotype (*2*). Other genotypes found in this study were grouped in 2 clusters that contained genotypes identified in Thailand (*5*) and Taiwan (*7*) that have not been linked to a reference serotype (Table).

Detection of *O. tsutsugamushi* in humans in Laos provides useful information on genotypes prevalent in this country. Our results were confirmed by using 2 target genes in 2 PCRs. No differences were found between the number of days of fever in 11 PCR-positive patients and number of days of fever in 52 PCR-negative patients. However, the PCR-negative patients may not have had bacteremia at the time of sample collection.

Diversity of *O. tsutsugamushi* genotypes found in Laos includes

genotypes closely related to genotypes from Thailand and Taiwan. This diversity raises doubt about usual concepts because it has been thought that *O. tsutsugamushi* genotypes are restricted to specific geographic areas and to specific mite vectors (*8*). Furthermore, these results might have clinical repercussions because sequence variations within the 56-kDa protein gene correlate with antigenic diversity of genotypes of *O. tsutsugamushi*. This finding is supported by data for sequences of the entire 56-kDa gene of different isolates (*6*) and for monoclonal and human and animal polyclonal antibodies used to map antigenic differences among isolates with known sequence variations (*9*).

Although our data are preliminary, diversity of nucleotide sequences of the 56-kDa protein–encoding gene in isolates from Laos might limit sensitivity and specificity of serologic methods. A recent study showed that addition of a serotype to the panel of *O. tsutsugamushi* antigens used for testing improved sensitivity of antibody detection in patients in Thailand (*10*). We demonstrated that, in analysis of sera in the diagnosis of scrub typhus contracted in Laos, antigen pools should contain at least Karp and Gilliam strain antigens. Furthermore, new genotypes identified in patients in Laos might be related to previously unrecognized type strains. However, cross-reactivity with Gilliam, Kato, and Kawasaki serotypes enabled serologic diagnosis in the initial study, including 1 patient infected with a Karp-related bacteria (*1*).

Phylogenetic studies based on larger fragments of sequences of the 56-kDa protein–encoding gene and of other genes of *O. tsutsugamushi* would help to better characterize the new genotypes identified in our study and their relationship with known serotypes. Expanding the panel of antigens used to test patients suspected of having scrub typhus to take into account local antigenic diversity would improve sensitivity of serologic assays for this disease.

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Table. Serologic results for 11 patients from Laos positive by real-time quantitative PCR for the 47-kDa outer membrane protein and a PCR for a fragment of the 56-kDa protein–encoding gene of *Orientia tsutsugamushi**

Patient	IgG/IgM titers, acute phase–late phase			GenBank accession no. of 56-kDa–amplified gene fragment	Highest % identity with GenBank sequences (reference)
	Gilliam	Kato	Kawasaki		
LaoUF74	64/1,048–64/1,048	128/256–128/256	128/256–128/256	EU168798	These 4 genotypes showed 100% identity with each other and 96.6% with strain BB23 from Thailand (*5*)
LaoUF317	2,048/1,024–ND	128/1,024–ND	2,048/1,024–ND	EU168799	
LaoUF366	2,048/512–2,048/512	128/32–128/32	2,048/512–2,048/512	EU168797	
LaoUF396	256/1,024–256/1,024	128/64–128/64	256/1,024–256/1,024	EU168796	
LaoUF220	2,048/512–2,048/1,024	2,048/512–2,048/512	1,024/256–2,048/512	EU168801	These 2 genotypes showed 100% identity with UT150 and UT332, which are related to Karp serotype isolates (*6*), BB29 from patients in Thailand (*5*), and TWyU81 from chiggers in Taiwan (*7*)
LaoUF244	1,024/128–ND	256/64–ND	128/64–ND	EU168800	
LaoUF83	128/1,024–ND	0/1,024–ND	0/128–ND	EU168795	95.9% with BB23 from Thailand (*5*)
LaoUF136	2,048/1,024–2,048/1,024	2,048/1,024–2,048/1,024	2,048/1,024–2,048/1,024	EU168804	99.5% with UT144, UT125, and UT196 from Thailand, which are related to Gilliam serotype isolates (*6*)
LaoUF186	128/1,024–2,048/1,024	128/1,024–2,048/1,024	128/1,024–2,048/1,024	EU168805	100% with BB23 from Thailand (*5*)
LaoUF187	256/1,024–256/1,024	256/0–256/0	0/0–256/1,024	EU168803	100% with UT144, UT125, and UT196 from Thailand (*6*)
LaoUF238	2,048/1,024–2,048/1,024	2,048/1,024–2,048/1,024	0/512–256/512	EU168802	100% with UT219, UT395, UT221, and UT213, which are related to Karp serotype–related isolates (*7*), and FPW2031 from Thailand (*6*)

*Ig, immunoglobulin; ND, not done. Specific microimunofluorescence assay (IFA) was performed in Marseille, France, by using whole-cell antigens of *O. tsutsugamushi* serotypes Kato, Gilliam, and Kawasaki. Serotype Karp was not available for serologic assays. An IFA result was positive if 1) titers were >128 for IgG and >64 for IgM, 2) seroconversion was observed in a convalescent-phase serum sample when an acute-phase serum sample was negative, or 3) there was a $\geq$4-fold increase in titers between acute- and convalescent-phase serum samples (0 indicates a titer <25).

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Clindamycin-Resistant Clone of *Clostridium difficile* PCR Ribotype 027, Europe

To the Editor: Since 2003, outbreaks of *Clostridium difficile*–associated disease (CDAD) associated with the emergence of a hypervirulent strain have been reported worldwide (*1*,*2*; www.eurosurveillance.org/em/v12n06/1206-221.asp). This strain has been associated with increased disease severity and attributable mortality. Patients infected with *C. difficile* 027 fail to respond to metronidazole therapy (*1*). Several typing methods have been applied to further characterize *C. difficile* PCR ribotype-027, including pulsed-field gel electrophoresis (PFGE) (North American pulsed field type 1) and restriction enzyme analysis (REA) (BI). PFGE and REA are widely used in the United States; PCR ribotyping is more commonly used throughout Europe. More recently, 2 multiple-locus variable-number tandem-repeat analysis (MLVA) protocols have been applied to type *C. difficile*, and these proved more discriminatory compared to other methods (*3*,*4*). Furthermore, MLVA can subgroup geographically diverse 027 isolates (G. Killgore et al., unpub data) as well as 027 isolates that are common to 1 institution (*5*).

We reported a case of *C. difficile* PCR 027 in Ireland, where the isolate had an identical antibiogram profile compared with those strains reported across Europe (*6*,*7*) (i.e., resistant to fluoroquinolones and erythromycin, susceptible to clindamycin). We have subsequently identified *C. difficile* 027 in 6 more healthcare settings. To date >100 Irish *C. difficile* 027 isolates have been characterized by analysis of their antibiogram profiles, toxinotyping, and 16S–23S rDNA PCR ribotyping. All *C. difficile* 027 isolates were resistant to moxifloxaxin, gatifloxacin,

ciprofloxacin (MIC >32 mg/L), and erythromycin (MIC >256 mg/L) but susceptible to metronidazole (MIC 0.25 mg/L) and vancomycin (MIC >0.5 mg/L). Clindamycin susceptibility varied between isolates from unrelated institutions. Isolates from 2 healthcare settings were susceptible to clindamycin (n = 11: MIC_{90} 4 mg/L). However, clindamycin-resistant PCR 027 isolates (n = 96: MIC_{90} >256 mg/L) were identified in the other 5 healthcare institutions. All clindamycin-resistant PCR 027 isolates were positive for the *erm*B gene, encoding the macrolide-lincosamide-streptogramin-B genotype.

A subset of clindamycin-sensitive and -resistant Irish 027 strains isolated throughout 2006 (n = 22) were further characterized by using a recently described MLVA protocol (*3*). Six clindamycin-susceptible isolates were selected from 2 healthcare settings. One hospital conducted active routine laboratory surveillance and molecular genotyping (n = 3). The second hospital submitted only random isolates (n = 3) for typing during a *C. difficile* outbreak. Sixteen clindamycin-resistant PCR 027 isolates were also included in the MLVA. Resistant isolates were selected from 5 healthcare settings. These included isolates from 2 *C. difficile* outbreaks with ongoing laboratory surveillance (n = 5, n = 6, respectively); a third hospital with ongoing laboratory surveillance (n = 3) and 2 hospitals that each submitted fecal samples from patients with severe cases of *C. difficile* disease (n = 1). The Stoke-Mandeville control strain R20291 was included for comparison.

MLVA determined that all strains within the clindamycin-resistant cluster were closely related and were single- or double-locus variants with a maximum 5 summed tandem-repeat difference (STRD). In contrast, the closest relationship between the clindamycin-resistant and the clindamycin-sensitive clusters was a triple-locus variant with an STRD of 17. The nonrelated reference strain of the Stoke-Mandeville outbreak (R20291) differed considerably from all Irish isolates but was more related to the clindamycin-sensitive cluster than to the clindamycin-resistant cluster (Figure). We thus linked a defined genetic marker with the clindamycin-resistant phenotype in *C. difficile* PCR-027. MLVA could clearly differentiate clindamycin-resistant and -susceptible isolates from the same geographic region and subgrouped them into 2 distinct clusters (Figure).

Although high-level resistance to fluoroquinolone antimicrobial agents has been well documented in PCR 027 (*1*,6), resistance to clindamycin is rare. Subsequently, clindamycin has been considered as a "protective" antimicrobial agent for the development of CDAD in an epidemiologic survey in the Netherlands (*8*). Currently, resistance to this agent in NAP 1/PCR 027 has been restricted to the United States. McDonald and colleagues reported that 19 (79%) of 24 NAP 1 isolates were classified as less susceptible (MIC 4 mg/L) or resistant (MIC 8 mg/L) to clindamycin when Clinical and Laboratory Standards Institute criteria were used (*2*). Unfortunately, MIC values were not reported, and the corresponding resistance genes were not investigated. In contrast, Canadian studies to date have not reported clindamycin resistance in this strain type. The MIC_{90} of Canadian NAP 1 isolates for clindamycin was 4 mg/L (*9*,*10*). Although outbreaks and sporadic cases of PCR 027 have been identified in several European countries, to date no clindamycin-resistant clone has been reported.

Detection of clindamcyin-resistant *C. difficile* PCR 027 strains is an important and worrying development. Resistance to this antimicrobal agent increases the risk for CDAD in patients, and its use may be an important factor contributing to the persistence and spread of PCR 027. A similar feature has already been observed when fluoroquinolones and cephalosporins are prescribed. Clindamcyin-resistant PCR

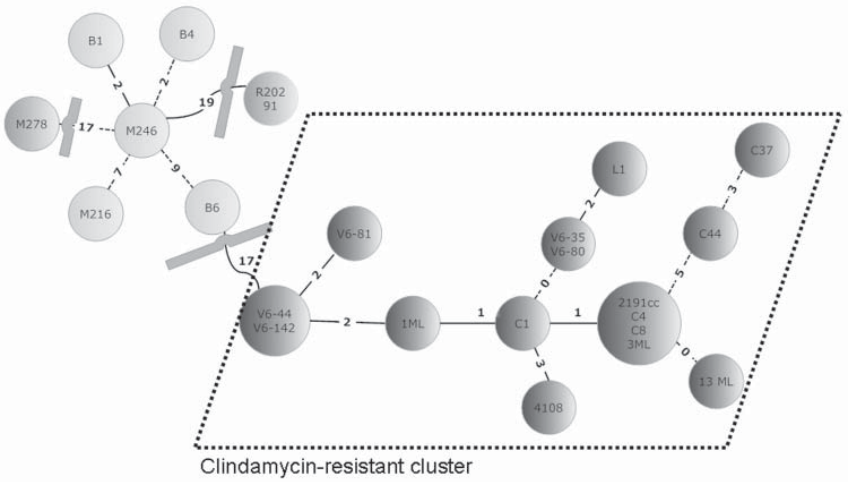

Figure. Minimal spanning tree of 23 *Clostridium difficile* isolates. In the circles, the individual isolates are mentioned. The numbers between the circles represent the summed tandem repeat differences (STRDs) between multiple-locus variable-number tandem-repeat analysis types. Straight lines represent single-locus variants, dashed lines double-locus variants. Curved lines represent triple-locus variants. Two related clusters can be discriminated: the light gray cluster (isolates B1, B4, M246, B6, and M216) and the cluster within dotted lines (isolates V6–44, V6–142, V6–81, 1ML, C1, 4108, V6–35, V6–80, L1, 2191cc, C4, C8, 3ML, C44, C37, and 13ML) The isolates in the light gray cluster are sensitive to clindamycin; isolates in the cluster surrounded by dotted lines are resistant. Two isolates (M278 and R20291) did not belong to a cluster but were more related to the sensitive cluster than to the resistant cluster. Genetically related clusters were defined by an STRD ≤10.

027 probably reflects the emergence of a new clone because MLVA clearly differentiates between clindamycin-susceptible and -resistant isolates.

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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.

Increasing Incidence of *Clostridium difficile*–associated Disease, Singapore

To the Editor: *Clostridium difficile*–associated disease (CDAD) has increased in incidence across North America and Europe (*1*). Recent reports document the emergence of an epidemic strain of *C. difficile*, NAP1/BI/027, associated with increased virulence (*2*,*3*). However, less information is available regarding CDAD epidemiology in Asia. We examined the incidence of *C. difficile* among hospitalized patients in Singapore from 2001 through 2006 and conducted a case–control study to evaluate risk factors for testing positive for *C. difficile* toxin (CDT) in our population.

Tan Tock Seng Hospital (TTSH) is a 1,200-bed, acute-care general hospital in Singapore that serves an urban population of 4 million. We calculated CDAD incidence using the number of patients testing positive for CDT per 10,000 patient days from 2001 through 2006. We used this calculation because CDT testing would have been ordered for clinical indications. CDT testing was performed by using the same ELISA (Premier Toxins A&B; Meridian Bioscience, Inc., Cincinnati, OH, USA) throughout the entire period of investigation.

Case-patients and controls were selected from patients hospitalized at TTSH from January 1 through December 31, 2004. Microbiology laboratory records were used to define 3 groups. Case-patients were defined as CDT-positive inpatients (group 1). Two sets of negative controls were defined: the first (group 2) consisted of patients who tested negative for CDT. However, because false-negatives could nullify differences between groups 1 and 2, we defined a second set of negative controls (group 3) from among 18,000 inpatients not tested for CDT.

Seventy patients were selected from each group by using a random number generator program. Forty-eight, 61, and 56 records were retrieved for groups 1, 2, and 3, respectively. Standardized forms were used to extract data from hospital medical records. Demographic data and hospitalization details, including ward type (6-bed, 4-bed, or single room), were collected. We examined antimicrobial drug use within 30 days of admission and within 30 days of CDT testing. We also evaluated the use of proton pump inhibitors (PPIs) and H2 blockers because these have been reported as risk factors (*1*,*4*–*6*). Outcomes ascertained included the time to discharge after CDT testing, and death within 30 days after CDT testing. The study was approved by the institutional ethics review board.

Characteristics of case-patients and controls were compared by using the Wilcoxon rank sum test for continuous variables and the Fisher exact test for categorical variables. Variables significantly associated with CDT in the univariate analysis were selected for inclusion in the multivariate regression model. A 2-sided p value <0.05 was considered significant for all comparisons.

CDAD incidence rose sharply from 1.49 cases per 10,000 patient-days in 2001 to 6.64 cases per 10,000 patient-days in 2006 (Figure). During the same period, the percentage of CDT-positive samples increased from 7% to 11%, while the number of samples tested increased from 906 to 3,508.

Comparing group 1 (CDT positive) with group 2 (CDT negative), a CDT-positive result was more likely to occur in those with prolonged hospital admissions (≥14 days) than in those who had shorter hospital stays (<7 days; odds ratio [OR] 2.59, 95% confidence interval [CI] 1.01–6.63). Of the 19 CDT-positive patients on PPIs and the 34 CDT-negative patients on PPIs, the median exposures were 14 and 7 days, respectively (p = 0.01). In multivariate analysis, exposure to broad-spectrum antimicrobial drugs was a borderline significant risk factor (adjusted OR 2.24, 95% CI 1.00–5.02, p = 0.05).

When group 1 (CDT positive) was compared with group 3 (not tested for CDT), quinolones (OR 6.67, 95% CI 1.85–24.03), anti-anaerobic antimicrobial agents (OR 7.29, 95% CI 2.39–22.26), and stay in a 6-bed ward (OR 3.15, 95% CI 1.01–9.82) were significant risk factors in multivariate analysis. Case-patients were more likely than controls to have a longer hospital stay after testing positive. The median hospital stay after CDT testing was 16 days for case-patients versus 11 days for controls (p = 0.03).

This study documents a 4-fold rise in CDAD incidence among hospitalized patients in Singapore from 2001 through 2006. The current incidence, 6.64 per 10,000 patient-days, is comparable to that reported by large hospitals in Canada (*7*), which indicates that CDAD has emerged as an important nosocomial infection in Singapore. This incidence rate, based on the number of patients (rather than the number of isolates) who had positive CDT test results, and the rise in sample positivity from 7% to 11% suggests that the higher rates are due to a true increased occurrence rather than merely more testing.

Possible factors driving the rise in CDAD include increased use of antimicrobial agents or changes in use patterns. The volume of quinolones and broad-spectrum antimicrobial drugs used at TTSH doubled between 2002 and 2005, consistent with other studies implicating quinolones as a risk factor in CDAD (*4*).

Rising incidence or virulence could herald the geographic spread of new *C. difficile* strains. Given the spread of NAP1/BI/027 strains in other parts of the world, this increased incidence in Singapore should heighten vigilance for the introduction of outbreak strains into Asia.

The findings from this study have implications for hospital management and infection control. Environmental contamination has been described as a mode of transmission (*1*). Potential crowding in 6-bed wards may increase spread of CDAD and may be particularly relevant in busy healthcare facili-

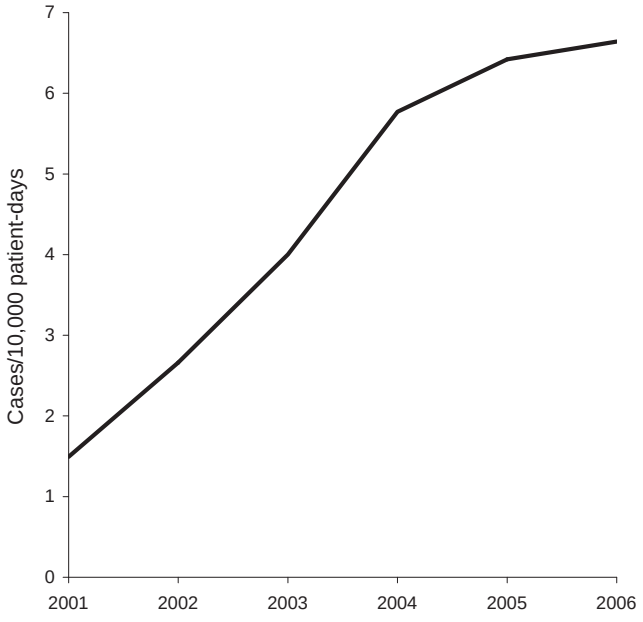

Figure. *Clostridium difficile*–associated disease incidence, Singapore, 2001–2006.

ties in Asia. CDAD is estimated to cost the healthcare system in the United States $3.2 billion annually (*8*). With longer hospitalization for persons after they test positive for CDT, as seen in our study, rising CDAD rates could increase hospital occupancy and result in excess healthcare expenditures.

CDAD in Asia is an emerging challenge that needs to be recognized. Its control will ultimately depend on priority being given to epidemiologic surveillance, infection control, and stewardship of antimicrobial agents.

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West Nile Virus in Golden Eagles, Spain, 2007

To the Editor: Although West Nile virus (WNV) has not been isolated in Spain, several recent studies provide evidence for its circulation in this country (*1*–*5*). We report isolation of WNV in Spain from 2 golden eagles (*Aquila chrysaetos*).

A captive-bred 2-year-old male golden eagle (GE-1) was released into the wild in central Spain. The bird's location was monitored daily by telemetry, and it remained within a radius of 100 km from its original release point. On September 15, 2007 (1 month after release), it was found moribund and was moved to a rehabilitation and captive breeding center for endangered raptors. Upon admission, the bird was in fair condition but debilitated and aggressive. It then became increasingly disorientated, showed a head tilt, and died 5 days after admission, despite intensive supportive care and treatment for secondary infections.

Eleven days after admission of GE-1, an adult male golden eagle (GE-2) and an adult female Bonelli's eagle (*Hieraaetus fasciatus* [BE-1]) living in pairs (with a golden eagle and a Bonelli's eagle, respectively) in enclosures were found disorientated, debilitated, and with impaired vision. Both birds where placed in isolation and received intensive supportive care; they slowly recovered. The respective pair of each bird (GE-3 and BE-2, respectively) remained asymptomatic. A magpie (MP-1) that had entered the golden eagle enclosure 5 days before admission of GE-1 was also placed in isolation, but remained healthy. After necropsy of GE-1, tissue samples (brain, kidney, and spleen) from this bird and oropharyngeal swabs from GE-2, BE-1, and MP-1 (obtained at day 11 after admission of GE-1) were subjected to virologic analysis.

Avian influenza and Newcastle disease were excluded by reverse transcription–PCR (RT-PCR) (*6*,*7*) of oropharyngeal and cloacal swabs from GE-1, GE-2, BE-1, and MP-1. Real-time RT-PCR specific for WNV (*8*) was conducted with brain, kidney, and spleen tissue homogenates from GE-1 and oropharyngeal swabs from GE-2, BE-1, and MP-1. All samples except that from MP-1 yielded specific WNV genome amplification products, which were confirmed after amplification and sequencing by using a previously described method (*9*).

Serum samples from clinically affected eagles (GE-1, GE-2, and BE-1), the magpie (MP-1), and the healthy Bonelli's eagle (BE-2) contained WNV-neutralizing antibodies detected by a virus neutralization test performed as described (*4*,*5*). A serum sample from GE-3 (asymptomatic) remained negative up to 74 days after admission of GE-1. Specificity of the neutralization test was assessed

by titration in parallel against a second, cross-reacting flavivirus (Usutu virus). Results showed that the highest titers were always obtained against homologous virus (WNV).

Virus isolation was conducted by placing filter-sterilized, clarified tissue homogenates (brain, kidney and spleen) from GE-1 and oropharyngeal swab eluate from GE-2 onto monolayers of BSR (baby hamster kidney) cells and Vero cells. The remaining 2 samples (oropharyngeal swabs from BE-1 and MP-1) were negative for virus. Isolates were identified by using real-time and conventional RT-PCR (*8*,*9*). WNV-specific cDNAs from the nonstructural protein 5–coding region of the genome (171 nt) were amplified by RT-PCR (*9*) from brain tissue of GE-1 (sample GE-1b), oropharyngeal swab of BE-1 (sample BE-1o), and first-passage infection supernatant of oropharyngeal swab from GE-2 (sample GE-2o). These samples were subjected to molecular analysis. Nucleotide sequences from the 3 samples were identical, except at 1 nt position in BE-1o (GenBank accession nos. EU486169 for GE-1b, EU486170 for GE-2o, and EU486171 for BE-1o). Phylogenetic analysis matched these isolates most closely with recent western Mediterranean WNV isolates within lineage 1a (Figure).

WNV was detected in 3 eagles of 2 species. The birds with the index and secondary cases had no direct contact. Transmission could have occurred through mosquito bites. The 2-year-old golden eagle died as a result of infection, and the 2 remaining infected eagles recovered. The 3 ill birds were potentially more susceptible because of stress (GE-1) or age (GE-2 and BE-1 were older birds). Serologic analysis detected WNV-specific antibodies in the affected birds and some contacts. Nucleotide sequence analysis showed high genetic identity among these new isolates, which cluster within lineage 1a of WNV.

Although information on WNV in Spain is scarce, its detection and relationship to the death of a raptor in the wild are of concern because many species of eagles, including the Spanish imperial eagle (*A. adalberti*), are endangered species. We recently found evidence of WNV infection in several Spanish imperial eagles sampled during 2001–2005 (*5*). Studies are ongoing to further characterize genetic and biologic properties of the new WNV isolates described to identify their genetic relationships with other WNV strains and to clarify the epidemiology of WNV in the study region.

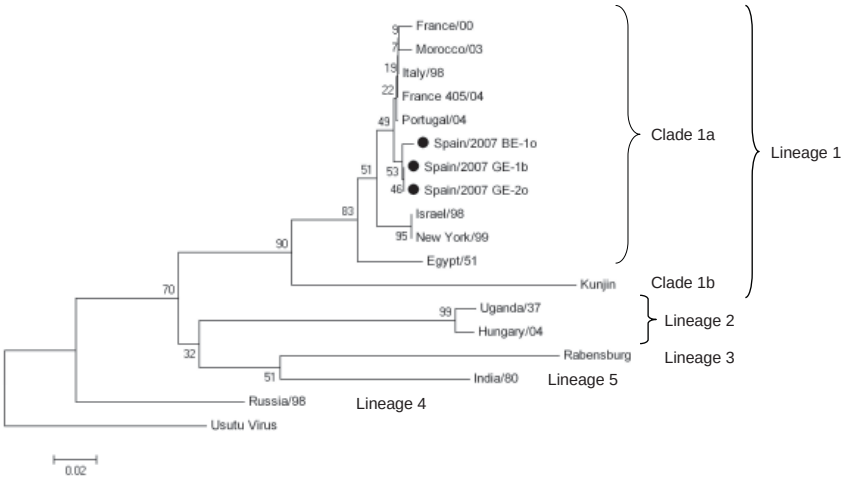

Figure. Phylogenetic tree of 18 partial nonstructural protein 5 West Nile virus nucleotide sequences (171 nt for each isolate, except 126 nt available for the Portugal/04 isolate) constructed with MEGA version 4 software (www.megasoftware.net). The optimal tree was inferred by using the neighbor-joining method. The percentage of successful bootstrap replicates (N = 1,000) is indicated at nodes. Evolutionary distances were computed with the Kimura 2-parameter method (with gamma correction). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. Branch lengths are proportional to the number of nucleotide changes (genetic distances). Scale bar shows number of base substitutions per site. Isolates sequenced in this study are indicated by solid circles. GenBank accession nos. are as follows: France/00 (AY268132), Morocco/03 (AY701413), Italy/98 (AF404757), France 405/04 (DQ786572), Portugal/04 (AJ965630), Israel/98 (AF481864), New York/99 (DQ211652), Egypt/51 (AF260968), Kunjin (D00246), Uganda/37 (M12294), Hungary/04 (DQ116961), Rabensburg (AY765264), India/80 (DQ256376), Russia/98 (AY277251), and Usutu virus (NC_006551) (outgroup).

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Merkel Cell Polyomavirus and Merkel Cell Carcinoma, France

To the Editor: Merkel cell carcinoma (MCC) is a primary cutaneous neuroendocrine tumor. This aggressive skin cancer is uncommon but increasing in frequency. During 1986–2001, incidence rate tripled; average annual increase was 8% (*1*). MCC shares epidemiologic features with Kaposi sarcoma, a malignant tumor associated with human herpesvirus 8 infection (*2*). In particular, MCC affects predominantly immunocompromised patients such as organ transplant recipients (*3*,*4*), patients with B-cell lymphoid tumors (*5*), and patients with AIDS (*6*). This similarity between MCC and Kaposi sarcoma may support the hypothesis of an infectious origin of MCC.

A new polyomavirus, provisionally named Merkel cell polyomavirus (MCPyV), has been recently identified in tumor tissue from patients with MCC. Furthermore, clonal integration of viral DNA within the tumor genome was observed in most of the cases (*7*). To assess the implication of MCPyV in MCC, we tested tumor biopsy samples collected from 9 patients with MCC. Patient median age was 65 years, and 2 patients were immunocompromised (patient 1 had a lymphoma, which was treated with rituximab; patient 7 had psoriatic rheumatism, which was treated with corticosteroids and methotrexate). As controls, biopsy samples from 15 patients with diverse proliferative or inflammatory skin or mucosa lesions were tested (Table).

DNA was extracted from fresh tissue samples by using the QIAamp DNA Mini Kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions. Paraffin was removed from previously formaldehyde-fixed, paraffin-embedded biopsy samples with xylene, and the samples were rehydrated with decreasing concentrations of ethanol. The extracts were tested for MCPyV DNA by PCR using 3 sets of primers initially described by Feng et al. (*7*) to target the predicted T-antigen (LT1 and LT3 primer pairs) and the viral capsid (VP1 primer pair) coding regions. Extracted DNA (5 μL) was added to 45 μL of the reaction mixture, which contained 5 μL 10× PCR buffer, 10 μL 5× Q-solution (QIAGEN), 2.5 mmol/L $MgCl_2$, 200 μmol/L each dNTP, 2.5 units Taq DNA polymerase (QIAGEN), and 15 pmol of each primer. Touchdown PCR conditions were as follows: 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s; annealing at 61°C (10 cycles), 59°C (10 cycles), and 57°C (15 cycles) for 30 s; extension at 72°C for 1 min; and a final extension step at 72°C for 10 min. Amplification products were subjected to electrophoresis in a 2% agarose, 1× Tris-borate-EDTA gel stained with ethidium bromide and examined under UV light. The sizes of the fragments amplified with the LT1, LT3, and VP1 primers pairs were 439, 308, and 351 bp, respectively. A negative control was included in each experiment; positive samples were confirmed by analyzing a second stored sample aliquot, and the amplified fragments were sequenced by using the same primers used for the amplification. The sequences were submitted to GenBank under accession numbers AM992895–AM992906. Total DNA level in sample extracts was measured by using the LightCycler control DNA kit targeting the β-globin gene (Roche

Diagnostics, Meylan, France).

MCPyV sequences were detected in 8 of the 9 patient samples and in none of the control samples (Table). Results for all 8 patients were positive with the LT3 primer pair, whereas they were positive for only 5 with the VP1 primer pair and only 1 with the LT1 primer pair (Table). Because the LT1, VP1, and LT3 primer pairs generate the longer, intermediate, and shorter DNA fragments, respectively, the difference in sensitivity could result from the deleterious effect of formaldehyde fixation on DNA; this effect would increase with the length of the fragment to be amplified. The negative result obtained for patient 8 might suggest that some MCC patients are not infected with MCPyV. This explanation is in accordance with the findings of Feng et al., who reported 80% prevalence of MCPyV in patients with MCC (*7*). Nevertheless, the single negative result observed in our study might alternatively be explained by insufficient tissue or by DNA degradation through the fixation and embedding process. Indeed, the level of β-globin gene DNA was much lower in the sample from this patient (441 pg/μL) than in samples from the other patients (median 13,500 pg/μL, interquartile range 8,902–19,750 pg/μL).

As observed with human papillomaviruses, a gene disruption caused by viral DNA integration into the host genome might be an alternative hypothesis to explain the lack of amplification of an MCPyV genome region (*8*). Sequences of the amplified PCR product were 99%–100% identical to those reported by Feng et al. (*7*), which indicates that this virus is genetically stable.

In summary, we detected MCPyV DNA sequences in 8 of 9 tumor samples from patients with MCC but in none of 15 control samples. Our results confirm the likely association of MCPyV with MCC. The epidemiologic characteristics as well as the carcinogenic role played by this newly discovered virus need to be more thoroughly investigated.

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Table. Merkel cell polyomavirus from 9 patients with Merkel cell carcinoma and 15 control patients, France*

Patient no.	Age, y	Sex	Diagnosis	Sample	PCR primer pair		
					LT1	LT3	VP1
1	57	M	MCC, primary	Fresh biopsy, buttock	+	+	+
2	61	F	MCC, primary	FFPE biopsy, buttock	–	+	–
3	57	F	MCC, primary	FFPE biopsy, lower eyelid	–	+	+
4	82	F	MCC, primary	FFPE biopsy, cheek	–	+	–
5	78	M	MCC, metastatic	Fresh biopsy, jugular lymph node	–	+	+
6	80	F	MCC, primary	Fresh biopsy, upper eyelid	–	+	+
7	65	M	MCC, primary	FFPE biopsy, temple	–	+	–
8	81	M	MCC, primary	FFPE biopsy, forearm	–	–	–
9	60	M	MCC, primary	FFPE biopsy, forearm	–	+	+
10	61	M	Hyperkeratosis	Fresh biopsy, foot	–	–	–
11	49	M	Seborrheic keratosis, penis	Fresh biopsy, penis shaft	–	–	–
12	60	M	Nonspecific lesion, esophagus	Fresh biopsy, lesion	–	–	–
13	40	M	Nasal papilloma	Fresh biopsy, papilloma	–	–	–
14	58	M	Anal condylomas	Fresh biopsy, condyloma	–	–	–
15	44	M	Epidermodysplasia verruciformis	Fresh biopsy, skin	–	–	–
16	19	M	Cutaneous warts	Fresh biopsy, wart	–	–	–
17	57	F	Cutaneous warts	Fresh biopsy, wart	–	–	–
18	64	F	Cutaneous nodule	Fresh biopsy, skin	–	–	–
19	6	F	Pharyngeal papillomatosis	Fresh biopsy, pharynx	–	–	–
20	27	M	Vocal cord polyp	Fresh biopsy, polyp	–	–	–
21	58	M	Lichen	Fresh biopsy, skin	–	–	–
22	63	M	Skin inflammation	Fresh biopsy, skin	–	–	–
23	72	M	Skin inflammation	Fresh biopsy, skin	–	–	–
24	57	M	Skin inflammation	Fresh biopsy, skin	–	–	–

*MCC, Merkel cell carcinoma; +, positive Merkel cell polyomavirus PCR amplification; FFPE, formaldehyde-fixed paraffin-embedded; –, negative Merkel cell polyomavirus PCR amplification.

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Worldwide Prevalence of Head Lice

To the Editor: Pediculosis capitis has been well-known since antiquity (*1*). Human infestation can result in psychological frustration for parents and children (*2*); furthermore, preventive and therapeutic practices, such as head shaving and the "no-nit" policy of excluding infected children from school, can also induce social stress.

We sought to synthesize the available evidence regarding the worldwide prevalence of lice infestation in the 21st century by conducting a literature search of PubMed and Scopus databases in which we searched for the term *pediculosis*. We also searched Google for the terms *head lice/pediculosis capitis* and individual country names and evaluated references of the articles and reports retrieved through this search. Eligible studies were archived from January 1, 2000, to January 18, 2008.

We retrieved 55 studies (online Technical Appendix, available from www.cdc.gov/EID/content/14/9/1493-Techapp.pdf). Most studies referred to schoolchildren, but some involved refugees, urban slums, child labor, jails, orphanages, and fishing communities.

Most studies had been conducted in Asia; Turkey was overrepresented. Prevalence varied from 0.7% to 59% and was higher in girls and women. Of the 29 studies, 24 involved schoolchildren; the other studies involved refugee children, child laborers, the general population, street children, jail inmates, and children accompanying their mothers in prison.

In Europe, prevalence varied from 0.48% to 22.4%. However, 1 study reported a much higher annual incidence (37.4%) in England (*3*). A study in the Ukraine showed increasing adult representation in the overall affected population (*4*). Six studies involved schoolchildren; the remaining studies involved refugees, homeless persons, and the general population.

Data from Africa, with the exception of 1 study in South Africa, were derived from Egypt. Prevalence varied from 0% to 58.9% and was higher in females. The study in South Africa (*5*) challenges the generally accepted concept that head lice infestation refers to lower socioeconomic status; of 2 schools, 1 in a low socioeconomic status area, populated by black students only, and the other in a high socioeconomic status area, populated by students of various races, head lice infestation was found only in the second school, solely among white pupils. Of 6 studies in Egypt, 4 involved diverse populations: urban poor preschool children, orphanage children, and the general population.

Most studies in the Americas were conducted in Brazil, although we also found data from the United States, Cuba, and Argentina. Prevalence varied from 3.6% to 61.4% and was higher in females. Of 7 studies, 4 involved populations other than schoolchildren to some extent: urban slum residents, fishing community residents, adolescents and adults sampled randomly from the general population, elderly nursing home residents, and persons living with repeatedly infested children. A recent study in Brazil (*6*) noted that prevalence rates determined by visual inspection are twice that of rates determined by hair analysis.

Only 1 study has been performed in Oceania. This study in Australia reported prevalence of 13% and that girls were more likely to have active infection.

Our review shows that pediculosis capitis is widespread throughout the world and does not discriminate on socioeconomic status grounds. The traditional perception of head lice as a parasitosis exclusively associated with schoolchildren of low socioeconomic status is challenged by some of the reports (online Technical Appendix).

Most studies underestimate overall prevalence by assessing it in a specific timeframe; to the contrary, head lice infestation is a dynamic process that can spread hypergeometrically in closed environments such as schools and in the community (*7*). The point-prevalence reported by Heukelbach et al (*8*) may represent a more accurate indicator.

Although socioeconomic status seems to be an indicator of the magnitude of lice infestation, more specific determinants are the dynamic processes of hygienic status and overcrowding. A recent study in Turkey compared 2 neighboring villages with different socioeconomic status. The only factor that was statistically significantly related to pediculosis capitis was size of the household; ≥6 inhabitants was associated with increased prevalence (*9*).

Another parameter that may indirectly influence overall prevalence and account for the leveling of the prevalence gradient between rich and poor is awareness of head lice and preventive and therapeutic practices. A study in Australia showed that although parents prefer to play a major role in prevention and treatment, they may lack insight into recent advances and dilemmas regarding these measures (*10*).

Variations in reported prevalence were found even in data from the same country. These differences can result from surveys being conducted during different seasons, various examination techniques, reporting of active infestation or presence of nits, and potential introduction of effective pediculicides.

Although head lice account for a substantial number of missed schooldays in children, among others, it is surprising that pediculosis capitis is not monitored and prevalence is not regularly reported. Although we cannot extinguish the parasite, effective monitoring and planning will enable us to limit the prevalence and distribution of this parasitosis.

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Texas Isolates Closely Related to *Bacillus anthracis* Ames

To the Editor: Forensic and epidemiologic investigation of the 2001 bioterrorism-associated anthrax attacks used multiple-locus variable-number tandem-repeat analysis (MLVA) to identify the attack strain as Ames (*1*). Strain identity was essential for subsequent molecular epidemiologic and forensic investigations of this biocrime. To more easily identify this particular strain, comparative whole-genome sequencing (*2*) and phylogenetic analyses were used to identify single-nucleotide polymorphisms (SNPs) that seem highly specific for Ames strain identification (*3*). Because *Bacillus anthracis* is a recently emerged clonal pathogen, these SNPs represent highly evolutionarily stable markers (*4*) that are amenable to many rapid and cost-effective analytical techniques.

MLVA and the Ames-specific SNP assay indicate that the Ames strain has been isolated from nature only 1 time, in southern Texas, USA. Several lineages of *B. anthracis* (*5*) have been ecologically established in North America. The A.Br.009 clade is the most successful and widely dispersed in North America, but it is not closely related to the Ames

strain (*5*), which is a member of the A.Br.001 clade. Although A.Br.001 is not as successful as A.Br.009, it appears to be ecologically well established in southern Texas. Analyses of outbreaks in this region from 1974 to 2000 found 190 culture-confirmed cases clustered mainly in 5 counties (*6*). A major epizootic in Texas in 2001 paralleled this trend. This outbreak (*6*) affected many deer species, horses, and bovids (total 1,637), which suggests that this clade is well established and not limited to cultivated areas and domesticated livestock. Previous molecular and epidemiologic analyses (*3*) of isolates from this region identified close, but not identical, matches to the Ames strain, which suggests that more intense surveillance in this region would likely yield more Ames and Ames-like isolates. Two recent (2006 and 2007) outbreaks in Texas confirmed this suggestion.

Isolates from the 2006 and 2007 outbreaks were initially screened by using an 8-marker MLVA system (MLVA8) as described by Keim et al. (*7*). The MLVA8 genotypes were identical to the *B. anthracis* Ames strain (GT62). Additional analysis by a 15-marker (MLVA15) variable-number tandem repeats (VNTR) system (*5*) again generated an MLVA15 genotype that was identical to the original Ames strain (A0462) and to the 2001 bioterrorism-associated attack strain (A2012) (Figure). Given the identical MLVA genotypes, could these natural strains be differentiated from the laboratory or biocrime Ames strain by using higher resolution genotyping?

We developed 6 Ames strain–specific SNPs to address the potential that the Ames strain might reappear naturally and hinder epidemiologic and forensic investigations (*3*). We found that 5 of 6 SNP loci could be used to distinguish between all known natural isolates and laboratory or biocrime isolates (Figure). Results were consistent with our previous identification of a *B. anthracis* isolate from a goat kid in Texas in 1997 (A0394) as being closely related to the Ames strain (*3*). However, the 2006 and 2007 isolates from Texas were even more closely related to the Ames strain because they also shared the MLVA15 genotype with Ames. In contrast, the 1997 goat isolate differed by a single mutational step at the BaVNTR16 locus (Figure). Hence, 5 of 6 SNP markers enabled differentiation among Ames and Ames near relatives even when VNTR profiles were identical.

Resolution of nearly identical genotypes might also be accomplished by using additional VNTRs (*8*) or hypermutable loci (*9*). However, we doubt that this approach would be better than whole-genome sequencing with interrogation of resultant SNPs because these markers would most likely result in topologic conflicts due to homoplasy (*10*). The available epidemiologic data from other isolates included in this clade show that although the Ames clade is well established in southern Texas, no subsequently recovered natural isolates completely match the original Ames isolate.

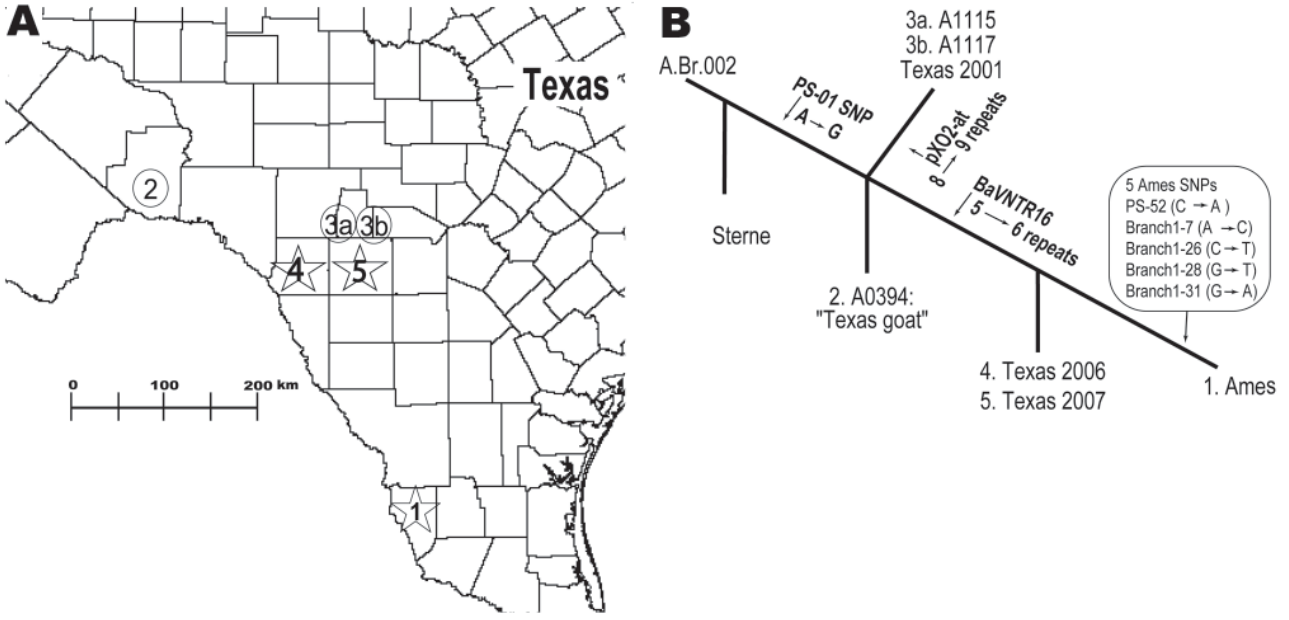

Figure. Geographic and phylogenetic relationships among strains closely related to *Bacillus anthracis* Ames strain. A) Spatial relationships among Ames-like isolates from southern Texas. 1, location of the original Ames strain, isolated from Jim Hogg County, Texas, in 1981; 2, closely related Texas 1997 goat isolate (A0394); 3a and 3b, Texas 2001 isolates; 4 and 5, most recent cases, i.e., Texas 2006 (Kinney County) and Texas 2007 (Uvalde County). B) Genetic relationships among isolates with variable-number tandem-repeat and single-nucleotide polymorphism (SNP) differences giving rise to that particular branch (arrows). The numbers at each branch terminus correlate with the numbers depicted on the map. SNP states are from ancestral to derived.

The precision of subtyping assays is a matter of importance and debate for epidemiologic and, recently, forensic investigations. Strain identity is commonly used to infer a common source even when spatial and temporal data are not congruent. Moreover, the definition of a strain is somewhat unclear and relies on analytical methods that vary widely. Therefore, isolates may be erroneously excluded or included into a strain definition and disease outbreak as illustrated with the Ames strain and 2 contrasting approaches to identification. MLVA15 ties naturally occurring isolates to bioterrorism-associated attacks, while specific SNP assays can distinguish among them.

MLVA is an unbiased approach and can be used on any set of *B. anthracis* strains, although, as in the 2006 and 2007 Texas outbreaks, it can be limited in resolving power. In contrast, our SNP assays have great resolving power but are useful only for differentiating the Ames strain, thus limiting their value to categorical inclusion or exclusion in outbreaks. Future rational use of a battery of different molecular signatures will yield far greater insights into strain identity than the application of 1 specific signature.

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Bluetongue in Eurasian Lynx

To the Editor: Bluetongue is an infectious disease of ruminants; it is caused by bluetongue virus (BTV), has 24 known serotypes, and is transmitted by several species of *Culicoides* biting midges. The disease mainly affects sheep and occurs when susceptible animals are introduced to areas where BTV circulates or when BTV is introduced to naive ruminant populations. The natural host range is strictly limited to ruminants, although seroconversion without disease has been reported in carnivores (*1*). We report BTV infection, disease, and death in 2 Eurasian lynx (*Lynx lynx*) and the isolation of BTV serotype 8 (BTV-8) from this carnivorous species.

The 2 Eurasian lynx, held in the same cage in a zoo in Belgium, became lethargic in September 2007; animal 1 died after 2 days, and animal 2 died in February 2008. Both had been fed ruminant fetuses and stillborns from sur-

rounding farms in an area where many bluetongue cases had been confirmed (*2*). Necropsy findings for animal 1 were anemia, subcutaneous hematomas, petechial hemorrhages, and lung congestion with edema. Necropsy findings for animal 2 were emaciation, anemia, enlarged and gelatinous lymph nodes, petechial hemorrhages, and pneumonia. For each animal, microscopic examination showed edematous vascular walls; enlarged endothelial cells; and evidence of acute to subacute vasculitis in muscle, myocardium, peritoneum, and lung. Tissue samples (spleen, lung, intestine) were analyzed by using 2 real-time reverse transcriptase–quantitative PCR techniques targeting BTV segment 5 and host β-actin mRNA as a control. BTV RNA was found in all samples from animal 1; cycle threshold values (*3*) ranged from 28.6 to 36.2. Tissues from animal 2 were negative for BTV RNA. Although the internal control was originally designed to detect β-actin mRNA of bovine or ovine species, clear positive signals were noted in all lynx samples, which indicated that this was a reliable control procedure. Infectious virus was subsequently isolated from the lung sample of animal 1 after inoculation of embryonated chicken eggs and amplification in baby hamster kidney–21 cell cultures (*4*). The specificity of the cytopathic effect, observed 48 hours after passage on baby hamster kidney–21 cells, was confirmed by real-time reverse transcriptase–quantitative PCR. Virus neutralization using specific reference serum (*5*) proved that the isolated virus was BTV-8. Anti-BTV antibodies were detected in lung tissue fluid from animal 2 (ID Screen Bluetongue Competition assay, ID VET, Monpellier, France) (*6*).

We describe a natural, wild-type infection of a carnivorous species. Although deaths have been documented in dogs accidentally infected with a BTV-contaminated vaccine (*7*), the 2 lynx in this report were neither vaccinated nor medically treated by injection. BTV-8 was first introduced to northern Europe in 2006 and has subsequently spread rapidly to many countries on that continent. During 2007, a total of 6,870 bluetongue cases were reported in Belgium (*2*); animal 1 died in September 2007, which corresponded to the peak of bluetongue outbreaks in that region. No deaths were reported during that period among other animals, including ruminants, held in the same zoo as the 2 lynx reported here. The time lapse between initial clinical signs and death could explain the failure to detect BTV-8 RNA in animal 2. Although speculative, the suspicion of bluetongue in this animal is based on the presence of anti–BTV-8 antibodies, vasculitis, and pneumonia, which have been found in dogs accidentally infected with BTV (*7*).

This report raises questions about the current knowledge of the epidemiology of bluetongue. Bluetongue in lynx indicates that the list of known susceptible species must be widened, at least for serotype 8. Although infection of a susceptible host by an insect vector is the only proven natural transmission mechanism for wild-type BTV, transplacental transmission of BTV-8, resulting in the birth of seropositive (*8*) or virus-positive calves (*9*), has recently been described in cattle. Although infection by an insect vector cannot be excluded, transmission by the oral route must be strongly suspected because the lynx described in this report had been fed ruminant fetuses and stillborn animals from surrounding farms. This possibility is supported by a previous suspicion that seroconversion to BTV in carnivores was a result of oral infection (*1*). The possibility of oral transmission is also supported by evidence of lateral transmission of BTV infection to cattle having occurred, in the absence of insect vectors, as a result of direct contact with newborn viremic calves born to infected dams that had been imported to Northern Ireland from a bluetongue-infected region of continental Europe (S. Kennedy, unpub. data). The role of wildlife, especially carnivores, in the epidemiology of bluetongue deserves further study to elucidate their role as either dead-end hosts or new sources of infection for livestock and to help determine the risks for wildlife populations.

Our findings clearly indicate that a novel transmission pathway enables the virus to cross species. Consequently, transmission to other species, including domestic animals, can no longer be excluded. Moreover, oral transmission is likely to have considerable implications for disease control, including vaccination, because BTV-8 is a fast-emerging virus with major financial consequences.

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Indigenous Dengue Fever, Buenos Aires, Argentina

To the Editor: For 2 decades dengue has increased in the Americas, with epidemic peaks every 3 to 5 years (*1*). The disease has reemerged in 3 South American countries bordering Argentina, namely, Bolivia, Brazil, and Paraguay.

Argentina had remained free from dengue for >80 years before the disease was reintroduced in 1998 (*2*) as a consequence of insufficient mosquito control and importation of cases from disease-epidemic areas. Since then, indigenous dengue circulation has only been reported in the northern provinces of the country, which are close to endemoepidemic countries. However, the principal dengue vector, the *Aedes aegypti* mosquito,, has spread southward to latitude 35°S near Buenos Aires (*3*).

We describe what might be the southernmost indigenous case of dengue fever documented in South America; this case occurred in 2007, an epidemic peak year for the disease on this continent (*1*). The patient was a pneumonologist who worked part-time at Muñiz Hospital, a referral infectious diseases treatment center in the Buenos Aires Federal District. She also provided healthcare at an outpatient clinic in Lanus, her town of residence, a suburb 6 km south of the Federal District. Febrile illness started suddenly in February 2007, midsummer season in Argentina. On day 5 of illness, fever was replaced by a short-lived rash and itching followed by asthenia and nausea that persisted for 2 days. The patient had not traveled or been accidentally exposed to patients' blood during the previous weeks. She had never been vaccinated against yellow fever. Dengue fever was only suspected retrospectively.

Serologic results provided supportive evidence of a recent dengue infection i.e., presence of immunoglobulin M, as determined by antibody-capture enzyme immunoassay, and immunoglobulin G seroconversion by 90% plaque reduction neutralization test on Vero cells (*4*). As shown in the Table, dengue virus serotype 3 was identified, and antibody results were negative for 3 other flaviviruses. Thus, this case fulfils Pan American Health Organization criteria for the diagnosis of dengue fever (*5*). Household contacts were seronegative.

For several years, conditions have been set for dengue virus circulation in Buenos Aires' urban and suburban areas because of the abundance of mosquitoes and disease in persons recently returning from neighboring countries. Risk for vector transmission is highest in the peripheral quarters of the city and towards late summer (*6*). Besides, Buenos Aires, like other Latin American metropolitan areas, is undergoing demographic changes that convey further risk for mosquito-borne disease transmission, namely, accelerated population growth mainly caused by informal settlements, deficient public health infrastructure and basic services, unregulated immigration from neighboring countries, and increased international mobility especially in or from neighboring countries (*1*).

Only imported dengue cases have been previously documented in Buenos Aires (*2*). According to official information, all 158 cases confirmed by antibody conversion in Buenos Aires Federal District and Province during 2007 were also imported (*7*). Of these, 50 occurred in the southern suburban district where our patient lives and works. In the summer of 2007, dengue infection was mainly introduced into the area by Paraguayan natives living in Buenos Aires who had recently visited their homeland. Dengue 3 serotype conversion was demonstrated in most of the cases investigated by plaque reduction neutralization assay, except for a few cases imported from Brazil, in which dengue 1 serotype was detected.

Table. Serologic findings of an autochthonous case of dengue fever, Buenos Aires, February 2007

Date (days after onset)	MAC-ELISA*	Plaque reduction neutralization test (90%)						
		Saint Louis encephalitis virus	West Nile virus	Yellow fever virus	Dengue 1 virus	Dengue 2 virus	Dengue 3 virus	Dengue 4 virus
2007 Jul 7 (16)	+	<20	<20	<20	80	<20	80	<20
2007 Apr 13 (53)	ND	<20	<20	<20	40	<20	640	<20

*Immunoglobulin M antibody-capture enzyme immunoassay with suckling mouse dengue virus antigen mixture of dengue 1, dengue 2, dengue 3, and dengue 4 serotypes. ND, not determined.

Most of the patients whose cases were diagnosed in Buenos Aires, including 5 who required hospitalization, were referred to Muñiz Hospital. Built a century ago, Muñiz Hospital comprises a number of independent pavilions surrounded by a spacious garden, where mosquitoes thrive, especially in summer. Thus, vector-borne infection in this case might have occurred either in Muñiz Hospital, in the Federal District, or in the southern city suburb, where the patient lives and works.

Until recently, dengue had not been suspected in patients with a fever living in the Buenos Aires area in the absence of a recent history of travel to an endemoepidemic area. Confirmation of our case was evidence of local circulation of dengue virus. Thereafter, serum testing became recommended in Buenos Aires for acute febrile illness, among other dengue surveillance interventions in the area. More recently, epidemiologic surveillance of febrile illness has been strengthened countrywide upon the recent reporting of yellow fever cases in Argentina (*8*).

No circulation of dengue virus was reported in Buenos Aires during the first 10 epidemiologic weeks of 2008. However, vector control measures should be strengthened to minimize the risk of infective persons triggering an epidemic of dengue or other flavivirus disease.

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Naegleria fowleri in Well Water

To the Editor: *Naegleria fowleri,* a protozoon found in hot springs and warm surface water, can cause primary amebic meningoencephalitis in humans. A survey of drinking water supply wells in Arizona determined that wells can be colonized and may be an unrecognized source of this organism that could present a human health risk.

N. fowleri is a free-living amoeboflagellate found in warm bodies of water such as ponds, irrigation ditches, lakes, coastal waters, and hot springs and can cause primary amebic meningoencephalitis. Humans come into contact with *N. fowleri* by swimming or bathing, particularly in surface waters. The ameba enters the nasal passages, penetrates the nasopharyngeal mucosa, and migrates to the olfactory nerves, eventually invading the brain through the cribriform plate (*1*). From 1995 to 2004, *N. fowleri* killed 23 persons in the United States (*2*), includ-

ing 2 children in the Phoenix, Arizona, area in 2002, who had been exposed to well water but had not consumed it (*3*). There have been 6 documented deaths in 2007, all in warmer regions (Arizona, Texas, Florida) (*4*).

Although *N. fowleri*'s presence in surface waters is well documented (*5*,*6*), no previous studies on its occurrence in wells have been conducted. We studied high-volume drinking water wells operated by municipal utilities or private water companies in the greater Phoenix and Tucson, Arizona, areas. Previous data from 500 wells in the region showed temperatures ranging from 13°C to 46°C. Typical well discharges ranged from hundreds to >3,780 L per minute. Well depths varied from 100 m to >300 m.

Well water samples were collected by using 1-L sterile polyethylene bottles at or near the wellhead before disinfection by well owners or utilities (*7*). In phase 1, samples were collected after wells were flushed until the water was clear. During phase 2, samples were collected as water was turned on from spigots at or near wellheads (initial) and after a 3-borehole volume had flushed through the system (purged). Additional wells were sampled during this phase. Samples were tested for temperature, pH, turbidity, chlorine residual, conductance, coliforms, heterotrophic bacterial plate counts (HPC), and *Escherichia coli* following standard methods (*7*).

To test for viable amebas, we spread aliquots on nonnutrient agar seeded with *E. coli* at 37°C (*3*,*8*). We placed scrapings from the advancing front of subsequent ameba plaques in distilled water to identify enflagellation (*5*); however, precise species identification was not possible. Live amebae were therefore harvested for PCR analysis to specifically identify *N. fowleri*. We chose PCR over the mouse pathogenicity test because other *Naegleria* species that are nonpathogenic in humans are lethal in mice (*8*). The genotype of isolates was not determined because all of the described genotypes found in the United States have been shown to be pathogenic in humans (*9*).

To concentrate trophozoites/cysts, we gently agitated samples for 2 minutes and then centrifuged and filtered them through polyethylene filters (2-μm pore; Millipore, Bedford, MA, USA). A 10-μL volume of concentrate was used as a template for nested PCR (*3*,*8*) (triplicate tests were conducted immediately and after a 2-week 37°C incubation). Positive and negative PCR products were frozen at –80°C, coded to prevent bias, and shipped to Francine Marciano-Cabral at Virginia Commonwealth University for confirmation by cloning and sequencing (*3*).

The general microbial quality of the wells was as follows: 73 (51%) had >500 HPC/mL; 8 (5.5%) were positive for coliforms; none were positive for *E. coli*. Oils used to lubricate well motors may result in the growth of HPC in well water (*10*). *N. fowleri* feeds on heterotrophic bacteria in water and could multiply in the well casing. This may explain *N. fowleri*'s colonization of wells.

The recent association in Arizona between unchlorinated drinking water and the transmission of *N. fowleri* suggests that groundwater has been an unrecognized source of this organism. PCR detected *N. fowleri* DNA in 11 (7.7%) of 143 wells. Of 185 total samples, 30 (16.2%) tested positive for *N. fowleri* (Table). The organism was most often detected after the wells had been purged (17.9% purged vs. 10.0% initial samples), suggesting that *N. fowleri* was present in the aquifer or was released from the well casing or column during pumping. The wells testing positive for *N. fowleri* ranged in temperature from 21.9°C to 37.4°C (average 29.0°C; median 29.5°C).

The live trophozoite form was confirmed in only 1 well, though 11 of 143 wells tested positive according to PCR. This discrepancy may be due to the low occurrence of trophozoites in water or to differences in assay volumes for detection of live trophozoites (0.75 mL) versus PCR (30 mL equivalent unconcentrated volume). PCR is also more sensitive, capable of detecting 100 organisms/L in an unconcentrated sample (*8*); however, PCR did not determine if the amebas were infectious. Although PCR can determine the species by using primers for a specific gene sequence not found in other *Naegleria* species, it cannot determine the life stage (cyst/trophozoite). Trophozoites are believed to be the infectious form of the organism (*1*); nonetheless, cysts can be equally harmful because they may revert to trophozoites under optimal conditions (*1*). The surprisingly common occurrence of *N. fowleri* in drinking water wells suggests that groundwaters may be an unrecognized human health threat.

Table. *Naegleria fowleri* in well water samples, Arizona

Sample type*	No. (%) positive
Initial	4/40 (10)
Purged	26/145 (17.9)
All	30/185 (16.2)

*Samples were collected before and after purging 3 borehole volumes. PCR was used to test samples.

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Popular and Scientific Attitudes Regarding Pandemic Influenza

To the Editor: Blendon et al. (*1*) described a survey of public attitudes regarding Americans' willingness and ability to follow the advice of public health officials during a severe influenza pandemic. The authors' results, however, can only be considered indicative if Americans' perceptions of pandemic influenza during the next pandemic are comparable to those associated with the hypothetical event they imagined while participating in the survey by Blendon et al.

By asking respondents to imagine a "severe outbreak" of "a new type of flu," the authors likely portrayed to survey participants an image of pandemic flu as an event starkly different from ordinary flu seasons. Although such a contrast reinforces popular perceptions of pandemic flu as a catastrophic event (*2*), it is not supported by historical studies which show that, in terms of deaths, recent pandemics have been comparable to (*3*) or less deadly than (*4*) ordinary influenza seasons.

A gap thus exists between the perceptions and reality of pandemic influenza. Although the authors described pandemic flu as an "unfamiliar crisis" that "many of the respondents may not have been familiar with," in actuality, 39% of survey respondents were ≥50 years of age and therefore had firsthand experience of 1 or more past pandemics. (The last 2 pandemics occurred in 1957 and 1968; a pandemic was predicted in 1976, but never materialized.) Whether those respondents were aware that they had lived through past pandemics is a question with important implications for the survey results, but unfortunately, this understanding was not queried by the authors. For example, would all of the 94% of respondents who reported a willingness to isolate themselves at home for 7–10 days if that were recommended by health authorities—in effect, "voluntarily" placing themselves in quarantine—also be willing to do so during a pandemic no more severe than ordinary influenza?

If even those who have experienced pandemics do not recall them as particularly memorable events, it calls for a rethinking of public communication strategies with respect to influenza. Perhaps a first step is to acknowledge that as the past 2 pandemics have not been public health crises, the next pandemic may likewise also not be a crisis.

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In Response: We agree with Doshi (*1*) that in our study, reported in Public Response to Community Mitigation Measures for Pandemic Influenza (*2*), we purposely asked respondents to imagine a "severe outbreak" of "a new type of flu," and that possible scenario was vastly different from ordinary flu seasons. Although previous pandemics have varied in their severity (*3*) and their concomitant illness and mortality rates, we were particularly interested in the public's response to community mitigation interventions (*4*) that would only be recommended if a severe 1918-like pandemic occurred (e.g., Pandemic Severity Index 4 or 5).

A great deal of cooperation from the public would be required to successfully implement community mitigation measures during a pandemic. The intensity of interventions must be matched with the severity of a pandemic to maximize the available public health benefit that may result from using these measures while minimizing untoward secondary effects. Socially disruptive measures such as dismissing children from schools, closing childcare programs, social distancing in the community and at the workplace, and cancelling large gatherings would likely reduce community transmission of pandemic disease, but would also create challenges for the public. Therefore, these interventions would only be recommended if the severity of the pandemic warranted their use. The survey was conducted to inform policy-makers who were, at the time, developing recommendations for community-based interventions. Thus, a severe pandemic was used as the scenario for this national survey to gauge the public's response to these proposed public health measures.

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etymologia

Merkel [mər′-kəl] *Cells*

Specialized cells found near the dermal-epidermal junction, characterized by numerous membrane-bound granules with dense cores. The cells were named after German anatomy professor Friedrich Sigmund Merkel, who experimented with osmium tetroxide staining and described these cells in 1875. First identified in the skin of a mole, they were later found in human skin. The cells are responsible for the highly malignant skin tumor known as Merkel cell carcinoma. An infectious cause for Merkel cell carcinoma has been proposed.

Sources: Dorland's illustrated medical dictionary, 31st edition. Philadelphia: Saunders/Elsevier; 2007; http://www.whonamedit.com; Merkel FS. Tastzellen und Tastkörperchen bei den Hausthieren und beim Menschen. Archiv für mikroskopische Anatomie. 1875;11:636–52; Foulongne, V, Kluger N, Dereure O, Brieu N, Guillot B, Segondy M. Merkel cell polyomavirus and Merkel cell carcinoma, France [letter]. Emerg Infect Dis. 2008;14:1491–2.

Food-Borne Parasitic Zoonoses: Fish and Plant-Borne Parasites (World Class Parasites)

K. Darwin Murrell and Bernard Fried, editors

Springer, New York, New York, USA, 2007
ISBN-10: 0387713573
ISBN-13: 978-0-387-71357-1
Pages: 686; Price: US $169

With >4,000 biomedical journals currently available, and more being added seemingly every month, one of the most overwhelming and daunting tasks for any health professional is to stay current with the literature. A welcomed lifeline has been the World Class Parasites series of publications that have made this challenge much easier to manage. The latest text (Volume 11), Food-Borne Parasitic Zoonoses: Fish and Plant-Borne Parasites, is a superb addition to this series. Murrell and Fried have brought together a stellar group of contributors in a comprehensive and up-to-date review of the helminthic zoonoses that are transmitted through fish and plant consumption. Given that the distribution and effect of these zoonoses remain woefully underappreciated, they are deserving of the justifiable attention brought by this book.

The first 9 chapters provide a thorough discussion of intestinal, liver, and lung flukes; fish-borne tapeworms; and tissue nematodes. Two superb chapters on immunologic aspects and molecular epidemiology of these parasites complete the text. There is much to extol in this volume. In an era of justifiable focus on molecular biology and genetics, it is heartening to see the broad-based and integrated approach taken by the editors and collaborators. This approach successfully accomplishes the stated objective of the editors to "… celebrate the diversity of approach that comprises modern parasitological research." The chapters are clearly written and well organized with a balance of both classic, historic articles as well as important recent work.

The editors and authors have obviously made a priority of ensuring that the material is widely accessible to scientists and health professionals in diverse disciplines. They have presented detailed information on the biology, life cycles, natural history, diagnosis, treatment, epidemiology, and control of the agents or infections discussed. Current diagnostic techniques are thoroughly addressed. Insightful discussion is provided at the end of each chapter on current gaps in knowledge and research needs that offers a coherent roadmap for future studies. The chapter on intestinal capillariasis by Cross and Belizario is particularly noteworthy. Abundant images of exceptional quality are included and this adds substantially to the text. More color images would have been of value, but they may have made the text prohibitively expensive. Some chapters could have had additional discussion of epidemiologic aspects and a few typos can be found, but these do not diminish the value of this outstanding work.

Food-Borne Parasitic Zoonoses: Fish and Plant-Borne Parasites is a text of exceptional quality that should have broad appeal and utility for health professionals, researchers, medical and public health students, and policy makers. Given the increasing interest in neglected tropical diseases, this book is a timely and commendable work that will promote efforts to reduce the effects of these parasitic agents.

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Epidemiologic Principles and Food Safety

Tamar Lasky, editor

Oxford University Press, New York, New York, USA, 2007
ISBN-10: 0-19-517263-9
ISBN-13: 978-0-19-517263-8
Pages: 272; Price: US $49.95

This text is an ambitious overview of the wide field of public health and food safety. Topics include foodborne pathogens, public health surveillance, basic epidemiologic principles, study design and outbreak management, food production, and food safety regulation. A substantial amount of attention has focused recently on the complexity of the food safety system in the United States, which includes a huge number of agencies, sometimes overlapping programs, databases and legal authority, and the inevitable consequence of inefficient and confusing communication. In light of these circumstances, this text provides a pertinent overview of the system that will be useful even to those very familiar with a particular aspect of the food safety continuum.

The descriptions of existing surveillance systems, sources of food consumption data, the federal regulatory system approach to risk assessment, food production and controls, and the regulatory environment are particularly clear, useful, and timely descriptions of sometimes vexingly complex systems. Clearly, such a breadth of topics cannot be addressed

in great detail in a single text, but many of the subjects are seldom summarized so cogently, and readers interested in exploring particular topics will find this text provides a nice foundation for delving more deeply into the literature. This book provides something for everyone—an excellent overview for students, epidemiologists, regulators, academicians, industry representatives, and others interested in a broad survey of the field.

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Bartolomé Esteban Murillo (1617–1682) The Young Beggar (detail) (c. 1650) Oil on canvas (1.34 m × 1 m) Photo: C. Jean. Louvre, Paris, France. Réunion des Musées Nationaux/Art Resource, New York, NY

"How comes it, Rocinante, you're so lean?" "I'm underfed, with overwork I'm worn"

—Cervantes

Polyxeni Potter

"There are only two families in the world, the Haves and the Haven'ts," wrote Miguel de Cervantes (1547–1616) at a time he viewed as "no golden age" in his native Spain (*1*). A brilliant satirist, Cervantes ridiculed the socially divisive mores of a gilded imaginary past held onto for too long and seeming all the more incongruous amidst the poverty and oppression of his own life.

Spain in the 17th century, its empire collapsed and population ravaged by three plague epidemics, was embroiled in conflict abroad and royal mismanagement at home. Its misadventures, chronicled in the plays of Lope de Vega and Calderón, also fueled the genius of Diego Velázquez, Francisco de Zurbarán, Jusepe de Ribera, and Bartolomé Esteban Murillo. They, too, advanced the cultural front by constructing from the rabble a Spanish school of painting for the ages.

Spanish baroque, as the school came to be known, expanded on similar art movements in the Netherlands and elsewhere in Europe. Immortalizing royals and street peddlers alike, it left a vivid record of the times. Among the greats of this period, Murillo, a native of Seville known as much for his good character as his artistic talent, painted the indigent and populated lofty religious scenes with ordinary human faces.

Orphaned in childhood, Murillo was raised by relatives (*2*). He apprenticed under Juan del Castillo, a leading painter of the 1630s and 1640s who, along with Alonso Cano, influenced his early work. He left this apprenticeship to go into business for himself, creating popular pictures for quick sale. As a street artist, he saw the poorest in the city, so his early paintings were sympathetic portraits of the ragged boys and flower girls of Seville. His break came with a commission to paint 11 works for a local Franciscan monastery. These paintings brought him fame, and soon he was affluent enough to marry well.

He traveled to Madrid, where he likely became familiar with the work of Velázquez, fellow Sevillian and painter to the king, and the work of Rubens, Titian, Veronese, and Tintoretto in the royal galleries. He returned to his hometown to found the Seville Academy of Art, paint major

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works, and enjoy great popularity, even outside Spain (*3*). His mature paintings were mostly religious. Some were genre scenes with children, a novel theme. A few were fine portraits. All attained such lightness and refinement they were labeled "vaporous."

Even now, a "Murillo" stands for a good painting in Spain. The artist made an impression, although his fortunes faltered over time. His works sold so well that the king limited their export. But they were copied too often. Poor imitations in a flooded market damaged his reputation, especially since he never signed or dated his paintings.

One legend has it that Murillo died poor; another, that he gave his wealth to charitable causes. His last work, the Espousal of St. Catherine, commissioned for a Capuchin monastery in Cadiz, was not completed. The painter fell from the scaffold and was severely injured. He was taken back to Seville, where his death was attributed to these injuries.

The Young Beggar, on this month's cover, exemplifies Murillo's technique. It showcases the masterful brushwork, use of chiaroscuro, meticulous attention to naturalism, and gentleness toward his subjects that attracted the attention of later artists, among them 18th- and 19th-century's Sir Joshua Reynolds, John Constable, and Édouard Manet.

"Masterless children," a common sight in Seville, were pitied and loathed by local society, which gave them alms with one hand and dismissed them with the other. Survival and socialization on the street suited them for servile tasks, which inevitably led to adult criminal opportunities, gambling, and prostitution (*4*). In 1593, the city was "full of small boys who wander about lost and begging and dying of hunger and sleep in doorways and on stone benches by the walls, poorly dressed, almost nude, and exposed to many dangers…and others have died of freezing by dawn" (*5*).

An unpublished parish-by-parish survey of the "honorable poor" of Seville in 1667 found that untended children aggregated in courtyards and abandoned buildings. Exposed to weather and pests, unwashed and malnourished, they were susceptible to infectious diseases, from ringworm to bubonic plague. When, against all odds, it snowed twice in 1624 and 1626, they were decimated. One study in the parish of San Bernardo showed that 27% of burials in 1617 to 1653 were of children.

Seville's population declined in the 17th century. A 1679 silk merchants' quarter survey showed that 40% of the buildings were vacant. Two serious plague epidemics and extensive emigration to the New World reduced the number of children. Charity, documented in literary works and prominent in the paintings of Murillo, was thought as noble and pious, but the ugliness of want (festered wounds, parasites, filth) so vivid in these paintings exposed the dark side. The poor had to register for licensed begging to be protected from impostors that would take "alms from the truly poor who cannot work" (*5*). City authorities used charitable funds to create "hospitals" for vagrants to be put away from the public eye.

A slant of light reminiscent of Caravaggio (1571–1610) illuminates Murillo's Young Beggar, allowing a glimpse of his life. Weary and resigned, the boy leans against the wall, his belongings strewn along with the remnants of a meal, scraps of fish and rotting fruit. He is delousing himself. The mild demeanor of the child is punctuated by the furrowed brow and soiled feet. Like Rocinante, he is underfed, overworked, and riddled with pests.

"The Haves and the Haven'ts" are still at it. The Haves contributing to charity, the Haven'ts flooding the streets, some 100 million of them around the globe: migrant workers in People's Republic of China and elsewhere, homeless in Los Angeles, Bristol, or Marseille. Authorities still sort the truly and deserving poor from impostors, while the ugliness of want (lice, tuberculosis, flu, HIV/AIDS, hepatitis, diphtheria) is not shrinking from public view (*6*,*7*).

The pathos in Murillo's Young Beggar lies in the child's complete abandon and his charm, unblemished by the circumstances. Well addressed in literature and art, the plight of the poor, its abatement and elimination, is a main concern of public health. Armed with time-honored public health practice: health education, prescription and device distribution, tuberculosis screening and treatment, improved hygiene, ivermectin use against scabies and body louse infestation, and systematic immunization, the public health worker can now join other dreamers championed by Cervantes in declaring, " 'My armour is my only wear, / My only rest the fray' " (*1*).

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Rise and Persistence of Global M1T1 Clone of *Streptococcus pyogenes*

Pyogenic Liver Abscess as Endemic Disease, Taiwan

Risk Factors for Nipah Virus Encephalitis, Bangladesh

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Epidemic and Endemic Lineages of *Escherichia coli* that Cause Urinary Tract Infections

Prophylaxis after Exposure to *Coxiella burnetii*

Ecologic Factors associated with West Nile Virus Transmission, Northeastern USA

Deaths from Norovirus among the Elderly, England and Wales

Norwalk Virus Shedding after Experimental Human Infection

Streptococcus pneumoniae, *Haemophilus influenzae*, and *Moraxella catarrhalis* associated with *Staphylococcus aureus* and Upper Respiratory Tract Infection

Pandemic Influenza and Excess Intensive-Care Workload

Estimating Community-associated *Salmonella*, *Campylobacter*, and Shiga Toxin–producing Infections, Australia

Cryptosporidium Species and Subtypes and Clinical Manifestations in Children

Optimized Control Strategy for *Echnicoccus multilocularis*

Automatic Outbreak Detection Algorithm versus Electronic Reporting System

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Molecular Surveillance for Multidrug-Resistant *Plasmodium falciparum*, Cambodia

Multistate Outbreak of *Escherichia coli* O157:H7 Associated with Spinach

Decision-making in Tropical Disease Control Programs

Decreased Tuberculosis Incidence and Declining Clustered Case Rates, Madrid

Complete list of articles in the October issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

October 30–November 1, 2008
Ninth International Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases Meeting (MEEGID IX)
University of California at Irvine
Irvine, CA, USA
http://www.th.ird.fr/site_meegid/menu.htm

February 12–13, 2009
The International Symposium on the Asian Tiger Mosquito
Rutgers University
New Brunswick, NJ, USA
http://www.rci.rutgers.edu/~vbcenter/atmsymposium.php

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Article Title

Preventing and Controlling Emerging and Reemerging Transmissible Diseases in the Homeless

CME Questions

1. Which of the following statements about reducing the risk for incident HIV and hepatitis infection among homeless individuals is most accurate?

A. Older homeless adults should be targeted for HIV prevention
B. Education and skills training can reduce the practice of unprotected sex among homeless women
C. Homeless people are less likely than other intravenous drug users to complete hepatitis B virus (HBV) vaccination
D. Homeless people should never receive the accelerated HBV vaccination schedule

2. Which of the following statements about tuberculosis and airborne diseases among the homeless is most accurate?

A. Most tuberculosis infections among homeless individuals are reactivations of established diseas
B. Sputum testing detects >90% of patients with tuberculosis
C. Screening for tuberculosis with chest x-ray may be the most cost-effective approach
D. Directly observed therapy in the acute hospital setting is associated with the highest completion rates

3. Which of the following statements about scabies and body louse infections in the homeless is most accurate?

A. The body louse is an efficient vector for multiple species of bacteria
B. Ivermectin is ineffective in treating scabies
C. A treatment regimen of clothing change and medical treatment has been demonstrated to eliminate scabies from a homeless shelter
D. The prevalence of body lice among sheltered homeless is approximately 5%

4. Which of the following statements about *Bartonella quintana* infection is most accurate?

A. *B quintana* is the most common louse-borne disease reported among urban homeless
B. *B quintana* does not cause endocarditis
C. Body lice are the natural reservoir for *B quintana*
D. Cefixime should be used for serious infections with *B quintana*

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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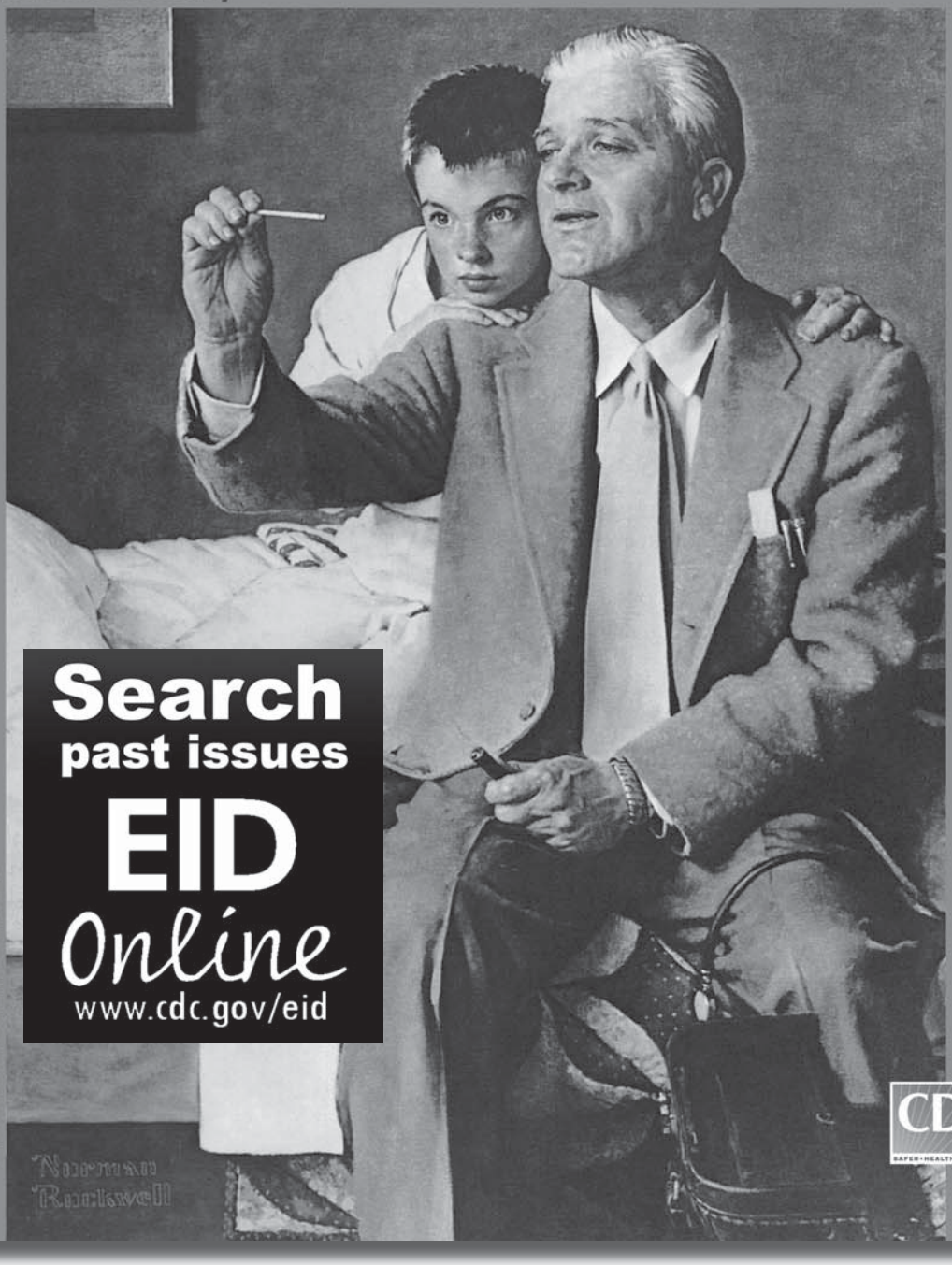

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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: 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.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

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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 www.cdc.gov/eid/ncidod/ EID/instruct.htm.

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Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, 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.

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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. 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. 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. 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 a 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 no more than 1,200 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 2); tables (not to exceed 2); and a brief biographical sketch. 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 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. 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.