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Managing Febrile Respiratory Illnesses during a Hypothetical SARS Outbreak

Kamran Khan,* Peter Muennig,† Michael Gardam,‡ and Joshua Graff Zivin†

Since the World Health Organization declared the global outbreak of severe acute respiratory syndrome (SARS) contained in July 2003, new cases have periodically reemerged in Asia. This situation has placed hospitals and health officials worldwide on heightened alert. In a future outbreak, rapidly and accurately distinguishing SARS from other common febrile respiratory illnesses (FRIs) could be difficult. We constructed a decision-analysis model to identify the most efficient strategies for managing undifferentiated FRIs within a hypothetical SARS outbreak in New York City during the season of respiratory infections. If establishing reliable epidemiologic links were not possible, societal costs would exceed $2.0 billion per month. SARS testing with existing polymerase chain reaction assays would have harmful public health and economic consequences if SARS made up <0.1% of circulating FRIs. Increasing influenza vaccination rates among the general population before the onset of respiratory season would save both money and lives.

On July 5, 2003, the World Health Organization (WHO) declared that human chains of transmission of severe acute respiratory syndrome (SARS) had ended. Since then, new cases of SARS have resurfaced in Asia, including several in the absence of laboratory exposures. This reemergence of the SARS-associated coronavirus (SARS-CoV) has sparked international concern and has prompted heightened surveillance by hospitals and health officials worldwide. Such concerns have been amplified by fears that a future SARS outbreak could coincide with respiratory infection season, when influenza infections and other febrile respiratory illnesses (FRIs) develop in large segments of the population.

Current SARS case-definition and case-exclusion criteria encompass clinical, epidemiologic, and laboratory features (1). Should the timely establishment of epidemiologic links between SARS cases be lost in a future outbreak, frontline healthcare providers would be forced to rely on clinical signs and symptoms or diagnostic testing to confirm or exclude infections with SARS-CoV (2). Unfortunately, the signs and symptoms of SARS are nonspecific and cannot be used reliably to differentiate SARS from other FRIs. Moreover, existing serologic tests for SARS-CoV cannot definitively exclude infection until at least 4 weeks has elapsed from the onset of symptoms and thus have no role in early clinical decision making (1). Although reverse transcriptase–polymerase chain reaction (RT-PCR) assays used to detect SARS-CoV can provide test results within a matter of hours, their suboptimal sensitivity makes them inadequate for ruling out SARS (3). Furthermore, since SARS infections would likely make up a minute fraction of FRIs circulating among the general population, the pretest probability, and thus the positive predictive value of RT-PCR tests, would be extremely low, even if future generation assays had better test sensitivity and specificity.

In 2003 and 2004, the emergence of SARS-CoV in China coincided with respiratory illness season, which suggests that the virus may resurface during winter months, like many other respiratory pathogens. Should this seasonal pattern recur, rapidly and accurately differentiating SARS infections from other FRIs would become a critical component of any future outbreak containment efforts (2,3). This distinction will also continue to be an important issue among travelers in whom FRIs develop after their return from SARS-affected areas. However, existing diagnostic limitations place frontline healthcare practitioners in a precarious position, since clinical decisions with potentially dangerous consequences must be made in the face of uncertainty. Recognizing such limitations, WHO recently called for the development of evidence-based clinical algorithms to help address these diagnostic dilemmas (4).
Methods

Overview and Definitions

A hypothetical cohort comprising all residents of New York City was entered into a decision-analysis model. The model is premised on a SARS outbreak during respiratory season where person-to-person transmission of SARS is documented and epidemiologic links between cases are poorly defined. The outbreak was designed to be consistent in size and duration with the Toronto outbreak (5). The analytic horizon of the analysis was defined as the expected lifetime of persons living in New York City during the 2004–2005 respiratory illness season. FRIs are defined herein as nonspecific infections caused by pathogens other than SARS-CoV for which the microbiologic origin cannot be determined on the basis of clinical grounds alone. The model was designed to identify the most effective and cost-effective uses of societal resources in managing FRIs of undetermined origin during a SARS outbreak.

The analysis was conducted in adherence with the reference case scenario as defined by the Panel on Cost-Effectiveness in Health and Medicine (6). All relevant costs and benefits were considered from the societal perspective of New York City, including those related to secondary transmission of SARS. Since costs and changes in health-related quality of life in the analysis were limited to a single respiratory season, no discounting was performed on these 2 parameters. However, all future years of life lost due to premature death from infections were discounted at an annual rate of 3%.

Decision-Analysis Model

A decision-analysis model was constructed by using DATA 4.0 (TreeAge Software, Williamstown, MA, USA) that examined 2 competing strategies in the context of a SARS outbreak coinciding with respiratory season: 1) home isolation for persons with FRIs of undetermined origin, pending fever and symptom resolution for at least 24 hours and 2) outpatient diagnostic testing of FRIs to ascertain a microbiologic diagnosis with subsequent test-driven management. A third complementary strategy entailing mass influenza vaccination among the general population before the onset of respiratory season was considered in conjunction with the above competing strategies.

Primary assumptions of the model were as follows: 1) epidemiologic linkages between SARS cases are not well defined; 2) SARS cannot reliably be distinguished from other FRIs on clinical grounds alone; 3) current SARS tests cannot definitively rule out infection early in the course of illness (1,7); 4) public nonadherence to home isolation guidelines during a SARS outbreak would be negligible (5,8); 5) positive SARS (RT-PCR) test requires isolation precautions pending confirmation of the diagnosis (2); 6) patients with confirmed SARS cases will be managed as inpatients pending resolution of the clinical illness; 7) patients with confirmed SARS cases require isolation precautions for 10 days after resolution of illness (2); 8) persons with FRIs of undetermined origin must be afebrile and symptom-free for 24 hours before returning to work; 9) negative SARS (RT-PCR) test alone will have no influence on SARS isolation precautions (2); 10) negative SARS (RT-PCR) test result combined with a positive test for another respiratory pathogen will result in the discontinuation of SARS isolation precautions (2); 11) in the absence of appropriate isolation precautions, persons with SARS will transmit infection to 3 additional persons (9,10); 12) SARS, influenza, respiratory syncytial virus, and community-acquired pneumonia are the primary causes of death from FRIs; 13) a future SARS outbreak would be managed by using existing healthcare infrastructure; and 14) no proven effective treatment for SARS currently exists.

A plausible range of high and low values for each variable was used to conduct sensitivity analyses, which examined the influence of parameter error on the results of the analysis. Selected variables in the model are listed in Tables 1 and 2.

Composition of FRIs

We used nationally representative data (25,27) in conjunction with studies published in the medical literature (11,28–30) to derive our base estimates for an “average” respiratory season. In our model, the microbiologic origin of an FRI was categorized into 1 of 4 mutually exclusive groups: 1) SARS-CoV and coronaviruses OC43 and 229E; 2) influenza viruses A and B; 3) a panel of common respiratory pathogens, including respiratory syncytial viruses A and B, parainfluenza viruses 1–3, human metapneumovirus, Bordetella pertussis, Chlamydia pneumoniae, Mycoplasma pneumoniae, Legionella pneumophila, and L. micdadei; and 4) all other causes.

In our base-case analysis, we assigned the proportion of FRIs due to SARS to be 0.01%, which was estimated assuming a SARS outbreak of similar size and duration to the Toronto outbreak. The proportion of FRIs due to influenza was derived from 2 large observational studies conducted over multiple respiratory seasons (11,28) and was corroborated by dividing the expected proportion of the U.S. population who get influenza each season (25) by the proportion of the U.S. population having influenzalike infections (27). The proportion of FRIs due to the common respiratory pathogen panel listed above was estimated from the medical literature (29,30). In our base-case scenario, we estimated that approximately one third of FRIs would be due to influenza, one third would be due to the
panel of common respiratory pathogens, and the remaining one third would be due to other miscellaneous pathogens not indicated above.

### Diagnostic Tests

We evaluated 3 categories of rapid diagnostic tests with optimal turnaround times of <24 hours. The first category constitutes RT-PCR assays capable of detecting SARS-CoV as well as coronaviruses OC43 and 229E (23,24). A second category includes 2 multiplex PCR assays, which, when used in combination, can detect 13 different respiratory pathogens, including influenza viruses A and B, respiratory syncytial viruses A and B, parainfluenza viruses 1–3, human metapneumovirus, *C. pneumoniae*, *M. pneumoniae*, *L. pneumophila*, *L. micdadei*, and *B. pertussis* (20–22). The third category comprises a widely available enzyme immunoassay capable of rapidly detecting infections with influenza A and B (19).

The sensitivity and specificity of these tests were obtained from the medical literature (19–24), while the positive predictive value of each diagnostic test was calculated by incorporating the estimated prevalence of specific pathogens into Bayes' equation.

### Influenza Vaccination

The effectiveness of the influenza vaccine was derived from the medical literature (31). To account for seasonal variation between circulating strains of influenza and the composition of the trivalent vaccine, we varied the effectiveness of the vaccine over a wide range of plausible values in our sensitivity analysis. The average seasonal effectiveness of the influenza vaccine was adjusted by assuming that the vaccine would be poorly matched to circulating influenza strains approximately twice every 10 years (31).

We used data from the U.S. Behavioral Risk Factor Surveillance System to estimate seasonal influenza vaccination rates among the population of New York City (33). In our sensitivity analyses, we evaluated the incremental costs and benefits of raising vaccination rates above this seasonal average.

### Management Algorithms

In our model, the home isolation strategy required persons with FRIs of undetermined origin to remain at home for at least 24 hours after resolution of illness. We assumed that adherence to public health guidelines in the setting of a widespread SARS outbreak would be near universal (5,8). Under this strategy, we assumed that persons would attempt to manage their illness at home by using self-care, visit a healthcare provider if the illness were serious or persistent, or proceed to a hospital if their illness became progressively severe.

The diagnostic evaluation strategy involved outpatient testing of persons with FRIs to ascertain a microbiologic origin. In this strategy, persons with FRIs of undetermined cause would observe home isolation precautions until the results of diagnostic tests were available. We assumed that a positive SARS RT-PCR test would require isolation precautions for the patient, public health intervention, and additional testing to confirm the diagnosis (2). We also assumed that a negative SARS RT-PCR test in conjunction

---

**Table 1. Selected costs in the decision-analysis model**

<table>
<thead>
<tr>
<th>Costs†</th>
<th>Low</th>
<th>Base</th>
<th>High</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccines and medications</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>influenza vaccine</td>
<td>$10.00</td>
<td>$27.78</td>
<td>$40.00</td>
<td>11</td>
</tr>
<tr>
<td>Antibiotics for FRI‡</td>
<td>$30.00</td>
<td>$64.72</td>
<td>$80.00</td>
<td>12</td>
</tr>
<tr>
<td>Medical care§</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ambulatory clinic visit</td>
<td>$40.00</td>
<td>$60.03</td>
<td>$80.00</td>
<td>13</td>
</tr>
<tr>
<td>Hospitalization for FRI</td>
<td>$5,000</td>
<td>$11,645</td>
<td>$15,000</td>
<td>14</td>
</tr>
<tr>
<td>Hospitalization for influenza</td>
<td>$7,500</td>
<td>$17,465</td>
<td>$25,000</td>
<td>14</td>
</tr>
<tr>
<td>Hospitalization for PUI</td>
<td>$15,000</td>
<td>$19,441</td>
<td>$25,000</td>
<td>14</td>
</tr>
<tr>
<td>Hospitalization for SARS</td>
<td>$20,000</td>
<td>$28,502</td>
<td>$40,000</td>
<td>14,15</td>
</tr>
<tr>
<td>Diagnostic tests</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rapid influenza test</td>
<td>$15.00</td>
<td>$26.66</td>
<td>$40.00</td>
<td>16</td>
</tr>
<tr>
<td>Multiple‡ RT-PCR</td>
<td>$50.00</td>
<td>$154.02</td>
<td>$200.00</td>
<td>Prodesse Inc., pers. comm.</td>
</tr>
<tr>
<td>SARS§ RT-PCR</td>
<td>$20.00</td>
<td>$54.80</td>
<td>$100.00</td>
<td>Prodesse Inc., pers. comm.</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient time (per hour)</td>
<td>$15.00</td>
<td>$24.55</td>
<td>$30.00</td>
<td>17</td>
</tr>
<tr>
<td>Contact investigation (per SARS contact)</td>
<td>$100.00</td>
<td>$222.94</td>
<td>$300.00</td>
<td>5,18</td>
</tr>
</tbody>
</table>

†FRI, febrile respiratory illness; PUI, person under investigation (for SARS); SARS, severe acute respiratory syndrome; RT-PCR, reverse transcription–polymerase chain reaction.

‡Medical and nonmedical costs were adjusted to 2004 U.S. dollars by using the Consumer Price Index.

§Antimicrobial drug costs are based on a 7-day course of oral levofloxacin.

Includes laboratory costs, transportation costs, and patient time.


#Detects SARS-associated coronavirus and coronaviruses OC43 and 229E.
with a positive test for an alternate respiratory pathogen would lead to the elimination of isolation precautions (2). If all test results were negative, we assumed that isolation precautions would remain in effect, since current SARS RT-PCR assays are not sufficiently sensitive to rule out SARS (2). We also assumed that persons with FRIs, for which the microbiologic origin was confirmed to be due to a pathogen other than SARS-CoV, would return to work only after resolution of their illness.

Under each strategy, we considered the possibility that persons with FRIs seeking medical care might receive antimicrobial drugs during their evaluation. We estimated

![Table 2. Selected probabilities in the decision-analysis model*](https://www.cdc.gov/eid/upload/1102/table2.png)

*RT-PCR, reverse transcriptase-polymerase chain reaction; SARS, severe acute respiratory syndrome; FRI, febrile respiratory illness; HRQL, health-related quality of life; HUI, Health Utilities Index.
†Refers to influenza viruses A and B, respiratory syncytial viruses A and B, parainfluenza viruses 1–3, human metapneumovirus, Legionella pneumophila, L. micdadei, Mycoplasma pneumoniae, Chlamydia pneumoniae, and Bordetella pertussis.
‡Detection of SARS-associated coronavirus and coronaviruses OC43 and 229E.
¶Febrile respiratory illnesses not due to SARS or influenza viruses A and B.
*In the absence of public health interventions.
this probability by using data from the National Ambulatory Medical Care Survey (32).

Illness and Death

Changes in health-related quality of life (HRQL), including the impact of isolation, due to SARS and other FRIs were derived by using the Health Utilities Index Mark 3 (HUI) (34). We used the HUI to minimize double counting of productivity losses, since HRQL scores generated from this instrument do not include productivity losses (William Furlong, pers. comm.). Parameters for the HUI were derived from a panel of 4 specialist physicians with clinical experience managing SARS patients in Toronto. These physicians did not directly value health states, but rather functioned as expert “describers,” who facilitated the mapping of health states to community-based preference scores from the HUI.

SARS, influenza, respiratory syncytial virus, and community-acquired pneumonia due to typical and atypical bacteria were assumed to be the primary contributors of death from FRIs on a population level. Mortality data for community-acquired pneumonia were obtained from the National Center for Health Statistics (35); data for SARS, influenza, and respiratory syncytial virus were obtained from the medical literature (24,26,36). We estimated that patients with SARS would each transmit infection to 3 other persons if appropriate isolation precautions were not observed (e.g., false-negative SARS RT-PCR test combined with a false-positive test for an alternate diagnosis) (9,10).

Costs and Charges

Costs attributable to transportation, ambulatory care (13), laboratory tests (16), influenza vaccination (11), antimicrobial agents (12), hospitalization (14,15), public health investigation (5,18), and patient time (17) were included in the analysis. Transportation costs to see a medical provider were derived by using U.S. national data and were adjusted to account for the estimated proportion of the population driving, using public transportation, or traveling by other means such as biking or walking. The base cost of an ambulatory care visit was estimated by using the national average 2000 Medicare reimbursement rates for a focused medical evaluation (CPT-code 99213); the cost of the rapid influenza test was derived from the Centers for Medicare and Medicaid Services (16). The costs of the SARS RT-PCR assay and the multiplex PCR assays used to detect the common respiratory pathogen panel were obtained from a test manufacturer and included 15 minutes of technician time (Prodesse Inc., pers. comm.)(18).

Influenza vaccination and antimicrobial drug costs were obtained by using average wholesale prices of pharma-ceuticals (11,12). The costs and frequency of adverse reactions to influenza vaccination were estimated from the medical literature and incorporated into the net costs and benefits of the vaccine (37).

Hospital charges and the average length of stay for patients with influenza and other respiratory infections requiring hospitalization were estimated from the Healthcare Cost and Utilization Project (14). The Medicare Provider Analysis and Review system was used to derive cost-to-charge ratios and subsequently convert hospital charges into societal costs (38). Per diem hospitalization costs for SARS were approximated by using ICD-9 code 769, “respiratory distress syndrome,” which was subsequently multiplied by the average length of stay for hospitalized patients with SARS (15). Public health costs, including contact investigation, were estimated from the Toronto SARS experience (5).

Patient time costs were estimated from data on the median salary of persons living in New York City and included time spent in travel and receiving medical care (17). When applicable, medical and nonmedical costs were adjusted to 2004 U.S. dollars by using the Consumer Price Index. The potential economic effects of a SARS outbreak on tourism or other commercial industries were not considered in the analysis.

Results

If SARS were to resurface during the 2004–2005 respiratory season and the timely establishment of epidemiologic links between SARS cases was not possible, our analysis estimates that the societal costs for New York City would exceed $2.0 billion for each month in which the SARS outbreak and respiratory season coincided.

In our base-case analysis, we found the use of multiplex PCR assays to detect infections with a broad panel of common respiratory pathogens to be the dominant strategy, saving $79 million and resulting in the gain of 8,474 quality-adjusted life-years (QALYs) relative to a strategy of home isolation. If SARS RT-PCR testing were used in conjunction with multiplex PCR assays in our base-case scenario, however, we estimate that costs would increase by about $87 million and have lower effectiveness than multiplex PCR testing alone. These findings are directly related to the very low positive predictive value of the SARS RT-PCR test under low prevalence conditions and the harm resulting from false-positive test results.

If SARS testing were unavailable, confirming an alternate diagnosis for an FRI would be the most effective and least expensive strategy, dominating a strategy of influenza testing alone or home isolation. However, if multiplex PCR testing were also unavailable, home isolation would be the least expensive strategy, albeit less effective than
testing for influenza alone. Rapid influenza testing would be accomplished at an incremental cost of $9.0 million but would result in gains of 5,286 QALYs (incremental cost-effectiveness ratio of $1,702 per QALY gained). If the described outbreak were to unfold, a campaign to increase influenza vaccination rates among the general population before the onset of respiratory season would save an estimated $5.0 million and lead to the gain of 128 QALYs for each percentage of New York City’s population vaccinated above the seasonal baseline.

The total costs, the number of QALYs gained, and the incremental cost-effectiveness of each strategy in the model is shown in Table 3. The results of sensitivity analyses are shown in Table 4 and Figure 1. Algorithms outlining optimal treatment strategies under different testing capabilities are shown in Figure 2.

Discussion

Our analysis indicates that current diagnostic limitations in discriminating SARS from other common FRIs could have enormous public health and economic consequences, particularly if epidemiologic links between SARS cases were to become tenuous. Under such conditions, we found that most costs would not be related to SARS infections themselves, but rather to procedural changes in the management of other FRIs due to the known or perceived presence of SARS.

We report 3 key findings with direct policy relevance. First, in our base analysis, the most efficient mechanism for discriminating SARS infections from other FRIs involves excluding SARS by confirming an alternate diagnosis. This approach is the most cost-effective strategy under low prevalence conditions since the positive predictive value of SARS RT-PCR tests would be extremely low, and false-positive SARS tests would have deleterious societal repercussions. While the Centers for Disease Control and Prevention supports an approach of excluding SARS by confirming an alternate diagnosis (2), caution is advised since SARS coinfection with other respiratory pathogens, including the human metapneumovirus, has been documented (39).

Second, we demonstrate that SARS testing under low prevalence conditions would be detrimental from both a public health and an economic perspective. In our analysis, the low positive predictive value of the SARS RT-PCR test translates into unnecessary costs from diagnostic testing, public health interventions, and lost opportunity costs for persons with false-positive test results. Moreover, negative consequences on quality of life would occur when persons are incorrectly diagnosed as having an infection with SARS. Our sensitivity analyses indicate that SARS diagnostic testing should not be performed unless the prevalence or pretest probability of SARS among persons presenting with FRIs exceeds 0.1%.

Third, the use of influenza vaccination as a means to distinguish SARS from influenza has been debated (40). In our analysis, we find that if SARS reemerged during respiratory season, higher rates of influenza vaccination among the general population would lead to both health benefits and economic savings. These savings would occur by reductions in influenza illness and death, reductions in costs related to the investigation and isolation of persons with FRIs, and increases in the pretest probability of SARS and, therefore, the positive predictive value of SARS diagnostic testing. The policy implications of these findings, however, must be carefully considered in the context of available influenza vaccine supplies and must ensure their prioritization for groups at high risk (40).

Our analysis has several limitations. Foremost was our inability to derive specific estimates of the proportion of FRIs due to specific pathogens. Since the seasonal composition of respiratory viruses and bacteria varies across regions and seasons, we attempted to derive estimates that best reflected seasonal averages. Although national

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**Table 3. Cost-effectiveness of strategies for managing FRIs of undetermined etiology**

<table>
<thead>
<tr>
<th>Available public health strategies</th>
<th>Costs ($ billion)†</th>
<th>QALY gained</th>
<th>Incremental cost-effectiveness (cost per QALY gained)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Home isolation</td>
<td>2.13</td>
<td>0</td>
<td>$1,702</td>
</tr>
<tr>
<td>Influenza testing</td>
<td>2.14</td>
<td>5,286</td>
<td></td>
</tr>
<tr>
<td>Home isolation</td>
<td>2.13</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Influenza testing</td>
<td>2.14</td>
<td>5,286</td>
<td>Dominated</td>
</tr>
<tr>
<td>Multiplex RT-PCR testing†</td>
<td>2.05</td>
<td>8,474</td>
<td>Savings</td>
</tr>
<tr>
<td>SARS + influenza testing</td>
<td>2.19</td>
<td>5,280</td>
<td>Dominated</td>
</tr>
<tr>
<td>SARS + multiplex RT-PCR testing†</td>
<td>2.14</td>
<td>8,429</td>
<td>Dominated</td>
</tr>
<tr>
<td>Multiplex RT-PCR testing†</td>
<td>2.05</td>
<td>8,474</td>
<td>Savings</td>
</tr>
</tbody>
</table>

†FRI, febrile respiratory illness; QALY, quality-adjusted life-year; RT-PCR, reverse transcription–polymerase chain reaction; —, reference category.

†Multiplex RT-PCR testing to detect influenza viruses A and B, respiratory syncytial viruses A and B, parainfluenza viruses 1–3, human metapneumovirus, *Bordetella pertussis*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Legionella pneumophila*, and *L. micdadei*. 
surveillance data on influenza are available, information on other common respiratory pathogens are more limited, since most of these pathogens are self-limited, non-reportable diseases, for which treatment is infrequently sought.

We estimated the sensitivity of current SARS RT-PCR assays to be ≈70% (4); however, we recognize that the type of specimen tested and the timing of collection can influence the test’s sensitivity (4,36). In our base-case scenario, in which SARS represented 0.01% of all circulating FRIs, changes in SARS RT-PCR test sensitivity had a negligible impact on overall societal costs and population health. If the pretest probability of SARS were to increase substantially above our baseline, however, SARS RT-PCR test sensitivity would have an increasingly important influence on the effectiveness of strategies involving SARS testing.

Our reported test sensitivity for the multiplex PCR assays, which detect common respiratory viruses and bacteria, is lower than values reported in the medical literature (20–22). Since estimates in the literature reflect experimental conditions and are essentially measures of test efficacy, we wished to estimate real-world effectiveness of these tests by taking into account factors such as ineffective specimen collection methods, delays in laboratory testing, or other related factors.

Our analysis demonstrates that influenza vaccination would lead to cost-savings, which has been reported in other studies of healthy adults in the pre-SARS era (31,37). However, the specific benefits quantified in our analysis would only be realized if the conditions of the model were to occur, i.e., the reemergence of SARS during a respiratory season, when epidemiologic links between cases are poorly defined.

Finally, our analysis does not adequately address the complexities of microbiologic coinfection in the development of FRIs. While our model allows for multiple positive test results, we assume that only 1 organism is responsible for causing an FRI. This issue is particularly relevant when considering SARS coinfection with other respiratory organisms (39). Nonetheless, in our analysis the effect of SARS coinfection on a population level is minimal given that SARS-CoV infections make up only 0.01% of all FRIs.

Speculation about the reemergence of SARS has prompted heightened surveillance by health officials worldwide. Given that SARS has resurfaced in each of the past 2 respiratory seasons in the absence of accidental laboratory exposures, SARS-CoV may reappear annually at times when FRIs are widely prevalent among the general population. Even if the world does not experience another large-scale, multinational outbreak, healthcare providers around the globe will continue to see patients with nonspecific FRIs who are incidentally returning from SARS-affected areas. This fact underscores the importance of having evidence-based guidelines to facilitate the timely and accurate distinction of SARS infections from other
FRIs of lesser public health importance. Our analysis provides guidance on the most effective and efficient use of resources when managing persons with FRIs of undetermined etiology when the epidemiologic history for SARS is either unavailable or unreliable. Our findings will help policy makers and healthcare practitioners make decisions based on available evidence and avoid decisions that are driven by fear and misinformation.

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Dr. Khan is an infectious diseases specialist with advanced training in preventive medicine and public health. He is an assistant professor of medicine at St. Michael’s Hospital, University of Toronto, where he conducts research on infectious diseases in new immigrant and refugee populations. His additional research interests include population mobility, the global movement of infectious diseases, health economics, and decision and cost-effectiveness analyses.

References


Address for correspondence: Kamran Khan, Inner City Health Research Unit, St. Michael’s Hospital, 30 Bond St, Toronto, Ontario, Canada M5B 1W8; fax: 416-864-5485; email: km.khan@utoronto.ca
Influenza A (H5N1) is endemic in poultry across much of Southeast Asia, but limited information exists on the distinctive features of the few human cases. In Thailand, we instituted nationwide surveillance and tested respiratory specimens by polymerase chain reaction and viral isolation. From January 1 to March 31, 2004, we reviewed 610 reports and identified 12 confirmed and 21 suspected cases. All 12 confirmed case-patients resided in villages that experienced abnormal chicken deaths, 9 lived in households whose backyard chickens died, and 8 reported direct contact with dead chickens. Seven were children <14 years of age. Fever preceded dyspnea by a median of 5 days, and lymphopenia significantly predicted acute respiratory distress syndrome development and death. Among hundreds of thousands of potential human cases of influenza A (H5N1) in Asia, a history of direct contact with sick poultry, young age, pneumonia and lymphopenia, and progression to acute respiratory distress syndrome should prompt specific laboratory testing for H5 influenza.

The 1997 outbreak of avian influenza in Hong Kong challenged the prevailing hypothesis that avian influenza viruses could infect humans only after passing through pigs or other intermediate hosts. In that outbreak, 18 persons were infected with influenza A (H5N1) virus, 6 died (1), and the epidemiologic and virologic evidence strongly suggested that direct contact with infected poultry was the route of transmission (1–3). All known influenza A virus subtypes that express hemagglutinins H1 to H15 and neuraminidases N1 to N9 are found in wild waterfowl (4,5), but only H1, H2, or H3 hemagglutinin subtypes had previously been known to cause human illness. Since 1997, avian outbreaks with some subtypes of influenza A viruses have been reported to cause mostly mild or inapparent infection in humans. For example, 2 mild clinical cases of H9N2 infection occurred in Hong Kong (6), and a large outbreak of conjunctivitis caused by H7N7 occurred in the Netherlands (7).

In late 2003 and early 2004, outbreaks of highly pathogenic avian influenza A (H5N1) virus infection were reported to cause lethal illness among poultry in at least 8 Asian countries (Cambodia, Indonesia, Japan, Laos, South Korea, China, Vietnam, and Thailand) (8). The first human cases were confirmed in Vietnam and Thailand in January 2004, and some clinical features of the first 5 Thai cases and 10 Vietnamese cases have been reported (9,10). Despite the fact that new outbreaks among poultry continued to be reported through the time of this writing (August 2004), human cases have not been recognized outside of Thailand and Vietnam. This finding may be in part because pneumonia is very common, and the distinguishing features of pneumonia caused by influenza A (H5N1) are not widely appreciated. We report the clinical details of 12 confirmed cases in Thailand and compare these with 21 suspected but unconfirmed cases and 577 reported cases that were later excluded. In addition, predictors of severe disease, pathologic features, and epidemiologic exposures are analyzed and discussed.

**Methods**

**Epidemiologic Investigations**

Nationwide surveillance to detect influenza A (H5N1) was initiated by the Thai Ministry of Public Health in December 2003, after outbreaks of sudden death in poultry were reported in some provinces in the central region.
Under this newly established surveillance system, all patients visiting the health services with pneumonia or influenzalike illness were asked if they had been exposed to ill poultry during the preceding 7 days or had resided in an area where abnormal poultry deaths occurred during the preceding 14 days. Influenzalike illness was defined according to the World Health Organization (WHO) recommendations, which require acute fever (temperature >38.0°C) and either cough or sore throat in the absence of other diagnoses. Patients admitted with pneumonia or influenza and either of these poultry exposures were reported through the provincial public health office to the regional disease prevention and control centers and also to Bureau of Epidemiology at the Ministry of Public Health. Throat or nasopharyngeal swabs and serum samples were collected for viral study at the Thai National Institute of Health, Department of Medical Sciences. Staff members from the provincial health office visited family members to confirm history of exposure and assess the household environment.

Patients with confirmed cases of H5N1 were defined as patients reported to the system who had laboratory evidence of influenza A (H5N1) infection. Suspected case-patients were defined as patients with reported exposure to ill poultry and severe pneumonia, or patients with exposure and laboratory evidence of influenza A infection not confirmed as H5N1. Excluded case-patients were all remaining patients reported through the system who did not meet the exposure criteria or who lacked laboratory evidence of influenza A (H5N1) infection, including those with infections caused by influenza A H3 or H1, as well as other laboratory-confirmed pneumonia pathogens.

We performed comparisons of dichotomous variables by using chi-square or Fisher exact tests, as appropriate, and t tests for continuous variables that were normally distributed, or Wilcoxon rank-sum tests for other continuous variables. We considered p values of <0.05 to be significant.

Laboratory Investigations

Respiratory specimens (including nasopharyngeal aspirates, nasopharyngeal swabs, nasal swabs, or throat swabs) were collected and stored in viral transport medium. Blood cultures were obtained from all patients on admission, and serum samples for mycoplasma titer and cold agglutinin testing were obtained when available. Paired serum samples taken at least 14 days apart, if available, were collected for serologic confirmation of H5N1 infection. An adequate sample was defined as any of the above respiratory specimens collected from day 2 to day 14 after onset of fever.

All specimens were submitted for testing at the National Institute of Health of Thailand, except 1, which was tested at Virology Laboratory at Siriraj Hospital, Mahidol University. Methods used for H5 identification were in accordance with those recommended by the WHO reference laboratories for influenza (11). Specifically, specimens in transport medium were tested by reverse transcription–polymerase chain reaction (RT-PCR) to detect nucleic acids of influenza A and B and injected onto a Madin-Darby canine kidney (MDCK) cell monolayer for viral isolation. Nasopharyngeal aspirates were agitated and centrifuged to separate the epithelial cells. Sediments of epithelial cells were tested for influenza A and B by immunofluorescence assay (IFA) with specific monoclonal antibodies. Specimens positive for influenza A were further tested for subtypes H1, H3, and H5 with specific monoclonal antibodies. The supernatant was tested by RT-PCR and viral isolation for the other types of specimens (12).

Specimens positive for influenza A by RT-PCR were further tested for subtypes H1, H3, and H5 by using specific primer sets. The H5-specific primer set was as follows: H5-1 GCC ATT CCA CAA CAT ACA CCC, and H5-2 TAA ATT CTC TAT CCT CCT TTC CAA, with an expected product size of 358 bp (12,13). If results were negative for all subtypes or positive for H5, they were confirmed by real-time RT-PCR using primer/probe H5 as follows: InfA_TH5_A, InfA_TH5_F, InfA_TH1_c, and InfA_TH5_fl (14). For viral isolation, if a cytopathic effect was observed, IFA was performed to identify the virus in infected cell cultures by using specific monoclonal antibodies to H1, H3, and H5. If a cytopathic effect was not observed in the first passage, the culture medium passed in MDCK for a second time. If no cytopathic effect occurred, the negative cell culture was confirmed by IFA with pooled viral monoclonal antibodies.

Specimens were considered positive for avian influenza virus if the viral culture was positive and was confirmed by IFA with H5-specific monoclonal antibody provided by the WHO, if epithelial cells in clinical specimens were IFA positive for H5, or if the RT-PCR was positive with H5 specific primers (RT-PCR or real-time RT-PCR). A specimen was negative for avian influenza virus if IFA, RT-PCR or real-time RT-PCR, and viral isolation (second passage) were negative.

Clinical Investigations

All potential case-patients reported through the surveillance system needed basic demographic, exposure, and clinical information recorded, as well as specimens submitted, for the purpose of case classification. Patients with suspected cases were reviewed in more detail by telephone or written correspondence with the attending physician. Laboratory-confirmed case-patients had a thorough review with standardized forms of all medical records, chest radiographs, and laboratory data by the attending physicians.
Respiratory failure was defined as requiring ventilatory support and cardiac failure as requiring inotropic drug support. Liver dysfunction was diagnosed when serum aspartate aminotransferase (AST) or alanine aminotransferase (ALT) was ≥8 times the upper limit of normal. Renal dysfunction was diagnosed when serum creatinine was ≥1.5 mg/dL. Bone marrow dysfunction was diagnosed when all 3 of the cell lines in the peripheral blood (erythrocytes, leukocytes, and platelets) were below the lower limit of normal. Leukopenia was defined as a total leukocyte count below the following age-specific cutoffs: 1–3 years <6,000, 4–7 years <5,500, and >8 years <4,500 cells/mm³. Lymphopenia was defined as an absolute lymphocyte count <1,500 cells/mm³, and thrombocytopenia was defined as a platelet count <150,000/mm³ (15).

The attending radiologist classified chest radiograph findings as normal, interstitial infiltrates, lobar infiltrates, or combinations of these by using standard criteria. Acute respiratory distress syndrome (ARDS) was defined when clinical deterioration was associated with chest radiographs showing diffuse bilateral infiltrates accompanied by severe arterial hypoxemia.

**Results**

From January 1 to March 31, 2004, a total of 610 cases were reported from 67 of 76 provinces in Thailand. After thorough review of the clinical, epidemiologic, and laboratory findings, we identified 12 confirmed and 21 suspected cases. The onset of illness of the first confirmed case was on January 3, and the last was on March 2 (Figure 1). A total of 577 cases were excluded, including 38 who had positive RT-PCR tests for influenza A (H3) infection, 48 seropositive for *Mycoplasma pneumoniae*, and 10 for Chlamydophila pneumoniae.

Table 1 compares characteristics of patients with confirmed, suspected, and excluded cases. Confirmed case-patients tended to be younger than suspected case-patients and more often had fatal disease than excluded patients (p < 0.0001). Reported poultry exposure was similar in all groups, but all confirmed patients had an adequate laboratory specimen, whereas 10% of suspected patients and 19% of excluded patients did not. All patients with an adequate laboratory specimen had testing completed.

Table 1. Characteristics of 12 confirmed, 21 suspected, and 577 excluded human cases of avian influenza A (H5N1) in Thailand, 2004

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Confirmed</th>
<th>Suspected</th>
<th>Excluded</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>12</td>
<td>21</td>
<td>577</td>
</tr>
<tr>
<td>Median age (y) (range)</td>
<td>12 (2–58)</td>
<td>33 (1–67)</td>
<td>12 (1–92)</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>67</td>
<td>71</td>
<td>59</td>
</tr>
<tr>
<td>Poultry contact (%)</td>
<td>58</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>Adequate* specimen (%)</td>
<td>100</td>
<td>90</td>
<td>81</td>
</tr>
<tr>
<td>Death (%)</td>
<td>67</td>
<td>38</td>
<td>4</td>
</tr>
</tbody>
</table>

* Adequate was defined as a respiratory specimen obtained 2–14 days after onset of fever.

Of the 12 confirmed cases, 7 were in children <14 years of age, and 5 were in adults (Table 2). Fever was often the first symptom, and dyspnea often occurred a median of 5 days after illness onset (range 1–16). During the initial evaluation at hospital, all patients were found to have fever, cough, and dyspnea, and almost half had myalgia and diarrhea. The hospital course was characterized by intermittent high fevers and persistent cough productive of thick sputum. One patient had a small amount of hemoptysis. Later in the course of the disease, organ failure or dysfunction was commonly observed, including respiratory failure in 9 (75%) patients, cardiac failure in 5 (42%), and renal dysfunction in 4 (33%).

Routine laboratory tests on admission showed leukopenia in 7 (58%) patients, lymphopenia in 7 (58%), and thrombocytopenia in 4 (33%) (Table 2). During the course of illness, elevated serum transaminase values were documented in 67% of patients, although they were >8 times normal in only 17%. Serum creatinine rose to >1.5 mg/dL in 4 (33%) patients. Blood cultures were negative in all patients. One adult patient was found to be HIV seropositive, and 1 pediatric patient had a mycoplasma titer of 1:160.

Admission leukocyte and platelet counts tended to be more depressed in the 8 patients who died than in the 4 patients who survived (Figure 2). ARDS was associated with a fatal outcome (p = 0.02), and depressed admission leukocyte and platelet counts were also associated with
ARDS development. The most pronounced difference was in the absolute lymphocyte count, with a mean of 995 in those with ARDS vs. 2,825 in those without (p = 0.002). A low absolute lymphocyte count on admission was also associated with death (mean of 1,056/mm³ in those who died compared to 2,247/mm³ in those who survived, p = 0.05). In addition, the median total leukocyte count was 3,700/mm³ for those who died compared with 6,010/mm³ for those who survived (p = 0.09), and the median platelet count was 145,000/mm³ in those who died and 243,000/mm³ in those who survived (p = 0.17).

All 12 patients had abnormal chest radiographs a median of 7 days after onset of fever (range 3–17 days). Two patients had interstitial infiltration, and 10 had patchy lobar infiltrates in a variety of patterns (single lobe, multiple lobes, unilateral or bilateral distributions). The radiographic pattern progressed to diffuse bilateral ground-glass appearance, with clinical features compatible with ARDS, in all 8 patients who died and in 1 patient who survived (Figure 3). A pneumothorax developed in 1 patient during mechanical ventilation. The median time from onset to ARDS development was 6 days (range 4–13).

Treatment for all patients included broad-spectrum antimicrobial drugs aiming to cover most of the usual and unusual respiratory pathogens. Eight patients were treated with corticosteroid drugs, including 2 patients who survived and 6 patients who died. Seven patients were treated with the neuraminidase inhibitor oseltamivir at various stages of illness. Treatment tended to have been started earlier in those who survived (a median of 4.5 days from onset compared with 9 days for those who died), and both survivors who were treated received the complete 5-day course of drug, whereas 2 of 5 patients who died received the complete 5-day course (Figure 4).

Pathologic tissues from the lungs and spleen of 3 patients were available for analysis in the current report. A fourth patient (number 6) was autopsied but is the subject...
of a separate report. The lungs showed diffuse alveolar damage, with hyaline membrane formation, reactive fibroblasts, and areas of hemorrhage. The spleen had numerous atypical lymphocytes but no viral inclusions (Figure 5).

All 12 confirmed patients resided in a village with abnormal chicken deaths (Table 3). Nine lived in a house whose backyard chickens died unexpectedly. Direct contact with dead chickens was reported in 8 patients, with a median of 4 days between the last exposure and the onset of symptoms (range 2–8 days). The details of exposures in these case-patients and in groups of matched controls are the subject of a separate investigation.

Discussion

The detection of a few human infections with influenza A (H5N1) in the context of an avian epizootic involving at least 8 countries has proven to be a considerable challenge.

The history of direct contact with sick and dying poultry, young age of many patients, pneumonia and lymphopenia, and progression to ARDS in spite of broad-spectrum antimicrobial treatment indicate that specific laboratory testing for H5 influenza should be sought. Ideally, such information should be routinely collected and used to minimize opportunities for recombination of this virulent new pathogen with existing human influenza viruses.

The optimal treatment for case-patients with suspected H5 infection is not known, but in vitro susceptibility testing suggests that resistance to adamantanes is a common feature of H5 isolates from 2004 (11), whereas these isolates remain susceptible to the neuraminidase inhibitors. Although no controlled data are available on which to base treatment recommendations, our observations were that the 4 patients who survived tended to have been treated with oseltamivir earlier in the course of their disease. We advocate using this agent in the early treatment of case-patients with suspected H5N1 influenza, in agreement with the recommendations of WHO (16). Controlled trials of oseltamivir and corticosteroid treatment would be helpful in confirming or refuting any specific benefit.

Approximately 1,820,387,000 persons live in the 8 countries in Asia that reported poultry epidemics with avian influenza A (H5N1) in 2004 (=30% of the world’s population). One community survey in Thailand found that 12%–61% of rural residents had regular contact with backyard birds (17). Thus, the 12 cases we report likely represent the end result of hundreds of thousands of potential exposures and an unknown number of human cases. Perhaps in part because few distinctive features of human disease caused by avian influenza have been reported, and specific diagnostic tests for H5 disease are not widely available, human cases have been few and have been reported only from Vietnam and Thailand.

Among >600 possible case-patients reported to the Thai Ministry of Public Health, most reported clear exposure to
sick poultry, and the demographic characteristics were similar among confirmed, suspected, and excluded groups. All confirmed patients had an adequate specimen submitted and processed, whereas 10% of the suspected patients and 19% of those excluded had inadequate specimens. The availability of properly collected specimens and use of specific laboratory tests for influenza A (H5N1) will be essential for monitoring the ongoing risk from this pathogen in East Asia.

Human infections with highly pathogenic avian influenza may be easy to miss in the context of the regular incidence of pneumonia in much of rural Asia, where the capacity to make specific etiologic diagnoses remains limited. We found certain features to be helpful, as have investigators in Vietnam (9). Eight of the 12 patients had direct exposures to ill poultry 2–8 days before onset. Seven of the 12 were young children, and routine laboratory testing at the time of admission to hospital identified marked lymphopenia in 8. Although the initial chest radiographs would not immediately identify these cases as unusual, deaths in children and younger adults from hospitalized, radiographically confirmed pneumonia typically range from 1% to 10% and from 1%-5% among patients with radiographically confirmed pneumonia in rural Thailand (18–20). Thus, the progression in 9 of the 12 patients to ARDS, followed by the death of 8 patients, separates these cases as a form of unusually severe pneumonia.

The disease may in fact be more severe than that seen in Hong Kong in 1997. Of the 34 cases officially reported to the WHO in 2004, 23 (68%) patients died compared to 6 (33%) of those in Hong Kong (p = 0.02). Several lines of evidence indicate that the H5N1 viruses have evolved to more virulent forms since 1997, with different antigenic structure (21), internal gene constellations (22), and an expanded host range (23,24). This virologic evolution may be a factor in the persistence of H5N1 viruses in the avian populations. Since the 1997 outbreak, Hong Kong has experienced a series of reintroductions of H5 viruses, despite instituting unusually stringent control measures, including the culling of all poultry in the territory, strict regulations of live poultry markets, and monthly “off days,” in which all markets are emptied and cleaned (22,25). H5 outbreaks in poultry have also recurred repeatedly in Thailand,
Vietnam, and elsewhere despite intensive control measures (26), and recurrences should be anticipated for the foreseeable future.

If H5 viruses do persist, they will likely continue to evolve, potentially to forms more easily transmitted from person to person. We identified no suspected or confirmed cases among Thai health personnel, supporting the experience from Vietnam and Hong Kong that efficient human-to-human transmission has not occurred (9,27). Serologic studies of healthcare workers and household contacts of patients in the 1997 Hong Kong outbreak provided evidence of occasional seroconversions associated with close exposures. These findings indicate that inefficient transmission is possible and reinforce the importance of infection control precautions (28,29). Studies of healthcare workers and poultry cullers in Thailand are under way to determine whether similar seroconversions may have occurred after exposure to patients with the 2004 viruses.

In addition to gradual mutational changes, H5 viruses have the potential to reassort with existing human influenza viruses to produce a strain with high virulence and efficient transmissibility. In this context, the known pattern of human influenza isolations in Thailand raises particular concerns about control of avian influenza during the months from June to August, when human influenza can be expected to peak (Figure 6).

Table 3: Brief history of exposure of the 12 confirmed case-patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Province/sex/age (y)</th>
<th>Exposure history</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Supanburi/M/2</td>
<td>Raised chickens in backyard. Chickens died unexpectedly 5 days before illness onset. Frequently played with chickens and had direct contact with carcasses.</td>
</tr>
<tr>
<td>2</td>
<td>Uttadit/F/27</td>
<td>Raised chickens in backyard, but chickens did not die. Two months before onset, ducks in a nearby area died unexpectedly.</td>
</tr>
<tr>
<td>3</td>
<td>Nakomratchasima/M/31</td>
<td>Raised chickens in backyard. Three days before onset, chickens started to die. The last patient died on the date he became sick. He buried all carcasses.</td>
</tr>
<tr>
<td>4</td>
<td>Lopburi/F/46</td>
<td>Raised 60 chickens in back yard. All chickens died unexpectedly 1 month before onset. She burned and buried carcasses without protection.</td>
</tr>
<tr>
<td>5</td>
<td>Khonkaen/M/5</td>
<td>Raised fighting cocks that died 4 days before onset. Reported direct contact with carcasses.</td>
</tr>
<tr>
<td>6</td>
<td>Kanchanaburi/M/6</td>
<td>No poultry in family. Helped slaughter one ill chicken 2 days before onset.</td>
</tr>
<tr>
<td>7</td>
<td>Sukhothai/M/6</td>
<td>Mother slaughtered 2 ill chickens in house 4 days before onset. No direct contact with chickens. Mother got sick on same day and died without laboratory confirmation.</td>
</tr>
<tr>
<td>8</td>
<td>Kanchanaburi/M/6</td>
<td>Chickens in backyard died unexpectedly. Grandfather slaughtered ill chickens. No direct contact with chickens but played near slaughtering area.</td>
</tr>
<tr>
<td>9</td>
<td>Supanburi/M/7</td>
<td>No poultry in family. Frequently played on ground near a chicken farm that reported unexpected poultry deaths.</td>
</tr>
<tr>
<td>10</td>
<td>Chaiyapoom/M/13</td>
<td>Helped raise chickens in backyard. Eight days before onset, chickens died unexpectedly and patient assisted with slaughtering.</td>
</tr>
<tr>
<td>11</td>
<td>Patumthani/F/39</td>
<td>Factory worker living in province A during weekdays but in province B on weekends. Fighting cocks lived at a neighboring house. Province B reported outbreaks 2 months before onset. No contact with live or dead chickens.</td>
</tr>
<tr>
<td>12</td>
<td>Supanburi/F/58</td>
<td>Raised 40–50 chickens in backyard. Chickens started to die 5 days before onset. Buried and slaughtered ill chickens every day until onset date.</td>
</tr>
</tbody>
</table>

After the official announcement of the first human case on January 23, a national public education campaign was carried out through the mass media and thousands of village health volunteers. Villagers, especially children, were informed to avoid exposure to ill poultry. According to the Department of Livestock, ≈40 million chickens in 160 affected villages of 41 provinces were slaughtered from January to May 2004. Within 2 months of implementing widespread poultry culling, quarantine measures, and the public education campaign, the number of potential cases reported to the surveillance system decreased dramatically and confirmed human cases ceased, despite interim improvement in the quality of surveillance and laboratory testing. The course of this outbreak reconfirms observations from the smaller 1997 outbreak in Hong Kong that early detection of human cases and aggressive public health and agricultural interventions can save lives (30).

We believe this outbreak of H5N1 is unlikely to be the last because of the formidable challenges in eradicating the virus, and the potential reservoir in waterfowl (31). We must be well prepared for a future surge of either small or

Figure 6. Seasonal variation in viral isolations of human influenza A (H3N2), A (H1N1), and B, in Thailand.
large outbreaks, early detection must be ensured, information shared, and control measures for both animals and humans promptly implemented.

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Dr. Chotpitayasunondh is a pediatric infectious disease specialist at Queen Sirikit National Institute of Child Health, Bangkok, Thailand. He serves as a senior medical consultant for the Thai Ministry of Public Health on emerging and reemerging infectious diseases, including SARS and avian influenza.

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Address for correspondence: Scott F. Dowell, Department of Disease Control Building 7, Ministry of Public Health, Tivanon Road, Nonthaburi 11000, Thailand; fax +66-2-580-0911; email sdowell@cdc.gov
To establish whether human-to-human transmission of influenza A H5N1 occurred in the healthcare setting in Vietnam, we conducted a cross-sectional seroprevalence survey among hospital employees exposed to 4 confirmed and 1 probable H5N1 case-patients or their clinical specimens. Eighty-three (95.4%) of 87 eligible employees completed a questionnaire and provided a serum sample, which was tested for antibodies to influenza A H5N1. Ninety-five percent reported exposure to ≥1 H5N1 case-patients; 59 (72.0%) reported symptoms, and 2 (2.4%) fulfilled the definition for a possible H5N1 secondary case-patient. No study participants had detectable antibodies to influenza A H5N1. The data suggest that the H5N1 viruses responsible for human cases in Vietnam in January 2004 are not readily transmitted from person to person. However, influenza viruses are genetically variable, and transmissibility is difficult to predict. Therefore, persons providing care for H5N1 patients should continue to take measures to protect themselves.

Direct transmission of H5N1 viruses of purely avian origin from birds to humans was first described during an outbreak among poultry in Hong Kong in 1997. In that outbreak, 6 of 18 confirmed human H5N1 case-patients died (1), and serologic evidence was found for asymptomatic infection in humans after exposure to infected poultry (2). Avian-to-human transmission of influenza viruses is believed to be infrequent because of host barriers to infection, such as cell receptor specificities, and because the acquisition by avian viruses of the ability for human-to-human transmission requires either genetic reassortment with a human influenza strain or genetic mutation (3). However, a study of household and social contacts of Hong Kong H5N1 case-patients found evidence, although limited, for human-to-human transmission (4). Further evidence was provided by a study of healthcare workers (HCWs), which found that significantly more HCWs exposed to patients with H5N1 infection were positive for H5 antibody than nonexposed HCWs (3.7% vs. 0.7%); 2 HCWs seroconverted after exposure to H5N1-infected patients, in the absence of known poultry exposure (5). These 2 studies provided the first evidence, although limited, of human-to-human transmission of H5N1 viruses of purely avian origin.

On December 12, 2003, influenza A H5N1 viruses were detected among poultry at a farm near Seoul, the Republic of Korea (6), and outbreaks of H5N1 in poultry were subsequently reported in 8 other Asian countries (Japan,
Indonesia, Vietnam, Thailand, Laos, Cambodia, China, and Malaysia); a situation that the Office International des Epizooties has called “a crisis of global importance” (7). Human case-patients infected with H5N1 related to these poultry outbreaks were identified in Vietnam and Thailand in January 2004, and on September 28, 2004, possible human-to-human transmission was reported in a family cluster in Thailand (8).

Concern is widespread that the current situation in Asia favors the emergence of a highly pathogenic influenza virus with the ability for efficient transmission from person to person, which would lead to an influenza pandemic. While experiences from Hong Kong in 1997 indicate that human-to-human transmission of purely avian H5N1 viruses is possible but not sustainable, genetic alterations over time may lead to subsequent H5N1 infections behaving quite differently. An understanding of the current and absolute risk for human-to-human transmission of circulating avian H5N1 viruses is vital to guide appropriate public health and infection control responses and to inform pandemic preparedness. Unfortunately, little data are available to quantify the transmissibility of the H5N1 strains currently circulating in poultry in Asia. To investigate the risk for human-to-human transmission of avian H5N1 viruses to hospital employees, we undertook a cross-sectional seroprevalence study among employees of 1 hospital in Vietnam, who were exposed to confirmed and probable H5N1 case-patients or their clinical samples.

Methods

From December 27, 2003, to January 19, 2004, 4 children, 4–12 years of age, with confirmed H5N1 infection and 1 with probable H5N1 infection were admitted and treated at the National Pediatric Hospital (NPH), Hanoi, Vietnam. Detailed information regarding the 4 confirmed H5N1 patients has been published elsewhere (9). Eligible study participants were hospital employees who had possible exposure to the patients with confirmed or probable H5N1 infections, such as by working in wards or entering rooms where H5N1 patients were admitted, or having handled clinical specimens from these patients. To allow sufficient time for seroconversion in any infected HCWs, the study took place 29 days after discharge of the last confirmed H5N1 patient. All eligible participants were provided with written and verbal information about the study and gave written consent for participation.

Definitions

We used the following definitions in our study: study period, from date of admission of first confirmed case-patient (December 27, 2003) to 29 days after discharge of the last confirmed case-patient (February 17, 2004); confirmed H5N1 primary case patient, a patient admitted to NPH, Hanoi, from December 27, 2003, to January 19, 2004, inclusive with a respiratory illness and influenza A H5N1 virus detected in clinical specimens by either viral culture or reverse transcriptase–polymerase chain reaction; probable H5N1 primary case patient, a patient admitted to NPH, Hanoi, from December 27, 2003, to January 19, 2004, inclusive with a respiratory illness and high titer of antibodies to influenza A/H5 detected in a single serum sample; possible H5N1 secondary case, a hospital employee who had fever (if measured ≥38°C), and at least 1 of 3 symptoms (cough, shortness of breath, sore throat), and contact with a confirmed or probable influenza A H5N1 case-patient, in the absence of exposure to poultry.

Serologic Testing

All participants were asked to provide a single blood sample. Serum samples were collected on February 17, 2004, immediately processed, stored at −25°C, and shipped frozen on dry ice to the Government Virus Unit, Department of Health, Hong Kong, China. Serum samples were tested for antibodies to influenza A H5N1 virus by microneutralization test as described by Rowe et al. (10) with H5N1 viruses A/Vietnam/1194/2004 and A/Vietnam/3212/2004. Serum was considered to be positive in the microneutralization test if an anti-H5 titer of >40 was obtained in 2 independent assays. Microneutralization antibody-positive serum was adsorbed with influenza A/H1N1 virus to eliminate the possibility of detecting antibody that was cross-reactive among influenza virus of different subtypes, and the microneutralization test was repeated. No change in antibody titer after adsorption indicated the presence of anti-H5 antibody, while a ≥4-fold reduction in microneutralization after adsorption was interpreted as evidence for significant cross-reaction. Microneutralization antibody-positive serum was subjected to Western blot analysis by using recombinant protein from A/HK/156/97 virus.
Results

Study Participants

Of 87 eligible staff members who had possible exposure to H5N1 patients, 83 (95.4%) completed a questionnaire and provided a serum sample (Table 1). The median age of employees was 37.4 years (range 22–55 years), and 53 (64%) were female. Most employees (97.6%) were residents of Hanoi City, Vietnam. Of the 83 employees, 51 (61%) were nurses or nurse’s aides, 19 doctors (23%), 7 (8%) laboratory employees, and 6 (7%) other. Thirty-seven (45.1%) worked in the intensive care unit (ICU), 30 (36.6%) in the infectious diseases department, 8 (9.8%) in the laboratory, 6 (7.3%) in radiology, and 1 in the hematology department. More than two thirds (68.3%) of the employees reported receiving influenza vaccine in 2004, and 1 person reported taking oseltamivir for treatment of influenzalike illness since December 27, 2003. No respondents took oseltamivir as prophylaxis against influenza infection. In total, 76.8% of participants reported contact with 2 or 3 influenza A H5N1 patients. Four hospital employees (4.9%) reported no contact with H5N1 patients; they were all laboratory personnel who had handled clinical material from H5N1 patients. Median duration of exposure to the hospitalized H5N1 primary case-patients reported was 82 hours, ranging from 1 to 299 hours (N = 78). Most participants reported always wearing protective masks (94.8%), gloves (61.5%), and eye-protection (31.6%) while caring for H5N1 patients (Table 2).

Clinical Symptoms

The figure summarizes the symptoms reported by hospital employees during the study period. Overall, 59 (72.0%) employees reported symptoms during the study period; 66.0% of these had onset of symptoms within 1 to 7 days after exposure to a H5N1 patient. Median duration of reported illness was 5 days (range 0–40 days). Three persons (5.4%) were too ill to work; none were admitted to the hospital. Two persons (2.4%) who worked in ICU met the possible secondary H5N1 case-patient definition. They reported contact with patients but not with sick poultry or pigs, and neither worked in the laboratory. Both reported receiving the 2003-2004 influenza vaccine and denied taking oseltamivir. Table 3 summarizes reported contact with poultry and pigs by participants. Approximately 1 quarter of participants (25.6%) reported the presence of poultry outside their homes, and 2 HCWs (9.5%) reported that poultry had died in the past month. The 2 possible H5N1 secondary case-patients did not report have poultry dying outside their homes within the previous month.

H5N1 Antibody Prevalence

Samples were obtained from all 83 participants, including the 2 with possible secondary cases, and none were positive for antibodies to influenza A H5N1. One sample initially had an antibody titer of 160 and 640 against A/Vietnam/1194/2004 and A/Vietnam/3212/2004, respectively. However, microneutralization tests using influenza A H1N1 viruses showed a high titer of 10,240, and microneutralization repeated after adsorption with influenza A H1N1 virus showed an 8-fold reduction in the antibody titer, which was interpreted as indicating a cross-reacting anti-N1 antibody.

Discussion

No evidence was found of nosocomial transmission of H5N1 viruses among 83 hospital employees with exposure...
to 4 confirmed and 1 probable H5N1 case-patients or their clinical samples. A number of possible factors may explain these findings: a lack of infectivity of the patients at the time of admission; the effective use of personal protective equipment (PPE) and infection control; low sensitivity of the antibody detection method; lack of susceptibility of HCWs, or a lack of transmissibility of this particular H5N1 strain.

No data are available on the duration of H5N1 virus shedding in children. However, for human influenza viruses, viral shedding at high titers is generally more prolonged in children, and virus can be recovered up to 6 days before and 21 days after the onset of symptoms. The H5N1 patients in this study were admitted with severe illness 3–7 days after onset of symptoms and PCR-positive specimens were obtained from the 4 confirmed case-patients on the day 1 (1 patient), day 2 (1 patient), and day 3 (2 patients) after admission. In addition, live virus was cultured from samples taken from 2 of the patients on days 1 and 3 after admission. In addition, live virus was cultured from samples taken from 2 of the patients on days 1 and 3 after admission. None of the patients were treated with oseltamivir because this was not available at the time (9). Two of the patients were treated orally with the nucleoside analogue ribavirin during their admission, 1 on day 4 after admission, and the other on day 1 (9). However, the 2 other confirmed case-patients and the probable case-patient did not receive antiviral treatment and, if human infection with H5N1 is associated with viral shedding, these patients would be expected to be contagious during their admission.

Most hospital employees (94.8%) reported that they always wore masks while caring for H5N1 patients, and often the reported type of mask was an N95 respirator. However, N95 respirators were first available in NPH on January 7, and some employees reported wearing N95s before this date. Therefore, reported PPE use in this study may be biased by inaccurate recall or a tendency to report behavior that HCWs know is recommended. Enhanced infection control practices and PPE were instituted on January 7, and the diagnosis of avian influenza was first confirmed on January 9. Therefore some HCWs in this study were likely exposed to H5N1 patients without optimal PPE or infection control.

Oseltamivir prophylaxis was not used by any of the staff in this study and therefore did not play a role in protecting HCWs. Whether the HCWs in the study were protected by cross-reactive immunity to other influenza A subtypes is hard to assess. One possible explanation for the observation that most confirmed H5N1 case-patients are reported in children or young adults is that older adults are protected by cross-reactive immunity from previous exposure to other influenza A viruses. This hypothesis requires further investigation.

Serum samples were taken from HCWs at least 29 days after last possible exposure and at a time when the antibody response to exposure would be expected to be detectable (4). Based on a small number of samples, the sensitivity of microneutralization test in detecting antibodies to H5N1 in children and adults is 88% and 80%, respectively, while the specificity is 100% and 93%, respectively (10). Also, the microneutralization assay utilized H5N1 strains isolated from human patients in North Vietnam, so the negative results are unlikely to be false negatives due to a poor match between antigen and antibody. False-positive results are perhaps more likely, and 1 sample was initially positive but appeared to be due to cross-reacting anti-N1 antibody.

Epidemiologic evidence from Vietnam and Thailand clearly indicates that sustained human-to-human transmission of H5N1 has not yet occurred. Most reports of
H5N1-infected patients have been sporadic, and despite the evidence from Hong Kong of human-to-human transmission and the occurrence of family clusters of H5N1 in Vietnam and Thailand, no evidence indicates that influenza A H5N1 has ever caused >1 generation of human-to-human transmission. Although this study has not distinguished the inherent transmissibility of the virus from the influence of infection control or host resistance, the data provides further reassurance that the risk for human-to-human transmission of currently circulating avian H5N1 viruses is low. Studies among household members of confirmed H5N1 case-patients will provide additional information on the risk for human-to-human transmission in the absence of infection control measures.

While the absolute risk for human-to-human transmission of avian H5N1 viruses may be low at this time, the high case-fatality proportion among recent human H5N1 patients demonstrates that the individual consequences of infection are very serious, and intensive measures to protect healthcare workers and laboratory staff against infection remain warranted. The risk of person-to-person transmission of H5N1 viruses could increase in the future. Consequently, every H5N1 case should be managed by clinicians and public health professionals with the assumption that human-to-human transmission can occur and that the risk for such transmission is unpredictable.

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Dr. Liem is director of the National Pediatric Hospital, Hanoi, Vietnam.

References


Mirna Du Ry van Beest Holle, Center for Infectious Disease Epidemiology, National Institute for Public Health and the Environment, PO Box 1, 3720 BA, Bilthoven, the Netherlands; fax: 31-30-2744409; email: mirna.du.ry@rivm.nl

The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.
Blood culture–negative endocarditis is common in Algeria. We describe the etiology of infective endocarditis in this country. Samples from 110 cases in 108 patients were collected in Algiers. Blood cultures were performed in Algeria. Serologic and molecular analysis of valves was performed in France. Infective endocarditis was classified as definite in 77 cases and possible in 33. Causative agents were detected by blood cultures in 48 cases. All 62 blood culture–negative endocarditis cases were tested by serologic or molecular methods or both. Of these, 34 tested negative and 28 had an etiologic agent identified. A total of 18 infective endocarditis cases were caused by zoonotic and arthropodborne bacteria, including *Bartonella quintana* (14 cases), *Brucella melitensis* (2 cases), and *Coxiella burnetii* (2 cases). Our data underline the high prevalence of infective endocarditis caused by *Bartonella quintana* in northern Africa and the role of serologic and molecular tools for the diagnosis of blood culture–negative endocarditis.

In Algeria, infective endocarditis is common. Vegetations graft primarily on lesions of rheumatic heart disease (1,2). The rate of blood culture–negative endocarditis in Algeria is as high as 76% (2), which leads to difficulty in antimicrobial treatment. Most cases of blood culture–negative endocarditis have been thought to be caused by previous antimicrobial therapy. Infective endocarditis prognosis is often obscured by delayed diagnosis and a lack of specific treatment. In Algeria, poor socioeconomic level and lack of medical follow-up of patients are among the factors associated with endocarditis. The concentration of medical infrastructures in the northern part of the country leads to the referral of patients with serious illnesses, such as endocarditis, to northern hospitals, especially within Algiers (Figure 1). Algiers, the capital and largest city with ~5 million inhabitants, has 7 hospitals, including 6 cardiology and 5 cardiac surgery wards. These wards receive patients with endocarditis, either for diagnosis and treatment or for corrective surgery of postendocarditis lesions. A retrospective analysis of Algerian infective endocarditis cases showed streptococci and staphylococci were the leading causes, followed by less frequent causes, such as enterobacteria and *Haemophilus* spp. (2). A high percentage of blood culture–negative endocarditis was noted. However, no study has evaluated the agents responsible for blood culture–negative endocarditis. New serologic and molecular tools, which have improved the etiologic diagnosis of infective endocarditis, have not been used to clarify the unknown role of fastidious bacteria (3–11). In our study, samples were collected from 110 patients with suspected cases of endocarditis. All samples were analyzed prospectively by using conventional microbiologic methods in Algiers. When available, cardiac valves and serum samples were stored to perform retrospective analysis at the Unité des Rickettsies (Marseille, France).

**Material and Methods**

**Patients**

Clinicians usually diagnose infective endocarditis by using the modified Duke criteria, which includes 3 major criteria (blood cultures typical of infective endocarditis, vegetations on echocardiography, and *Coxiella burnetii* serologic testing with immunoglobulin [Ig] G phase I titer ≥1:800) and 7 minor criteria (positive blood cultures, fever, previous heart disease, arterial embolism, positive results on serologic examination for endocarditis bacterial pathogens, immunologic disorders, and atypical but compatible findings on echocardiography) (12). Definite infective endocarditis is diagnosed if any of the following conditions is met: 2 major criteria exist; 1 major criterion and 3 minor criteria; or 5 minor criteria. Possible infective endocarditis is considered if 1 major criterion and 1 minor criterion or 3 minor criteria exist. On the basis of these criteria, we could locate 110 cases in 108 patients with
definite or possible infective endocarditis in 5 cardiology wards and 2 cardiac surgery wards in Algiers during a 42-month period (June 2000–December 2003). For each patient, an information sheet with epidemiologic, clinical, echocardiographic, and biologic data was filled out. A minimum of 3 blood cultures were sampled per patient. Thirty-eight cardiac valve specimens from 38 (35.4%) patients were sampled and stored at –80°C. Thirty-seven cardiac valve specimens from another 30 (27.3%) patients were formalin-fixed for pathologic testing. Sixty-one serum samples from 61 (55.5%) patients were available.

**Blood Cultures**

Either Castaneda Aer/Anaer (Bio-Rad, Marnes-La-Coquette, France) or broth for blood culture (Institut Pasteur d’Algérie, Algiers, Algeria) were used as blood-culture medium and were incubated at 37°C. If signs of culture appeared, a blood sample was taken from the culture bottle and Gram staining on Columbia blood agar (BioMérieux, Marcy L’Etoile, France) and chocolate agar (BioMérieux) was performed. Agar plates were incubated in 5% CO₂ at 37°C. In the event of culture, the microorganism was identified by API identification tests (BioMérieux). At day 15 of incubation, if cultures remained negative, an enrichment of each bottle was processed on Todd-Hewitt broth (Institut Pasteur d’Algérie) supplemented with Polyvitaminic Supplement (Bio-Rad) at 35°C for 15 days in 5% CO₂. We performed direct Gram staining and identified colonies as described above.

**Axenic Culture**

Thirty-eight excised cardiac valves were examined. If macroscopic lesions of infective endocarditis were detected, we attempted to divide the valve into 3 parts to be used for bacteriologic analysis, storage at –80°C, and histologic analysis. Portions of valve tissue were ground with a mortar and pestle and cultured on Columbia blood agar and chocolate agar supplemented with Polyvitaminic Supplement (Bio-Rad) at 35°C for 15 days in 5% CO₂. We performed direct Gram staining and identified colonies as described above.

**Cell Culture**

Cell cultures were performed in France. Specimens from 12 cardiac valves positive on polymerase chain reaction (PCR) for *Bartonella quintana* or *Brucella melitensis* were spread onto cells grown within a shell vial as previously described (13,14). After 3 weeks of incubation at 37°C, the bacteria were detected by using Gimenez staining, a direct immunofluorescence test incorporating polyclonal antibodies directed against *Bartonella*, and by PCR targeting the 16S rRNA sequence.

**Molecular Biology**

For the 38 cardiac samples stored at –80°C, molecular analysis was performed in France. After 18 hours of proteinase K digestion at 55°C, DNA was extracted from tissue by using the MagNA Pure LC instrument (Roche Molecular Biochemicals, Mannheim, Germany) and MagNA Pure LC DNA Isolation Kit III (Roche Molecular Biochemicals), as described by the manufacturer. A PCR-positive valve sample taken from a patient with *Staphylococcus aureus* endocarditis was used as a positive control. A mixture of all reagents used for DNA extraction and DNA extracted from normal heart tissue were processed as negative controls. One negative control was included for every 5 samples tested. PCR amplification and sequencing were performed, as previously described (15), by using primers in Table 1. PCR targeting the 16S rRNA sequence was systematically performed. When a negative result occurred, additional PCR was performed targeting the 18S and 28S rRNA internal transcribed spacer to search for fungal infections. All positive PCR products were sequenced. The sequences were compared to those available in GenBank. Positive PCR results were considered as certain, when congruence existed between the results obtained with PCR and those obtained with pyridoxal (Sigma-Aldrich). In cases of broth turbidity, microscopic examinations were performed as described above. If culture was positive, the strain was identified.
other analyses. With a positive result interpreted as a possible case, we performed additional PCR, targeting a second gene with genus-specific primers (Table 1). When the PCR was positive and the sequence gave the same result, the case was reclassified as certain. When the second PCR was negative, we performed a PCR targeting a third gene. When both PCRs targeting the second and the third gene were negative, the result was classified as negative.

Histologic and Immunohistologic Analysis

Thirty-seven valve samples underwent fixation by formalin and were paraffin-embedded. Valve specimens were cut to 3-µm thickness serial sections. Hematoxylin-eosin-saffron, periodic acid-Schiff, Giemsa, Brown-Hopps/Brown-Brenn Gram, Grocott-Gomori methenamine silver, and Warthin-Starry stains were used (16). On the basis of the histologic findings, valve specimens were divided into 3 groups: A, B, and C. Group A samples showed histologic features of infective endocarditis consisting of vegetations or polymorphonuclear leukocyte-rich valvular inflammation. Group B specimens showed valvular inflammation composed of mainly inflammatory mononuclear cells, macrophages, and lymphocytes without vegetations and microorganisms. Group C samples were devoid of inflammation, vegetations, or microorganisms. When *Bartonella* endocarditis was suspected, immunohistochemical analysis was performed on valve sections with an anti-*Bartonella* rabbit polyclonal antibody as previously described (17).

Serum Sample Analysis

Serologic Testing

*Brucella* serologic analysis was performed by Rose-Bengale agglutination (Bio-Rad, Marnes-La-Coquette, France) for 61 serum samples from 61 patients in Algiers, and the samples were stored at −20°C for further study. The confirmation was observed by Wright Serology (Bio-Rad). In the case of endocarditis, specific antibody titers exceeded 1:800. *Bartonella* and *C. burnetii* serologic testing was performed in France on all 61 samples. For *Bartonella* serologic testing, *B. quintana* and *B. henselae* were used as antigens in a microimmunofluorescence (MIF) assay performed as previously described (18). A patient was considered to have *Bartonella* endocarditis when IgG titers >1:800 were observed (18). The species identification was performed with Western blot performed before and after serum cross-adsorption as previously described (19). For *C. burnetii* serologic testing, immunoglobulin (Ig) G, IgM, and IgA antibody titers were estimated by using an MIF test as previously described (20). A diagnosis of chronic endocarditis was made when a patient had an IgG phase I titer >1:800 (20). A Light Cycler nested PCR was performed on positive serum samples for *Bartonella* and *C. burnetii* as previously described (21,22).

Results

Patient Characteristics

Our prospective study led to identification of 110 cases from 108 patients. The 110 episodes were classified as 77 (70%) definite infective endocarditis and 33 (30%) possible infective endocarditis (12). A second episode of infective endocarditis developed in 2 patients during our survey. The patients included 64 men and 40 women with a mean age of 35.3 years (range 17–72 years). The series included 4 children, 2 boys (6 and 8 years of age) and 2 girls (9 and 14 years of age). Among the patients, 34 came from rural areas, 61 lived in urban areas, 1 was in prison, and no information could be obtained for 12. Among 96 patients whose living conditions were known, 59 (61.5%) lived in poor and crowded families of at least 10 persons. Among the 110 cases, 87 (79%) episodes were diagnosed on native valve and 23 (21%) on prosthetic valve. The mitral valve was affected in 31 (28.2%) cases, the aortic in 29 (26.3%), and both in 41 (37.2%). The tricuspid valve was affected in 3 (2.7%) patients, and 4 (3.6%) had aortic, mitral, and

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Eubacteria</em></td>
<td>16S rRNA</td>
<td>S36F 5’ CAGCACGCGGCGGAATAC</td>
<td>RP2 5’ AGGGTACTCCTGTTACGACTT</td>
</tr>
<tr>
<td><em>Staphylococcus</em> spp.</td>
<td>RpoB</td>
<td>SphF 5’ AAACCIATACGGCAATTTGTT</td>
<td>StphR 5’ GTTTACTGACTGAGCACG</td>
</tr>
<tr>
<td><em>Streptococcus</em> spp.</td>
<td>RpoB</td>
<td>StrpF 5’ AARYTGGMCGCTGAAGAAT</td>
<td>StrpR 5’ GTIATTTTCTATACACCATG</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp.</td>
<td>RpoB</td>
<td>StrpF 5’ AARYTGGMCGCTGAAGAAT</td>
<td>StrpR 5’ GTIATTTTCTATACACCATG</td>
</tr>
<tr>
<td><em>Streptococcus</em> spp.</td>
<td>SOD</td>
<td>d1 5’ CCITAYCITATAYGAYGCTYTGARCC</td>
<td>d2 5’ ARRTAGTACGTGTYTCCCAAACRTC</td>
</tr>
<tr>
<td><em>Enterobacteriaceae</em></td>
<td>RpoB</td>
<td>CM7 5’ AACGATTCGCCGTTGCGCTGGG</td>
<td>CM31b 5’ CCGAAAACACGCTGGAAG</td>
</tr>
<tr>
<td><em>Mycoplasma hominis</em></td>
<td>PfsY</td>
<td>MH1F 5’ GTTTGTTATCGACAAAG</td>
<td>MH1R 5’ GSGTGTGTATCGACAAAG</td>
</tr>
<tr>
<td><em>Coxiella burnetii</em></td>
<td>IS111</td>
<td>Trans3 5’ CAACTGTGTTGGAATGTAG</td>
<td>Trans5 5’ TTACAGTGACCAATAGCCG</td>
</tr>
<tr>
<td><em>Bartonella</em> spp.</td>
<td>ITS</td>
<td>ITSF 5’ GCGACTGGGGTGAAATGGG</td>
<td>ITSR1 5’ AGGGTGGGATATCATC</td>
</tr>
<tr>
<td><em>Bacillus</em> spp.</td>
<td>RpoB</td>
<td>Bc55F 5’ TCTCTGTGAACTGCTGTTT</td>
<td>Bc260R 5’ TGAAGGTCACGGYACCTTCAA</td>
</tr>
<tr>
<td><em>Corynebacterium</em> spp.</td>
<td>RpoB</td>
<td>C2700F 5’ GWATGAAACCATYGGBACAGGT</td>
<td>C3130R 5’ TCCATYTCRCCRAARCGCT</td>
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<tr>
<td><em>Fungi</em></td>
<td>18S-28S ITS</td>
<td>FCU 5’ TCCGTAGGTGAACCTGCGG</td>
<td>RCU 5’ GTCGGTCTTTCTACGATCG</td>
</tr>
</tbody>
</table>

RESEARCH

Table 1. Primers used for broad-range 16S rRNA polymerase chain reaction (PCR) and, according to species identified by sequencing, primers targeting a second gene for confirmation of positive 16S rRNA PCR results and primers used for fungal PCR.
tricuspid involvement. We reported 1 case with mitral and pulmonary valves affected, with the persistence of an arterial canal, and 1 patient on a pacemaker.

**Blood Cultures**

Blood cultures identified 48 microorganisms (Table 2). Of the 22 Streptococcus spp. cultures, 5 Streptococcus mitis, 6 Streptococcus sp., 3 S. agalactiae, 3 Gramulicatella adiacens, 2 α-Streptococcus, 1 S. oralis, 1 S. intermedius, and 1 Gemella morbillorum were identified. Seven Staphylococcus aureus and 5 coagulase-negative Staphylococcus were observed. One Haemophilus influenzae, 1 H. aphrophilus, 1 Haemophilus sp., 1 Kingella kingae, and 1 Actinobacillus actinomycetemcomitans were identified among the HACEK group (Haemophilus, Actinobacillus, Cardiobacterium, Eikenella, Kingella). One Brucella melitensis, a zoonotic agent, was isolated.

**Serum Analysis**

Using serologic testing, infective endocarditis could be diagnosed in 11 (18%) of 61 serum samples. A positive *Brucella* serologic result with titers of 1:3,200 was observed for 2 patients (1 sample was also culture positive). Two other patients had a typical profile of Q fever endocarditis (Phase I: IgG 1:3,200; IgM 1:25; IgA 1:1,600/Phase II: IgG 1:6,400; IgM 1:25; IgA 1:1,600 for 1 patient and Phase I: IgG 1:6,400; IgM 1:800; IgA 1:50/Phase II: IgG 1:12,800; IgM 1:800; IgA 1:100 for the other patient). Among these 2 patients, *C. burnetii* Light Cycler nested-PCR performed on serum samples was positive for the sample from 1 patient. A positive *Bartonella* serologic result, with IgG ≥1:800, was observed for 7 patients (Table 3). The Western-blot analysis of the 7 serum samples allowed the specific diagnosis of *B. quintana* (Figure 2). Of these 7 patients, *B. quintana* Light Cycler nested-PCR performed on serum samples was positive for 5 patients (Table 3).

**Cardiac Valve Analysis**

Axenic culture of cardiac valves was positive for 9 samples. The growth of 2 coagulase-negative *Staphylococcus*, 2 *Streptococcus* sp., 1 *Streptococcus* mitis, 1 *S. intermedius*, 1 *Corynebacterium* sp., and 1 *Candida kruzei* was observed. Another sample was polymicrobial. Cell culture allowed the growth of *B. quintana*, an arthropodborne disease agent, from 3 valve samples (Tables 2 and 3). The numbers of valve specimens classified into groups A, B, and C were 21, 5, and 11, respectively. With the exception of *Bartonella* endocarditis, the samples with histologic features of infective endocarditis had vegetations in most cases, moderate fibrosis, calcifications in some cases, and numerous inflammatory infiltrates composed predominantly of polymorphonuclear leukocytes and abundant neovascularization. By using special stains, microorganisms were visualized in 16 samples from group A, gram-positive cocci and gram-negative bacilli in 8 cases each. In samples from group B, the inflammatory infiltrates were rare and focal and consisted mainly of macrophages and lymphocytes with discrete neovascularization. The specimens from group C showed noninflammatory

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**Table 2. Distribution of 110 infective endocarditis cases* diagnosed in Algeria using blood culture, cardiac valve culture, serologic testing, cardiac valve polymerase chain reaction (PCR), and PCR on serum samples.**

<table>
<thead>
<tr>
<th>Identified microorganisms</th>
<th>Blood culture (N = 110)</th>
<th>Cardiac valve culture (N = 38)</th>
<th>Serologic testing (N = 61)</th>
<th>Cardiac valve PCR (N = 38)</th>
<th>PCR on serum sample (N = 9)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Streptococcus spp. and related genera</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bartonella quintana</em></td>
<td>0/1†</td>
<td>0/3</td>
<td>5/2</td>
<td>10/0</td>
<td>2/3</td>
<td>12/2</td>
</tr>
<tr>
<td><em>Streptococcus</em> spp.</td>
<td>2/10</td>
<td>0/3</td>
<td>NP</td>
<td>2/1</td>
<td>NP</td>
<td>11/3</td>
</tr>
<tr>
<td>HACEK†§</td>
<td>0/4</td>
<td>0/0</td>
<td>NP</td>
<td>1/1</td>
<td>NP</td>
<td>5/1</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp.</td>
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<td>0/0</td>
<td>2/0</td>
<td>2/0</td>
<td>0/0</td>
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<tr>
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<td>NP</td>
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<tr>
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<tr>
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<td>1/0</td>
<td>NP</td>
<td>1/0</td>
</tr>
<tr>
<td><em>Candida</em> spp.</td>
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<td>0/1</td>
<td>NP</td>
<td>1/0</td>
<td>NP</td>
<td>1/0</td>
</tr>
</tbody>
</table>

Negative samples for definite infective endocarditis/negative samples for possible infective endocarditis

<table>
<thead>
<tr>
<th>Positive samples/tested samples</th>
<th>Blood culture</th>
<th>Cardiac valve culture</th>
<th>Serologic testing</th>
<th>Cardiac valve PCR</th>
<th>PCR on serum sample</th>
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<tbody>
<tr>
<td>0/22</td>
<td>0/4</td>
<td>NP</td>
<td>7/0</td>
<td>NP†</td>
<td>24/0</td>
</tr>
</tbody>
</table>

*77 definite and 33 possible.
†NP, not performed.
‡If we consider that *Bartonella quintana* was misidentified as *Haemophilus influenzae*.
§HACEK, *Haemophilus, Actinobacillus, Cardiobacterium, Eikenella, Kingella*.
degenerative damage with extensive fibrosis and often calcifications. The histologic features of *Bartonella* endocarditis were different from the other infective endocarditis. Samples from 7 cases with *Bartonella* endocarditis were examined. The valve tissues showed degenerative damage with extensive fibrosis. The valve tissues were poorly inflamed with rare mononuclear inflammatory cell infiltrates composed of lymphocytes and macrophages and discrete neovascularization. Vegetations, present in all samples, were small in size. In all cases, the Warthin-Starry stain detected *Bartonella*, mainly in vegetations as small bacillary organisms (Figure 3).

The 16S rRNA PCR was positive for 29 cardiac valves (Tables 2 and 4). *B. quintana* was detected on 10 specimens (Table 3). Among the *Streptococcus* spp. and related genera, 3 *Streptococcus* sp., 1 *S. mitis*, 1 *S. mutans*, 1 *S. gordonii*, 1 *S. pneumoniae*, and 1 *Granulicatella adiacens* were detected. Two *Staphylococcus aureus* and 1 coagulase-negative *Staphylococcus* were identified. Among the 2 bacteria from the HACEK group, 1 *H. paraphrophilus* and 1 *Cardiobacterium hominis* were identified. PCR performed with a second gene confirmed the previous PCR results with 1 exception. One *Streptococcus* sp. was not retrieved by PCR targeting a second or third gene and was considered as contamination. The PCR targeting the 18S–28S rRNA ITS allowed the detection of 1 *Candida parapsilosis*. Finally, *Bartonella* spp. were also specifically visualized in vegetations by immunohistochemistry in all the cases of *B. quintana* endocarditis (Figure 3).

**Causative Microorganisms and Discordant Results**

The overall distribution of causative microorganisms and their identification, depending on the diagnostic tools used, are displayed in Table 2. An etiologic agent could not be determined for 10 (13%) of definite cases and 28 (76%) of possible cases. For the 2 patients with recurring infective endocarditis, the cause for the first episode was different than that of the second episode. One patient had endocarditis caused by *Streptococcus oralis*, and 1 year later, endocarditis caused by *K. kingae* developed. For the other patient, no etiologic diagnosis was established for the first episode, during which a valve removal was necessary. Four months after cardiac surgery, the patient had endocarditis...
caused by *Staphylococcus epidermidis*. Nine discrepant results were also observed and are summarized in Table 4.

**Discussion**

Endocarditis cases with fastidious agents escape microbiologic diagnosis classically applied in Algerian laboratories. For the first time, we established a profile of the microbiologic etiology of infective endocarditis in Algeria. Our conclusions concerning PCR results were submitted to a rigorous strategy of validation. All of the controls must be correct for validating each assay. The result was considered true if confirmation was obtained by successfully amplifying bacterial DNA when targeting another gene, the PCR result was congruent with the results of other diagnostic tools, or both.

Of the 77 cases of definite infective endocarditis, the cause was found for 67 (87%) cases. The diagnosis was performed on the basis of positive blood cultures for 44 cases. For 20 (26%) cases, no etiologic diagnosis was obtained in Algeria but was performed in France on the basis of cardiac valve PCR, and *Bartonella* and *Coxiella burnetii* serologic testing. These data show improvement in the etiologic diagnosis of endocarditis when molecular or serologic tools are used. The rate of remaining infective endocarditis without cause is comparable to the prevalence in western countries (16). As in other countries, the etiologic distribution is dominated by the bacteria responsible for infective endocarditis, such as *Streptococcus* spp. and related genera, *Staphylococcus* spp., and bacteria from the HACEK group. The difference in comparison to other countries is that blood culture–negative endocarditis is mainly linked to zoonotic and arthropodborne agents.

For the 33 cases of possible infective endocarditis, the number of etiologic diagnoses was fewer than those for definitive infective endocarditis. However, in this group, some cases are infective endocarditis and others are not. If we consider a *Bartonella* serologic result ≥1:800 as a major criterion (5), the 2 possible cases of *B. quintana* infective endocarditis will be classified as definite. Therefore, *Bartonella* serologic results should be taken into account in future revisions of the Duke criteria. Of the 48 case-patients with positive blood cultures, 19 had additional samples tested through a second analysis (serologic or molecular methods). Of the 19, 11 had negative results, 5 were concordant, and 3 were discordant. Of these 48 cultures, 1 corresponds to brucellosis.

Of the 62 blood culture–negative endocarditis cases, samples from all were tested by serologic or molecular methods. Of these, 34 were negative, and 28 had an etiologic agent identified. Seventeen of those were due to zoonoses or arthropodborne bacterial diseases.

Discrepancies were observed between the results obtained by using the various techniques. Some discrepancies

![Figure 3. A) Section of an aortic valve from a patient with Bartonella endocarditis. Note the extensive fibrosis of the connective valve tissue (arrowhead), the vegetation (*), and the low inflammatory infiltrate of the valve tissue (hematoxylin-phloxine-saffron, original magnification 100x). B) Resected valve with Bartonella quintana infection showing darkly stained bacilli consistent with Bartonella. Note the numerous clusters of argyrophilic bacteria present in the valvular vegetation (Warthin-Starry silver, original magnification 1,000x). C) Immunohistochemical detection of B. quintana in a resected valve from a patient with Bartonella endocarditis. Note the extracellular distribution of the bacterial colonies (*) in the valvular vegetation (polyclonal antibody and hematoxylin counterstain, original magnification 250x).](Images/.../BacterialZoonosesandInfectiveEndocarditisAlgeria Российской Федерации)
resulted from culture contamination with the cutaneous flora. A significant rate of contamination has been already reported, and the low specificity of valve culture that we observed confirms these results (23–25). One discrepancy was caused by identification problems at the species level for *Streptococcus* species. This fact has been previously reported (7). Another discrepancy was linked to a *Candida* species misidentification by phenotypic analysis, which was corrected by using molecular tools. The last discordant case corresponded to a patient for whom blood cultures were positive for *H. influenzae*. When serum samples were analyzed, a diagnosis of *B. quintana* endocarditis has been established in the presence of positive *Bartonella* MIF, Western blot, and PCR. We do not know if *B. quintana* was misidentified as *H. influenzae*, which is possible as both are slow-growing, hemin-dependent, small, gram-negative bacteria (26). We believe that as fastidious, small, gram-negative bacteria growing in blood agar, the 2 organisms may be confused.

In Algeria, cases of infective endocarditis caused by zoonotic and arthropodborne disease agents, such as *Coxiella burnetii*, *Brucella melitensis*, and *Bartonella quintana* are frequently observed and correspond to one quarter of the performed diagnoses. *B. quintana* would be one of the most common agents of infective endocarditis in our Algiers series (15.6% of definite infective endocarditis). The prevalence of endocarditis caused by *Bartonella* varies depending on the country. In Canada, *Bartonella* causes 3% of endocarditis cases (27). In Sweden, no *Bartonella* endocarditis was identified in an analysis of 334 infective endocarditis cases (28). In the United Kingdom, *Bartonella* endocarditis accounts for 1.1% of infective endocarditis cases (29). In Germany and in France, *Bartonella* endocarditis accounts for 3% of all infective endocarditis (A. Sander et al. unpub. data) (27). The frequency of *Bartonella* endocarditis is <1% for Sweden and higher in France, Germany, the United Kingdom (3%), and North Africa (15%). Such differences may be linked to differences in living conditions.

Homeless people are at risk for *B. quintana* endocarditis (30,31). Indeed, *B. quintana*, like *Rickettsia prowazekii*, the agent of epidemic typhus, is transmitted by body lice. Those who live in extreme poverty are often the persons who are infested. The recent description of typhus in Algeria confirms that poor socioeconomic conditions still exist in this country (32–34). In our studies, *B. quintana* endocarditis cases occurred in patients living in poor conditions. Although the only known reservoir for *B. quintana* is humans, the bacterium has recently been associated with fleas (35). Moreover, some cases of *B. quintana* infections have been linked to contact with cats and cat fleas in patients who were not homeless and did not have body lice (36).

*Brucella melitensis*, well known in northern Africa, where bucellosis is endemic in certain areas, accounts for 2.6% of all infective endocarditis cases for which an etiologic agent was identified (7).
logic diagnosis has been performed (37). Be cause *C. burnetii* detection requires specialized tests not normally found in most laboratories, it is not often diagnosed in Algeria (38). Two cases were retrospectively detected.

Importance of infective endocarditis caused by zoonotic and arthropodborne agents in Algeria leads to 2 considerations. First, specific serologic tests need to be used for diagnosis. Indeed, 25% of our etiologic diagnoses correspond to microorganisms for which the diagnosis is usually based on serologic testing. Secondly, the therapeutic impact of *Brucella* and *Coxiella* diagnosis is important because the antimicrobial treatment of endocarditis caused by these agents must include doxycycline. The 2 patients with Q fever endocarditis died during their hospitalization because of inadequate antimicrobial therapy. Finally, the high rate of blood culture–negative endocarditis was not linked to prior antimicrobial therapy but rather to fastidious microorganisms for which serologic testing (as for zoonotic and arthropodborne disease agents) or molecular analysis (as for *Mycoplasma hominis* [39] and *Corynebacterium* spp.) are diagnostic tools.

Our study underlines the need to perform serologic analysis to determine for the etiology of infective endocarditis. *Bartonella* serologic testing is an important tool for diagnosis of blood culture–negative endocarditis and should be taken into account in future revisions of the Duke criteria. This study made it possible to show that zoonotic and arthropodborne disease agents cause one quarter of infective endocarditis in Algeria; *B. quintana* caused 13% of our cases.

Acknowledgments

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Dr. Benslimani is a physician working at the E.H.S. Dr Maouche, Algiers, Algeria. Her research interests include the clinical features and diagnosis of endocarditis.

References


Address for correspondence: Didier Raoult, CNRS UMR 6020, Unité des Rickettsies, IFR, 48 Université de la Méditerranée, Faculté de médecine, 27 Boulevard Jean Moulin, 13385 Marseille cedex 5, France; fax: 33-491-83-03 90; email: Didier.Raoult@medecine.univ-mrs.fr
A flavivirus (strain 97-103) was isolated from Culex pipens mosquitoes in 1997 following floods in South Moravia, Czech Republic. The strain exhibited close antigenic relationship to West Nile virus (WNV) prototype strain Eg-101 in a cross-neutralization test. In this study, mouse pathogenicity characteristics and the complete nucleotide and putative amino acid sequences of isolate 97-103, named Rabensburg virus (RabV) after a nearby Austrian city, were determined. RabV shares only 75%–77% nucleotide identity and 89%–90% amino acid identity with representative strains of WNV lineages 1 and 2. Another RabV strain (99-222) was isolated in the same location 2 years later; it showed >99% nucleotide identity to strain 97-103. Phylogenetic analyses of RabV, WNV strains, and other members of the Japanese encephalitis virus (JEV) complex clearly demonstrated that RabV is either a new (third) lineage of WNV or a novel flavivirus of the JEV group.

West Nile virus (WNV), a member of the Japanese encephalitis virus (JEV) group within the genus Flavivirus, family Flaviviridae, is the most widespread flavivirus, occurring in Africa, Eurasia, Australia, and North America. Other members of the JEV group flaviviruses are Cacipacore virus (CPCV), Koutango virus (KOUV), JEV, Murray Valley encephalitis virus (MVEV), Alfuy virus (ALFV), St. Louis encephalitis virus (SLEV), Usutu virus (USUV), and Yaounde virus (YAOV) (1). Although initially WNV was considered to have minor human health impact, the human and equine outbreaks in Europe (Romania, Russia, France, Italy), Africa (Algeria, Tunisia, Morocco), and Asia (Israel) within the last 10 years, and especially the virus’s emergence and spread in North America since 1999, put it into the focus of scientific interest. The distribution and ecology of WNV, as well as clinical features, pathogenesis, and epidemiology of West Nile disease have been reviewed (2–6). Phylogenetic analyses showed 2 distinct lineages of WNV strains (which themselves subdivide into several subclades or clusters), isolated in different geographic regions (7–10).

The presence of WNV in central Europe has been known for some time. Serologic surveys have detected specific antibodies to WNV in several vertebrate hosts in Austria, Czech Republic, Hungary, and Slovakia during the past 40 years, and several virus strains were isolated from mosquitoes, rodents, and migrating birds (3). Human cases of West Nile fever were reported in the Czech Republic in 1997 (11) and in Hungary in 2003 (12). Although these countries are important transit areas or final destinations for migratory birds from the African continent, and hence may play an important role in the circulation and conservation of different WNV strains, genetic information about the strains isolated in central Europe has not been available. We report the complete genome sequence and phylogenetic analyses, as well as antigenic and mouse virulence characteristics, of a unique flavivirus strain (97-103), closely related to WNV, which was isolated by intracranial injection of suckling mice with homogenates of female Culex pipens mosquitoes collected 10 km from Lanzhot, Czech Republic, after a flood in 1997 (11,13,14). The collection site was very close to the Czech-Austrian border, ≈2 km from the small Austrian town of Rabensburg. Consequently, the isolate 97-103 was later tentatively called Rabensburg virus (RabV). Another antigenically identical or very closely related strain (99-222) was isolated from Cx. pipiens mosquitoes in the same location 2 years later (14).

**Methods**

Isolates 97-103 (passage 5 in suckling mouse brain [SMB]) and 99-222 (passage 4 in SMB) were freeze-dried in October 2000 (14). Viral RNA was extracted from 140 µL of virus resuspended in diethylpyrocarbonate

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*University of Veterinary Medicine, Vienna, Austria; †Szent István University, Budapest, Hungary; ‡Institute of Vertebrate Biology ASCR, Brno, Czech Republic; and §United Arab Emirates University, Al Ain, United Arab Emirates

1This study will be presented at the International Conference on Emerging Infectious Diseases, February 26-March 1, 2005, Al Ain, United Arab Emirates.
(DEPC)-treated water, using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. For amplification of the complete genome, oligonucleotide primers were designed with the help of the Primer Designer 4 for Windows 95 program (Scientific and Educational Software, version 4.10) and were synthesized by GibcoBRL Life Technologies, Ltd. (Paisley, Scotland, UK). A complete list of the 35 primers used in reverse transcription–polymerase chain reaction (RT-PCR) and sequencing reactions is available upon request. Reverse transcription and amplification were performed with a continuous RT-PCR method with the Qiagen OneStep RT-PCR Kit (Qiagen) following the manufacturer’s instructions. Reverse transcription (at 50°C for 30 min) was followed by a denaturation step at 95°C for 15 min, and 40 cycles of amplification (94°C for 40 s, 57°C for 50 s, 72°C for 1 min). Reactions were completed by a final extension for 7 min at 72°C, and the amplicons were kept at 4°C until electrophoresis was carried out. The reactions were performed in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler (Perkin-Elmer Corp., Wellesley, MA, USA). After RT-PCR, the amplicons were electrophoresed in agarose gel, stained with ethidium bromide, and bands were visualized under UV light. Gels were photographed with a Kodak DS Electrophoresis Documentation and Analysis System (Eastman Kodak Company, New Haven, CT, USA). Product sizes were determined with reference to a 100–bp DNA Ladder (Promega, Madison, WI, USA). Fluorescence-based direct sequencings were performed in both directions on the PCR products with the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit (Perkin-Elmer) and an ABI Prism 310 genetic analyzer (Perkin-Elmer) automated sequencing system (15).

The nucleotide sequences were identified by BLAST search against GenBank databases and were compiled and aligned with the help of the Align Plus 4 for Windows 95 (Scientific and Educational Software, version 4.00) and ClustalX Multiple Sequence Alignment (version 1.81) programs. Phylogenetic analysis was performed with the Phylogeny Inference Program Package (PHYLIP) version 3.57c. Distance matrices were generated by the Fitch program, with a translation/transversion ratio of 2.0. Phylogenetic trees were delineated by using the TreeView (Win32) program version 1.6.6.

**Results**

Both virus strains were identified as WNV by complement fixation and neutralization tests (11,13). Strain 97-103 was compared antigenically in detail with the Egyptian Eg-101 topotype strain of WNV (16), a representative of WNV lineage 1 (clade 1a). In plaque-reduction cross-neutralization tests (PRNT) with homologous and heterologous antisera (produced by injection of ICR mice with 3 intraperitoneal doses at weekly intervals), the serum raised against Eg-101 neutralized both the homologous virus and 97-103 at a titer of 512, while the strain 97-103 specific serum was effective against strain Eg-101 only at a titer of 64, although it neutralized the homologous virus at 512. The average 4-fold difference in cross-PRNT titers indicates certain antigenic heterogeneity of the 2 strains, and the 97-103 isolate was therefore regarded as a subtype of WNV (14).

Virulence of RabV strains 97-103 and 99-222 was determined by intracranial and intraperitoneal injection of specific-pathogen-free (SPF) outbred ICR mice. Central nervous system symptoms (e.g., pareses of hind limbs) developed in suckling mice, which died 7–15 days after intracranial injection (Table 1). Adult mice did not show any clinical symptoms and survived the experimental infection. On the other hand, the WNV topotype strain Eg-101 caused fatal illness in intracranially injected mice, killing them within 4 to 6 days after infection, regardless of their age (11,13). After intraperitoneal injection, strain Eg-101 killed all suckling mice but a <10% of adult mice; RabV strains 97-103 and 99-222 killed approximately one third of suckling mice, and the average survival time was 11 days (range 10–14 days). Thus, both Rabensburg virus strains exhibit clearly lower virulence for mice than the Egyptian WNV topotype strain. In addition, average survival time of sucking ICR mice injected intracranially with RabV was significantly longer than with strain Eg-101.

The genome of strain 97-103 Rabensburg virus (RabV) was investigated by RT-PCR and subsequent direct sequencing of the amplicons. Initially, oligonucleotide primers designed on the consensus sequences of lineage 1 and 2 WNV strains were applied to the viral nucleic acid of RabV. On the basis of the sequence information obtained from these PCR products, specific primer pairs were designed to produce overlapping amplicons covering

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**Table 1. Survival time (days) of sucking mice injected intracranially with Rabensburg virus isolates 97-103 and 99-222**

<table>
<thead>
<tr>
<th>Suckling mouse brain (SMB) passage no.</th>
<th>Strain 97-103</th>
<th>Strain 99-222</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average survival time</td>
<td>Range</td>
</tr>
<tr>
<td>SMB0*</td>
<td>12.1</td>
<td>12–13</td>
</tr>
<tr>
<td>SMB1</td>
<td>8.5</td>
<td>7–10</td>
</tr>
<tr>
<td>SMB2</td>
<td>8.5</td>
<td>7–11</td>
</tr>
<tr>
<td>SMB3</td>
<td>8.1</td>
<td>7–9</td>
</tr>
</tbody>
</table>

*Represents the original mosquito suspension during virus isolation attempts.
the entire genome. The RT-PCR products were sequenced, and the sequences were compiled, resulting in a 10,972–nucleotide (nt) sequence that represented the complete genome of the virus. The sequence was identified by BLAST search against GenBank databases. The highest identity rates of RabV to other flaviviruses (78%–90%) were found with certain regions of WNV strains of lineage 1 and 2.

From the second isolate (99-222), 5 genomic regions have been amplified and sequenced so far, showing a total of 3656 nt. They represent partial coding sections from the core (C), anchored C, premembrane (PreM), and membrane (M) protein regions (between nucleotide positions 117 and 752); NS3 protein region (between nucleotide positions 5294 and 5536, and between nucleotide positions 5565 and 6343); NS4b and NS5 regions (between nucleotide positions 7321 and 8112); and NS5 protein region (between nucleotide positions 9095 and 10305).

Partial sequence analysis of isolate 99-222 showed >99% identity to 97-103. Aligned to strain 97-103, only a few nucleotide substitutions were observed, in the following positions: C<sub>609</sub> to T; C<sub>720</sub> to A; G<sub>5727</sub> to A (resulting in an amino acid change Met to Ile); T<sub>5961</sub> to C (resulting in an amino acid change Ile to Thr); T<sub>5961</sub> to C; C<sub>9630</sub> to A; and G<sub>9843</sub> to T.

Similar to other flaviviruses (17), the nucleotide sequence of RabV contains 1 open reading frame (ORF) encoding the viral proteins as a large polyprotein precursor. The ORF starts at nucleotide position 97, and codes for a 3,433-amino acid (aa) polypeptide. The putative amino acid sequence of the polyprotein precursor gene of RabV 97-103 has been translated; based on the amino acid alignment with WNV, the putative mature proteins, conserved structural elements, and putative enzyme motifs were localized. The anchored C protein is located between nt 97 and 465; within this region, the C protein is located between nt 97 and 411. The PreM protein is encoded from nt 466 to nt 966, with the M protein between nt 742 and 966. The envelope (E) protein is encoded between nucleotide positions 967 and 2469, followed by the non-structural proteins NS1 (nt 2470–3525), NS2a (nt 3526–4218), NS2b (nt 4219–4611), NS3 (nt 4612–6468), NS4a (nt 6469–6846), 2K (nt 6847–6915), NS4b (nt 6916–7680), and NS5 (nt 7681–10395), respectively.

### Table 2. Sequences of West Nile virus (WNV) strains and other members of the Japanese encephalitis virus group used for phylogenetic analyses

<table>
<thead>
<tr>
<th>Virus name</th>
<th>Code</th>
<th>Accession no.*</th>
<th>Year</th>
<th>Host</th>
<th>Geographic origin</th>
<th>WNV lineage, clade</th>
</tr>
</thead>
<tbody>
<tr>
<td>WNV HNY1999</td>
<td>NY99a</td>
<td>AF202541</td>
<td>1999</td>
<td>Human</td>
<td>New York</td>
<td>1a</td>
</tr>
<tr>
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<td>NY99b</td>
<td>AF196835</td>
<td>1999</td>
<td>Flamingo</td>
<td>New York</td>
<td>1a</td>
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<tr>
<td>WNV IS98STD</td>
<td>Is98</td>
<td>AF481864</td>
<td>1998</td>
<td>Stork</td>
<td>Israel</td>
<td>1a</td>
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<tr>
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<td>Is98</td>
<td>AF404757</td>
<td>1998</td>
<td>Horse</td>
<td>Italy</td>
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<td>Ro96</td>
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<td>1999</td>
<td>Human</td>
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<td>1999</td>
<td>Human</td>
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<td>1997</td>
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<tr>
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<td>1960</td>
<td>Culex annulirostris</td>
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<td>Laboratory strain</td>
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<td>1937</td>
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<td>Dermacentor marginatus</td>
<td>Caucasus</td>
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<td>Rabensburg virus (97-103)</td>
<td>RabV</td>
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<td>Czech Republic</td>
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<tr>
<td>Saint Louis encephalitis virus</td>
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<td>Koutango virus</td>
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<td>Yaounde virus</td>
<td>YAOV</td>
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*Partial nucleotide sequences (NS5 protein region) are indicated in italics.
†Unknown, tentative speciation.
intramolecular bonds in the E and NS1 protein, putative integrin binding motif of the E protein, catalytic triad and substrate binding pocket of the trypsin-like serine protease, RNA helicase motif of the NS3 protein, and RNA-dependent RNA polymerase motif of the NS5 protein; 15).

To investigate the phylogenetic relationship of RabV to other WNV isolates, multiple nucleotide and putative amino acid sequence alignments were made involving 16 WNV strains (listed in Table 2). Although several complete WNV nucleotide sequences from previously published studies (10,18) have been deposited in the GenBank databases, only selected representatives of lineages and clades have been included in our alignments, in order to obtain more precise and demonstrative trees.

RabV exhibited 73%–77% nucleotide identity rates to the different WNV strains (Table 3). The relationships between the strains are demonstrated in Figure 1. The 2 lineages of WNV are obviously separated in the tree. Clade 1a viruses form a tight cluster with close genetic relationship among the members. Kunjin virus, the representative of clade 1b, appears as a separate branch of lineage 1. Unfortunately, no complete genome sequence information is available on clade 1c (Indian strains); thus, they are not represented in the tree. The prototype Uganda strain B956 (WNFCG) of lineage 2 is grouped together with the Sarafend strain, a laboratory strain with uncertain origin and passage history. Two viruses proved to be clearly distinct with significant genetic distances to all other WNV strains and also from each other: RabV and strain LEIV-Krnd88-190 (in the phylogenetic trees designated Rus98). The latter virus was isolated from Dermacentor marginatus ticks in the northwest Caucasus Mountain valley in 1998 and was regarded as a new variant of WNV (19–21). Because these 2 viruses differ considerably from all WNV strains, the issue is raised about whether classifying these 2 viruses as separate members of the JEV group might be more appropriate.

To elucidate this question, a comprehensive phylogenetic analysis was performed on all representatives of the JEV group. Because only partial common sequence information of the NS5 protein gene region is currently available from SLEV, ALV, CPCV, KOUV, and YAOUV (22), the phylogenetic analysis had to be restricted to this region (Figure 2). Within the investigated genome stretch, RabV showed 77%–78% identity to lineage 1 and 2 WNV strains, 77% identity to strain LEIV-Krnd88-190, and 71%–76% identity to other representatives of the JEV group. In the phylogenetic tree (Figure 2), the separation of the 2 unique strains (RabV and LEIV-Krnd88-190 = Rus98) from WNV is clearly visible. Although RabV

Table 3. Nucleotide and amino acid identity rates between RabV* and other flaviviruses

<table>
<thead>
<tr>
<th>Code</th>
<th>WNV lineage and clade</th>
<th>Identity to RabV (%)</th>
<th>Nucleotide</th>
<th>Amino acid</th>
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<td></td>
<td></td>
<td></td>
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<td>Partial†</td>
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<tr>
<td>NY99a</td>
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<td>78</td>
<td>90</td>
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<td>77</td>
<td>78</td>
<td>90</td>
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<td>77</td>
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<td>90</td>
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<td>90</td>
</tr>
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<td>77</td>
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<td>USUV</td>
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<td>68</td>
<td>72</td>
<td>75</td>
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<td>SLEV</td>
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<td>–</td>
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<td>ALFV</td>
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<td>YAOUV</td>
<td>–</td>
<td>–</td>
<td>75</td>
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</table>

*RabV, Rabensburg virus; JEV, Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; USUV, Usutu virus; SLEV, St. Louis encephalitis virus; ALFV, Alfuy virus; CPCV, Cacicapore virus; KOUV, Koutango virus; YAOUV, Yaounde virus.
†Partial alignment between nucleotide positions 9067 and 10101.
‡Partial alignment between amino acid positions 2591 and 3335.
exhibits the closest relationship to the WNV representatives, similar identity rates (76%) exist between MVEV and USUV, as well as between JEV and ALFV, and these viruses have been taxonomically classified as separate viruses. The Rus98 virus clusters together with KOUV, a virus isolated originally from a Kemp’s gerbil (Tatera kempi) in Senegal 1968 and subsequently recovered from other rodent species and several genera of ticks (Rhipicephalus, Hyalomma, Alectorobius) in central Africa (23). The Rus98 strain was also isolated from ticks.

The putative amino acid sequence of RabV was also compared with the corresponding sequences of representatives of WNV lineages and clades, as well as with other JEV group viruses on the available polypeptide sequence regions. RabV shared 89%–90% identity on the complete polypeptide precursor region with the WNV strains, 87% identity with the Rus89 strain, and 75%–76% identity with JEV, USUV, and MVEV. The alignments of the partial amino acid sequences of the NS5 region (between aa 2991 and 3335) showed 94%–96% identity rates with the WNV strains, 95% with strain Rus98, and 78%–90% with the other members of the JEV group (Table 3). Phylogenetic trees, based on the amino acid alignments, displayed nearly identical topology to nucleotide sequence–based trees (data not shown). The complete genome sequence of RabV (flavivirus strain 97-103) has been deposited in GenBank under accession no. AY765264.

**Discussion**

WNV strains of different lineages exhibit considerable genomic diversity (76%–77% nucleotide identity only). At the same time, WNV is not sharply delimited genomically from the other members of the JEV group. The available partial sequences of the NS5 gene region from other viruses of the group show 71%–76% nucleotide and 78%–90% amino acid identities to WNV strains. The closest relatives of WNV are KOUV and YAOV (10,22–24).

Lineage 1 of WNV comprises strains from several continents and is subdivided into at least 3 clades. In clade 1a, several subclades or clusters are formed by closely related strains, such as strains isolated 40–50 years ago in Europe and Africa; strains isolated 20–30 years ago in Africa; strains isolated within the last 10 years in Europe and Africa; and strains isolated within the last 5 years in the United States and Israel. Clade 1b consists of the Australian isolates (Kunjin), while clade 1c contains strains from India. Lineage 2 is composed of WNV strains that have been isolated, so far exclusively, in the sub-Saharan region of Africa and in Madagascar (18). The genetic distance between the 2 lineages is relatively great in contrast to that within some representatives of lineage 1 that were isolated in distant geographic locations and within considerable time intervals. While the viruses in clade 1a share 95.2%–99.9% nucleotide and 99.3%–100% amino acid identity to each other, and also 86.6%–87.8% nucleotide and 97.4%–97.7% amino acid identity to the clade 1b viruses, the overall identity rates between lineage 1 and 2 are only 75.7%–76.8% on nucleotide level and 93.2%–94.0% on amino acid level (18), identity rates that resemble those between RabV and either lineage 1 or lineage 2 WNV strains. Besides genomic differences, antigenic variability can be observed in cross-neutralization analyses and monoclonal antibody binding assays (8,18).

The results of the phylogenetic analyses indicate that viruses closely related to WNV are present in central Europe and southern Russia. Although these viruses have initially been identified as WNV, they can be regarded, on
The antigenic and biologic differences between RabV and the WNV reference strain Eg-101 also support this opinion. Isolation of RabV in 1997 was obviously not an isolated event; rather, flaviviruses of the RabV type seem to be present or persist in this area, as demonstrated by the isolation of an almost identical virus strain (99-222) 2 years later (14). The ecology of RabV needs further investigation. Other unanswered questions concern the pathogenicity and host spectrum of the virus, especially regarding possible human infections.

To summarize, a novel flavivirus strain of unknown human pathogenicity, repeatedly isolated from Cx. pipiens mosquitoes in central Europe, has been molecularly characterized, including determination of its complete nucleotide and deduced amino acid sequences. Based on the analysis of the virus and comparison with related viruses including phylogenetic relationships, we suggest that RabV be classified either as a new (third) lineage of WNV or as a novel flavivirus within the JEV group.

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Dr. Bakonyi is a lecturer in virology at the Faculty of Veterinary Science, Budapest, and also works as a guest researcher at the University of Veterinary Medicine, Vienna. He is interested in the molecular diagnosis and epidemiology of animal and human viruses.

References


Address for correspondence: Norbert Nowotny, Zoonoses and Emerging Infections Group, Clinical Virology, Clinical Department of Diagnostic Imaging, Infectious Diseases and Clinical Pathology, University of Veterinary Medicine, Vienna, A-1210 Vienna, Austria; fax: 43 1 250772790; email: Norbert.Nowotny@vu-wien.ac.at
Surveillance for lyssaviruses was conducted among bat populations in 8 provinces in Thailand. In 2002 and 2003, a total of 932 bats of 11 species were captured and released after serum collection. Lyssavirus infection was determined by conducting virus neutralization assays on bat serum samples. Of collected samples, 538 were either hemolysed or insufficient in volume, which left 394 suitable for analysis. These samples included the following: *Pteropus lylei* (n = 335), *Eonycteris spelaea* (n = 45), *Hipposideros armiger* (n = 13), and *Rousettus leschenaulti* (n = 1). No serum samples had evidence of neutralizing antibodies when tested against rabies virus. However, 16 samples had detectable neutralizing antibodies against Aravan virus, Khujand virus, Irkut virus, or Australian bat lyssavirus; all were specifically associated with fruit bats *P. lylei* (n = 15) and *E. spelaea* (n = 1). These results are consistent with the presence of naturally occurring viruses related to new putative lyssavirus genotypes.

Rabies is an acute encephalitis caused by a lyssavirus. On a global basis, bats have been associated with several different genotypes of lyssavirus (1–5). Two human infections with Australian bat lyssavirus (ABLV) have been reported, the clinical signs of which were consistent with classical rabies infection, namely a diffuse, nonsuppurative encephalitis (3). A serosurvey for agents similar to ABLV among bats in the Philippines detected a prevalence of 9.5% (22/231) (6). Six of 14 species (fruit- and insect-eating bats) were seropositive for reactivity against ABLV. These included *Taphozous melanopogon* (4/30), *Mineopterus schreiberi* (4/11), *Philetor brachypterus* (1/13), *Scotophilus kuhlii* (4/63), *Pteropus hypomelanus* (3/14), and *Rousettus amplexicaudatus* (6/50) (6). However, Asian bat lyssaviruses (1,2,4) were unavailable at that time to check for cross-reactivity.

Canine rabies is enzootic in Thailand. No bat-associated rabies or lyssavirus deaths in humans or other animals (7). This lack of data for other agents, however, does not exclude their existence (1). Rabies statistics in humans and animals are underreported (8). Moreover, without a history of dog bite, rabies may be dismissed, or clinical manifestations of bat-related cases may be variable (8). In the context of bat lyssavirus as an emerging global infectious disease, baseline data are necessary to allow for future public health assessment of its impact. This active surveillance sought to determine whether bats in Thailand had evidence of lyssavirus infections.

Methods

Collection of Specimens

From March 2002 through August 2003, bats were collected from 8 provinces throughout central, eastern, and southern Thailand (Figure 1). Sites were chosen on the basis of local reports of known bat colonies or after investigation by the Royal Department of Forestry, Ministry of Agriculture. Insectivorous bats in caves were captured during the day by using fine-mesh, long-handed butterfly nets. Larger fruit bats were captured with nets near sunset, as the bats flew for foraging activities, or before dawn when returning to their roosts (Figure 2). Thick leather gloves were worn when bats were handled and transferred into individual cotton pouches for transportation and processing.

Of the 932 bats collected, all were identified to 11 different species of both insectivorous and frugivorous bats (Table 1). Forty percent were female. All bats appeared healthy. At least 110 bat species (>20 million) are believed to be present in Thailand, according to estimates from a

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*Thai Red Cross Society, Bangkok, Thailand; †Ministry of Agriculture, Bangkok, Thailand; ‡Chulalongkorn University, Bangkok, Thailand; and §Centers for Disease Control and Prevention, Atlanta, Georgia, USA*
Survey for Bat Lyssaviruses, Thailand

Royal Department of Forestry survey in 2003. Eighty five percent are insectivorous; the rest are frugivorous.

Bats were anesthetized by administering a 0.2- to 0.5-mg intramuscular injection of ketamine hydrochloride. Animals were identified to sex and by species, based on gross morphology, as described (9). Animals were marked by hair or claw clipping. Blood, obtained from wing veins or by direct cardiac puncture, was transferred from the collecting syringe into 1.5-mL microtubes (Axygen Scientific, Union City, CA, USA) and stored in an icebox until centrifugation. Serum was frozen at –20°C during transportation and stored at –70°C. After recovery from sedation, bats were allowed to fly to their roosts. Sixteen of 932 died during the capture process. No additional bats died after the procedure, according to residents living near roosts.

Serologic Testing for Neutralizing Antibodies

Serum specimens were obtained from blood samples after clotting. In general, 394 samples from 4 different species were of sufficient volume and quality (Table 2). The samples originated from Chonburi (n = 167), Ayuttaya (n = 105), Chachoengsao (n = 36), Singburi (n = 81), and Surattani (n = 5). For adequate volume during testing, they were diluted 1:5 in Eagle’s minimum essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2% fetal bovine serum (Invitrogen). Serum samples were heat-inactivated for 30 min at 56°C before testing.

Initially, all 394 samples were screened in a modified rapid fluorescent focus inhibition test (6) against rabies virus (RABV, strain CVS-11) and ABLV (pteropid subtype; 40 50% tissue culture infective dose), with World Health Organization standard serum as a source for positive control antibody with 50% endpoint dilution of 1 IU = 1:20. Approximately 50 µL of diluted serum at 1:5, 1:10, and 1:20 dilutions was incubated with 50 µL of ABLV in 96-well microtiter plates for 90 min at 37°C in a CO2 incubator. Murine neuroblastoma cells (50 µL) were added to each serum-virus mixture, which was incubated for 20 h. Culture medium was removed after incubation, and the plates were fixed with 90% acetone, air-dried, and then stained with fluorescein isothiocyanate–conjugated anti-rabies monoclonal antibodies (Fujirebio Diagnostic, Inc, Malvern, PA, USA). Samples were considered positive if the number of fluorescent foci was reduced by 50% at the 1:5 dilution.

Those samples that demonstrated positive or suspicious activity were additionally tested against a broader panel of lyssaviruses, including Aravan, Khujand, and Irkut virus isolates. Twofold serum dilutions, from 1:25 to 1:100, were tested, and virus doses varied from 32 to 100 infectious units. These reactions were performed by using drops of cell culture medium on 4-well (6-mm) Teflon-coated glass slides (Cell-line/Erie Scientific Co., Portsmouth, NH, USA), incubated in a moist chamber for 48 h.

Direct Fluorescent Antibody (DFA) and Mouse Inoculation (MI) Testing of Brains

Brains from 16 dead bats (2 P. lylei and 14 P. hypomelanuss) were collected in iceboxes at the capture sites for transportation and then were stored at –70°C until testing. Each brain was tested for lyssavirus antigen by DFA. Multiple impressions were prepared, and slides were fixed in acetone, allowed to dry at room temperature, and stained with commercial fluorescein isothiocyanate–conjugated anti-rabies monoclonal antibodies (Fujirebio Diagnostic, Inc). These brain impressions were examined with a fluorescent microscope.

For MI testing, pooled 20% brain suspensions from all 16 bats were prepared by mixing ≈0.5 g of each bat brain
in 32 mL of normal saline solution. No antimicrobial preparations were added. The mixture was left to sediment at room temperature for 30 min, and the supernatant was used to inject into the brains of 1-month-old Swiss albino strain mice. Approximately 0.03 mL of each suspension was injected into each of 30 mouse brains. They were kept in 6 glass jars (5 in each) with a diameter of 15 cm and were observed for 60 days.

Results

Serologic Testing

All 394 serum samples were negative against RABV, but 16 (4%) were positive or suggestive of ABLV (Table 2). Further tests of these samples demonstrated neutralizing activity against Aravan, Khujand, or Irkut viruses or ABLV (Table 3). These 16 samples originated from 2 species, *P. lylei* (n = 15) and *Eonycteris spelaea* (n = 1), collected at Chonburi (n = 9), Singburi (n = 4), Ayuttaya (n = 2), and Chachoengsao (n = 1) Provinces (Table 2).

Chonburi is adjacent to Chachoengsao Province in the east, whereas Singburi and Ayuttaya are both located in the central part of the country (Figure 1). Approximately 5% of positive bat serum specimens were found in 2 eastern provinces (Chonburi, 9/158 and Chachoengsao, 1/36) versus 3% in 2 central provinces (Singburi, 4/81, and Ayuttaya, 2/105). Antibody-positive bats were dispersed throughout the collection period (March 2002 through August 2003). Most (15 of 16) positive samples came from *P. lylei*. One of 45 *E. spelaea* (versus 15 of 335 *P. lylei*) tested positive.

DFA and MI Testing

Sixteen bat brains tested by DFA had no detectable lyssavirus antigen. After intracerebral injection, 4 of 30 mice died, on days 11, 12, 14, and 21, respectively. None of these 4 brains tested positive with DFA for evidence of lyssavirus antigens.

Discussion

This study presents evidence of neutralization of lyssaviruses other than RABV and ABLV by sera from Thai bats. These findings are consistent with the presence of naturally induced antibodies against ≥1 lyssavirus genotype in the Thai bat populations studied.

Lyssaviruses are classified into groups on the basis of their genetic, antigenic, and relative pathogenic attributes. At least 7 putative genotypes and 2 major phylogroups are recognized on the basis of their overall phylogenetic

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**Table 1. Bat species captured in Thailand**

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<th>Species</th>
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<th>Rayong</th>
<th>Ayuttaya</th>
<th>Chachoengsao</th>
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<td>124</td>
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</table>
relatedness (1). Phylogroup I includes RABV (genotype 1), Duvenhage virus (DUVV) (genotype 4), European bat lyssavirus (EBLV) 1 (genotype 5), EBLV-2 (genotype 6), and ABLV (genotype 7). Phylogroup II includes Lagos bat virus (LBV) (genotype 2) and Mokola virus (MOKV) (genotype 3) (10). In this study, neutralization titers to new putative genotypes, namely, Irkut, Khujand, and Aravan viruses, and of much lesser degree to ABLV but not to RABV, were evident. Khujand virus is related to genotype 6, while Aravan virus is related to Khujand virus, with moderate similarity to genotypes 4, 5, and 6 (2,4). ABLV is more closely related to RABV (3). When a comparative phylogenetic analysis was performed, Irkut virus was recognized as a member of a cluster joining lyssavirus genotypes 4 and 5 (76% bootstrap support) (1).

This preliminary study demonstrates that which virus is used for a serologic test is critical. All Thai samples were negative to RABV and most to ABLV, findings which help explain why lyssavirus infection has not previously been reported in Thai bats. A relatively low prevalence of lyssavirus infection in Thai bats in the current study (4% as compared to 9.5% in the Philippines survey [6]) may be explained by the fact that as many as 43 samples had a 1:5 (some of them, both 1:5 and 1:10) dilution considered unreadable because of the effect of hemolysis. Moreover, another 13 samples with equivocal result were seropositive for ABLV after subsequent testing. Further testing of these additional 13 samples against Irkut, Khujand, and Aravan viruses was not possible because of insufficient volume. Therefore, the actual positive number might be 29 (7.3%) of 396. Nevertheless, without a Thai lyssavirus isolate, concluding to which virus these bats have been exposed is difficult. These data also suggest that several lyssaviruses are in circulation throughout Thailand as well as other Asian countries, such as in the Philippines, Central Asia, and portions of Russia (1,2,4,6).

Further studies throughout the year should be expanded to other species of bats, as well as a focus upon bats such as P. lylei and in locations with the highest prevalence of neutralizing antibodies. Whether P. lylei is the single most important species is not known. Surveillance among sick and dying bats and collection of their brains would assist in identifying infecting viruses.

Public health authorities need to be aware of the potential for bats to transmit lyssaviruses, and to increase surveillance and public education. Attention should focus on the protective efficacy of commercially available vaccines and immune globulins against these novel nonrabies lyssaviruses after exposure, before fatal human infection occurs.

Table 2. Bat sera screened and positive for neutralizing antibodies (positive/screened)

<table>
<thead>
<tr>
<th>Species</th>
<th>Chonburi</th>
<th>Singburi</th>
<th>Ayuttaya</th>
<th>Chachoengsao</th>
<th>Suratthani</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hipposideros armiger</td>
<td>1/8</td>
<td>0/23</td>
<td>0/5</td>
<td>0/45</td>
<td>0/1</td>
<td>1/13</td>
</tr>
<tr>
<td>Eonycteris spelaea</td>
<td>0/22</td>
<td>0/23</td>
<td>0/5</td>
<td>0/45</td>
<td>0/1</td>
<td>0/45</td>
</tr>
<tr>
<td>Rousettus leschenaulti</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Pteropus lylei</td>
<td>8/136</td>
<td>4/58</td>
<td>2/105</td>
<td>1/36</td>
<td>0/5</td>
<td>15/335</td>
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<tr>
<td>Total</td>
<td>9/167</td>
<td>4/81</td>
<td>2/105</td>
<td>1/36</td>
<td>0/5</td>
<td>16/394</td>
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Table 3. Neutralization of lyssaviruses by Thai bat sera*†

<table>
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<tr>
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<th>Aravan</th>
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<th>Irkut</th>
<th>ABLV</th>
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</tbody>
</table>

*ABLV, Australian bat lyssavirus; CVS, challenge virus standard.
†Boldface indicates statistical significance.
Acknowledgments

We thank our colleagues at the Thai Red Cross Society, Ministry of Agriculture, and Chulalongkorn University for their input and expertise, Denny Constantine and Richard Luce, and staff members in the Viral and Rickettsial Zoonoses Branch of the Centers for Disease Control and Prevention. Special thanks to the family of Joachim-Sutthiporn Bulian for the field information.

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Dr. Lumlertdacha is a staff member in the Rabies Diagnostic and Quarantine Unit, Queen Saovabha Memorial Institute, Thai Red Cross Society. His areas of interest are rabies epidemiology, zoonoses, including surveys of Nipah virus and lyssaviruses, and the diagnosis of rabies in animals.

References


Address for correspondence: Boonlrt Lumlerdacha, Queen Saovabha Memorial Institute, Thai Red Cross Society, Rama 4 Rd, Bangkok 10330, Thailand; fax: 662-2540212; email: Qsmibld@yahoo.com
The presence of the nucleic acid of the spotted fever group (SPG) and typhus group (TG) rickettsiae was investigated in 200 serum specimens seropositive for SFG rickettsiae by multiplex-nested polymerase chain reaction with primers derived from the rickettsial outer membrane protein B gene. The DNA of SFG, TG, or both rickettsiae was amplified in the 24 serum specimens, and sequence analysis showed *Rickettsia conorii*, *R. japonica*, and *R. felis* in the specimens. *R. conorii* and *R. typhi* were found in 7 serum specimens, which indicated the possibility of dual infection in these patients. These findings suggest that several kinds of rickettsial diseases, including boutonneuse fever, rickettsialpox, *R. felis* infection, and Japanese spotted fever, as well as scrub typhus and murine typhus, are occurring in Korea.

Spotted fever group (SFG) rickettsioses are associated with arthropods, such as ticks, mites, and fleas (2). SFG comprises several divergent lineages: the *R. rickettsii* group, *R. japonica*, *R. montana*, the *R. massiliae* group, *R. helvetica*, *R. felis*, and the *R. akari* group (2). Recently, the nucleic acids of *R. japonica* and *R. rickettsii* were found in *Haemaphysalis longicornis* in Korea (7). A previous seroepidemiologic study demonstrated that SFG rickettsioses were highly likely in Korea (8). No clinical human case of SFG rickettsioses, however, has been reported in Korea until now.

In this study, to check whether SFG rickettsioses were present in humans, serum specimens from patients with acute febrile disease were studied by using molecular sequence–based identification techniques. We report the presence of the rompB gene of SFG rickettsiae, similar to *R. akari*, *R. conorii*, *R. japonica*, and *R. felis*, in serum specimens from Korean patients with acute febrile disease. The nucleic acids of both *R. conorii* and *R. typhi* were found to coexist in 7 serum specimens. This study presents the first molecular evidence of SFG rickettsioses in humans.

**Materials and Methods**

**Rickettsial Strains**

The following strains were obtained from the American

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**Materials and Methods**

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The following strains were obtained from the American

**Serum Samples and Serologic Testing**

The serum specimens analyzed in this study were obtained from South Korean patients with acute febrile illness from 1993 to 1999. The specimens were submitted to the Institute of Endemic Disease at Seoul National University’s Medical Research Center for laboratory diagnosis for scrub typhus, leptospirosis, and hemorrhagic fever with renal syndrome caused by hantavirus. Some of the serum specimens were used for the nucleic acid detection study of SFG rickettsial agents. The rationale for selecting the samples for polymerase chain reaction (PCR) analysis included the presence of immunoglobulin (Ig) M antibodies with titers from 1:40 to 1:160 against any of the tested antigens in the samples. Serologic testing was performed by indirect immunofluorescence assay (IFA) with a panel of 4 SFG rickettsial antigens, *R*. *japanica*, *R*. *akari*, *R*. *conorii*, and *R*. *sibirica*, as previously described (8).

**Oligonucleotide Primers**

The oligonucleotide primers used for priming the PCRs are shown in Table 1. The primers were developed on the basis of the *rompB* gene sequences of *R*. *conorii* strain Seven (GenBank accession no. AF123721), and the citrate synthase (*gltA*) gene sequence of *R*. *prowazekii* (GenBank accession no. M17149) was synthesized. The selection of the primers was based on the “primer 3” program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/), to obtain the optimal melting temperature and GC content and to avoid hairpin loop structures. The selected sequences were analyzed through the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target rickettsia group</th>
<th>Gene</th>
<th>Position</th>
<th>Nucleotide sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rompB OF</td>
<td>SFG and TG</td>
<td>rompB†</td>
<td>3,620–3,643</td>
<td>GAAACGGAGTAGATCGTTTCTGTTAA</td>
</tr>
<tr>
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<td>SFG and TG</td>
<td>rompB</td>
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<td>GCTTTATACCAACTAACCACCA</td>
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<td>rompB SFG IF</td>
<td>SFG</td>
<td>rompB</td>
<td>3,652–3,674</td>
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<td>rompB SFG/TG IR</td>
<td>SFG and TG</td>
<td>rompB</td>
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<td>TG</td>
<td>rompB</td>
<td>3,828–3,850</td>
<td>AAGATCTTCCGTAGTTGGAACAA</td>
</tr>
<tr>
<td>RpCS.877p§</td>
<td>SFG and TG</td>
<td>gltA‡</td>
<td>877–895</td>
<td>GGGGACTGCTGCTCGCGG</td>
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<td>RpCS.1,258n§</td>
<td>SFG and TG</td>
<td>gltA</td>
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<td>RpCS.896p</td>
<td>SFG and TG</td>
<td>gltA</td>
<td>896–915</td>
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<td>RpCS.1,233n§</td>
<td>SFG and TG</td>
<td>gltA</td>
<td>1,233–1,215</td>
<td>GGGCGCTGCTGATTACCC</td>
</tr>
</tbody>
</table>

*SGF, spotted fever group; TG, typhus group; OR, outer reverse primer; OF, outer forward primer.
†Oligonucleotide primer sequences derived from *Rickettsia conorii* genes (accession no. AF123721).
‡Oligonucleotide primer sequences derived from *R. prowazekii* genes (accession no. M17149).
§Primer sequences derived from (9).

**Detection of rompB Gene in Human Sera**

DNA for PCR analysis was extracted from 200 µL of serum samples by using QIAamp Blood Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. SFG and typhus group (TG) rickettsia *rompB* gene in human sera were detected with multiplex nested PCR. The primary amplification of the specimen was performed in a final reaction volume of 50 µL. The reaction mixture contained 5 µL of prepared DNA sample, 20 pmol of *rompB* outer forward primer (OF) and outer reverse primer (OR), 200 µM of deoxynucleoside triphosphate mixture (dTTP, Takara, Otsu, Japan), 1 x PCR buffer, 1.25 U Taq polymerase (Takara EX Taq, Takara), and distilled water. First, PCR reactions were incubated at 95°C for 5 min, subjected to 35 cycles of 95°C for 15 s, 54°C for 15 s, and 72°C for 30 s, and final extension at 72°C for 3 min in a GeneAmp PCR system 9600 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). After this, 2 µL of the amplified product was again amplified in a nested fashion with inner primer sets (rompB SFG IF, rompB SFG/TG IR, and rompB TG IF). The nested PCR reaction mixture contained 10 pmol of each primer in a PCR premix tube (AccuPower PCR PreMix, Bioneer Corp., Daejon, Korea) that contained 1 U of Taq DNA polymerase, 250 µmol/L each of dNTP, 50 mmol/L of Tris-HCl (pH 8.3), 40 mmol/L of KCl, 1.5 mmol/L of MgCl2, and gel loading dye. The volume was then adjusted to 20 µL with distilled water. Nested PCR reactions were incubated at 95°C for 5 min, subjected to 35 cycles of 95°C for 15 s, 56°C for 15 s, and 72°C for 30 s, and final extension at 72°C for 3 min. PCR amplification of the *gltA* gene of SFG and TG rickettsiae was performed by using the oligonucleotide pairs RpCS.877p and RpCS.1,258n for the primary PCR amplification and RpCS.896p and RpCS.1,233n for the secondary amplification. The primary PCR cycling condition consisted of incubation at 95°C for 5 min, then 35 cycles each of 15 s at 95°C, 15 s at 54°C, and 30 s at 72°C, followed by a final extension cycle of 3 min at 72°C. The nested PCR cycling condition consisted of incubation at
95°C for 5 min, then 35 cycles each of 15 s at 95°C, 15 s at 54°C, and 30 s at 72°C, followed by a final extension cycle of 3 min at 72°C. To avoid cross-contamination, 3 separate rooms with entirely separate equipment and solutions were used. Thus, the handling and treatment of samples and the addition of a template, the handling of DNA-free PCR reagents, and the post-PCR work were strictly separated. Aerosol-resistant tips (Axigen Scientific, Inc., Union City, CA, USA) were used for the handling of all reagents in the PCR study. The amplification products were visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.5 µg/mL) and using 1 x TAE migration buffer (pH 8.0; 40 mmol/L Tris-acetate, 1 mmol/L EDTA).

Restriction Fragment Length Polymorphism (RFLP) Analysis

The PCR products were purified by using an AccuPrep PCR purification kit (Bioneer Corp.), according to the manufacturer’s instructions. Restriction endonuclease digestions were performed with 10 µL of amplified products by using AluI (New England Biolabs, Beverly, MA, USA). The digested DNA was resolved by electrophoresis through a 10% polyacrylamide gel at 100 V for 4 h in a 1 x TAE buffer (pH 8.0; 90 mmol/L Tris-borate, 2 mmol/L EDTA), and was visualized after staining with ethidium bromide.

Cloning, Sequencing, and Analysis of Nucleotide

All positive PCR products were cloned by using pGEM-T Easy Vector System I (Promega). Verifying whether the clones contained inserts was accomplished by digestion of plasmid DNA with EcoRI (New England Biolabs) and separation in 1.5% agarose gels. Plasmids containing DNA inserts were sequenced for both strands by using Big Dye Terminator Sequence Kit and ABI Prism 377 Automated DNA Sequencer (Perkin-Elmer Applied Biosystems), according to the manufacturer’s protocol. The obtained sequences, except for the primer regions, were aligned with the corresponding sequences of other rickettsiae deposited in the GenBank database to identify known sequences with a high degree of similarity using multisequence alignment programs, the Phydit software (10), and the MegAlign software package (Windows version 3.12e; DNASTAR, DYNASTAR Inc., Madison, WI, USA). Phylogenetic trees were generated by using the neighbor-joining algorithms and the Jukes and Cantor matrix. Bootstrap analysis was performed to investigate the stability of the trees obtained through the neighbor-joining method. The percentages of similarity were determined using the FASTA network service (European Bioinformatics Institute Fasta Service; available from http://www.ebi.ac.uk/fasta).

Nucleotide Sequence Accession Numbers Used

GenBank accession numbers of the rompB gene sequences used for sequence comparisons are AB003681 for R. japonica, AF123705 for R. aeschlimannii, AF123706 for R. africae, AF123707 for R. akari, AF123708 for Astrakhan rickettsia strain A-167, AF123709 for R. australis, AF123711 for R. honei strain RB, AF123712 for Israeli tick typhus rickettsia, AF123714 for R. massiliiae, AF123715 R. mongolotimonae, AF123716 for R. montanensis, AF123717 for R. parkeri, AF123719 for R. rhipicephali, AF123721 for R. conorii strain Seven, AF123722 for R. sibirica, AF123723 for R. slovaca, AF123725 for R. helvetica, AF182279 for R. felis, AF211820 for R. prowazekii strain Florida, AF211821 for R. prowazekii strain Virginia, AF123718 for R. prowazekii, AF161079 for R. prowazekii, AF479763 for R. amblyommii strain WB-8-2 rompB pseudogene, AY260451 for R. heilongiangensis, AY260452 for R. huliniensis, L04461 for R. typhi crystalline surface layer protein (slpT) gene, and X16353 for R. rickettsii. The GenBank accession number of the gltA gene sequence used for developing primers is M17149 for R. prowazekii.

Results

Multiplex Nested PCR Amplification of rompB Gene

Nested PCR assay, with primer pairs rompB OF and rompB OR in primary reactions and rompB SFG IF, rompB SFG/TG IR, and rompB TG IF in multiplex-nested reactions, was performed to identify the unknown rickettsial agents in the seropositive serum specimens and to differentiate between SFG and TG rickettsiae in terms of size. When the primers previously mentioned were used, the nested PCR assay generated ≈420 bp for SFG rickettsiae and about 230 bp for TG rickettsiae. The negative controls consistently failed to yield detectable PCR products, whereas the positive controls always gave the expected PCR products. Overall, 200 serum specimens from febrile patients from all areas of South Korea were tested. After the nested PCR was performed, the expected rompB gene products were obtained from 24 seropositive serum samples. Figure 1 shows the result of electrophoresis of 24 PCR-amplified samples. Of the 24 amplified products, 16 showed the electrophoretic pattern of 1 DNA band of ≈420 bp, which corresponded to SFG. The amplified size of only 1 sample was ≈230 bp for TG. The 7 other amplified products showed an electrophoretic pattern of 2 bands of ≈420 bp for SFG and 230 bp for TG. Therefore, the 23 amplified products corresponding to SFG rickettsial agents were named H1 product, while the 8 products corresponding to TG were named H2 product. The H1 products included H1 to H24 (except H19), while the H2 products were H3-2, H7-2, H8-2, H13-2, H14-2, H15-2, H18-2, and H19 (Figure 1).
RFLP Analysis and Sequencing Analysis

RFLP analysis of the 23 H1 products corresponding to SFG rickettsial agents using AluI demonstrated that the restriction patterns of 17 H1 products were identical with that of R. conorii, 2 with that of R. akari, 1 with that of R. japonica, and 3 with that of R. felis (Figure 2). RFLP analysis of the 8 H2 products corresponding to TG rickettsial agents by using AluI showed that the restriction patterns of all the H2 products were identical with that of R. typhi (Figure 3).

Sequencing Analysis

To identify the SFG and TG rickettsiae detected in human serum specimens, nucleotide sequences of the PCR-amplified products were determined and compared with partial rompB gene sequences of various rickettsial agents obtained from the GenBank database. Table 2 shows the similarity between the partial rompB gene sequences of various rickettsial agents and 6 of the sequenced H1 products (clones H1, H3, H5, H10, H20, and H22). Clones H1, H3, and H20 showed 100%, 99.72%, and 98.87% degrees of similarity to R. conorii, respectively. Clone H10 showed 100% similarity to R. japonica, and clone H5 showed 100% similarity to R. akari. In particular, clone H22 showed 99.44% similarity to R. felis. All the compared H1 products showed low levels of similarity (70.90%–74.01%) to the TG species. The clones that clustered partially with the rompB gene of R. conorii were differentiated in 3 groups by their levels of similarity: group 1 (12 H1 products with 100% similarity), group 2 (4 H1 products with 99.72% similarity), and group 3 (1 H1 product with 98.87% similarity). Clones H22, H23, and H24 clustered as the R. felis group. Table 3 shows the similarity between the partial rompB gene sequences of various rickettsial species and H2 product sequences. All H2 products showed low levels of similarity (67.05%–69.94%) to SFG rickettsial species, such as R. sibirica, R. akari, R. conorii, R. felis, and R. japonica. They also showed high levels of similarity (93.64%–100%) to TG rickettsial species, such as R. prowazekii and R. typhi. The H2 products’ levels of similarity to R. typhi ranged from 99.42% to 100%. A neighbor-joining analysis based on partial rompB gene sequences demonstrated that 17 H1 products formed a cluster with R. conorii, 2 with R. akari, 1 with R. japonica, and 3 with R. felis (data not shown). The analysis of the
8 H2 product sequences showed that the sequences of all H2 products formed a cluster with R. typhi and were separated from the SFG rickettsial strains (data not shown).

Nested PCR Amplification of gltA Gene

The results of the multiplex nested PCR of the rompB gene were confirmed by a second PCR assay with specific primer pairs RpCS.877p and RpCS.1,258 in primary reactions and RpCS.896p and RpCS.1,233 in nested reactions. The primer sets generated ≈338 bp for SFG and TG rickettsiae. The expected size of the gltA gene fragment was generated in 22 of 24 samples that were positive for the PCR detection of the rompB gene (Figure 4). All positive PCR products were cloned, and their sequences were determined. Since the PCR assay using primer sets for the amplification of the gltA gene could not discriminate between the SFG rickettsia and TG rickettsia by size difference, the sequences of 3 clones for each PCR product were determined. The results of the sequencing analysis for gltA-PCR amplifications were identical to those of the analysis of the rompB-PCR product (data not shown). Seven samples that were positive for both the rompB genes of R. conorii and R. typhi were also positive for both of their gltA genes (data not shown).

Discussion

SFG and TG rickettsial infections occur worldwide and may cause serious diseases in humans. These pathogenic bacteria are transmitted to people by arthropod vectors, such as ticks, fleas, and lice. In this study, multiplex-nested PCR was conducted to detect and identify SFG and TG rickettsial antigens in patient sera with positive results from the serosurvey. The rompB gene domain II region, which is a highly conserved region of rompB, was targeted for PCR amplification for the specific detection of SFG and TG rickettsiae. Amplified DNA sequences were analyzed by using nucleotide-sequencing methods, and RFLP analysis was used to confirm the PCR results. The results indicated the presence of several SFG rickettsiae, R. conorii, R. akari, R. japonica, and R. felis, in the serum specimens. The results were also confirmed by a second PCR with specific primer pairs for the gltA gene and by sequence analysis of its DNA amplicons.

Table 2. Similarity matrix between partial rompB gene sequence of various rickettsial strains and nested polymerase chain reaction (H1 products)

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<th>4</th>
<th>5</th>
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<th>7</th>
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<th>H5</th>
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*1, partial rompB of Rickettsia akari (AF123707); 2, R. conorii (AF123721); 3, R. felis (AF182279); 4, R. japonica (AB003681); 5, R. sibirica (AF12322); 6, R. prowazekii (AF211820); 7, R. typhi (L04661).
†H, H1 products amplified from patient sera.
‡The similarity values (%) on the lower left are the levels of similarity between partial rompB gene sequences.
For the first time, SFG rickettsiae in human serum specimens in South Korea have been reported. *R. akari* is a member of the spotted fever group rickettsiae and is a causative agent of rickettsial pox, a disease transmitted by the bite of *Allodermamyssus sanguineus*, a mite ectoparasite of the domestic mouse (*Mus musculus*) (2). The disease was first described in New York City in 1946. *R. akari* was isolated from the Korean vole in 1957. The previous seroepidemiologic study conducted by the authors on 3,401 patients with febrile disease indicated that the seropositive rate was 16.24% for the rickettsial antigen through IFA. *R. conorii* is an etiologic agent of the Mediterranean spotted fever or boutonneuse fever (2). Our previous study indicated that the seropositive rate was 14.34% for the antigen. *R. japonica*, the causative agent of Oriental spotted fever, was first isolated from a patient with febrile, exanthematous illness in Japan in 1985 (2). Previous studies showed the presence of nucleic acids of *R. japonica* and *R. rickettsii* in *H. longicornis* by PCR. Our seroepidemiologic study demonstrated that the seropositive rate...
was 19.9%. Although no clinical human case of SFG rickettsioses has been reported in Korea until now, this study’s findings strongly suggest the prevalence of SFG rickettsiosis in Korea.

*R. felis* is an emerging pathogen responsible for fleaborne spotted fever and had been considered a member of the TG rickettsiae based on its reactivity with anti-*R. typhi* antibodies. A genetic analysis of the 16S rRNA, citrate synthase, *rompA*, and *rompB* genes, however, placed *R. felis* as a member of SFG. *R. felis* has been reported in various countries, including the United States, Mexico, Brazil, Germany, and France (11,12). In Asia, the first case of *R. felis* infection was reported in 2003 (13). *R. typhi* was also among those detected in the SFG rickettsiae in the febrile disease patients’ sera. Fleas are also found to be vectors for *R. typhi* (2). Of major importance to the epidemiology of the rickettsioses caused by *R. typhi* and *R. felis* is the maintenance of both rickettsial agents in their hosts by transovarial transmission, and the fact that neither organism is lethal for fleas (14).

Finally, we report the presence of both *R. conorii* and *R. typhi* in serum from Korean patients. Sera from patients with SFG rickettsiosis have been reported to react with TG rickettsiae by using serologic analysis methods (15). The serum specimens from patients with TG rickettsiosis were also demonstrated to contain cross-reactive antibodies against SFG rickettsiae (15,16). In a previous study, approximately one third of specimens seropositive for antibodies against SFG rickettsiae had antibodies against TG rickettsiae (unpub. data). Therefore, the multiplex-nested PCR was designed to detect and differentiate SFG rickettsial agents from TG rickettsial agents in the patient serum specimens with positive results from the serosurvey with SFG rickettsial antigens. SFG rickettsiae and TG rickettsiae were differentiated in terms of the size of amplified products. PCR results also confirmed the RFLP and sequencing analysis. In sera taken from 7 patients, both SFG and TG rickettsial antigens were detected, which indicated dual infection. Previously, a case of dual infection with *Ehrlichia chaffeensis* and an SPG rickettsia was reported in a human patient. Cases of dual infection with *Bartonella clarridgeiae* and *B. henselae* in cats have also been reported, as well as infection with the 2 different genotypes of *B. henselae* (17,18). A recent report suggested that coinfection of *R. felis* with either *B. clarridgeiae* or *B. quintana* in fleas may cause dual infection in a human that comes in contact with flea feces (14). These reports support this study’s findings regarding the dual infection of SFG and TG rickettsiae in 7 patients. The differences between *R. conorii* and *R. typhi* vectors, however, still cannot be explained, and further studies are needed.

In conclusion, this study confirmed, by using PCR-based amplification methods, that several SFG rickettsiae, *R. conorii*, *R. akari*, *R. japonica*, and *R. felis*, existed in the sera of Korean patients with febrile episodes. Our findings indicate that SFG rickettsiae, including *R. felis*, should be used in serologic tests on Korean patients suspected of having rickettsiosis. TG rickettsiae existed in 8 patients, and 7 of them were also infected with *R. conorii*. The evidence of double infection is expected to help describe the cross-reactivity between the patient sera of SFG rickettsioses and TG rickettsioses.

This study was supported by a grant from the Korea Health 21 Research and Development Project, Ministry of Health and Welfare, Republic of Korea (01-PJ10-PG6-01GM01-0004). This study was conducted in the Department of Microbiology, College of Medicine, Konkuk University.

Dr. Choi is a postdoctoral fellow in the Department of Microbiology, College of Medicine, Konkuk University,
Choong-cheongbuk-do, Korea. This work is part of her doctoral thesis. Her research focuses on the serologic and molecular epidemiology of various rickettsial diseases and the development of diagnostic tools.

References


Address for correspondence: Ik-Sang Kim, Department of Microbiology and Immunology, Seoul National University College of Medicine and Institute of Endemic Disease, Seoul, 110-799, Republic of Korea; fax: 82-43-851-9329; email: molecule@plaza.snu.ac.kr
The possible presence of *Pneumocystis* among healthy adults was examined by detecting *Pneumocystis jirovecii*-specific DNA in prospectively obtained oropharyngeal wash samples from 50 persons without underlying lung disease or immunosuppression. *Pneumocystis* carriage, defined by detecting *Pneumocystis* DNA by nested polymerase chain reaction in 2 independent analyses plus successful mitochondrial large subunit ribosomal RNA typing by direct sequencing, was found in 20% of cases. All carriers were asymptomatic, anti-HIV negative, and had normal total lymphocyte and CD4+ cell counts. A second sample obtained in the 6-month follow-up was positive in 2 of 9 available carriers. Genotype analysis showed different polymorphisms; 85A/248C (40%) and 85C/248C (30%) were most frequently observed. This study provides the first evidence that *P. jirovecii* DNA can be frequently detected in the respiratory tract of immunocompetent adults, which agrees with the hypothesis that the general population could be a reservoir and source of this infection.

*Pneumocystis jirovecii* (formerly known as *Pneumocystis carinii* f. sp. *hominis*) (1) is the causative agent of *Pneumocystis* pneumonia (PCP), one of the most frequent and severe opportunistic infections in immunocompromised patients (2). *Pneumocystis* organisms represent a large group of species of atypical fungi with universal distribution and pulmonary tropism, and each species has a strong specificity for a given mammalian host species (3,4).

Despite the advances made in understanding human *Pneumocystis* infection, many aspects about its epidemiology and natural history remain unclear. Serologic studies have shown that specific antibodies to the pathogen can be detected in most children early in life (5–7), which indicates frequent exposure to this organism. Based on this finding, disease in immunocompromised persons has long been thought to result from reactivation of latent infection acquired in childhood. However, animal and human studies have shown that elimination of *Pneumocystis* often occurs after infection (8–9), which implies that the persistence of latent organisms is limited.

Alternatively, the possibility that *Pneumocystis* can be transmitted from person to person has been raised after the reports of cluster outbreaks of PCP among solid-organ transplant and oncology patients (10,11). Evidence supporting the active or de novo airborne acquisition of the organism from human sources has accumulated in the last few years, including evidence for different *Pneumocystis* genotypes in different episodes of PCP in the same patient (12,13). Also, *Pneumocystis* DNA was detected in the upper respiratory tract of healthy participants after close contact with patients with PCP (14–16) and in air samples from the rooms of PCP patients (17,18). *Pneumocystis* has also been found in immunosuppressed patients without PCP (19); these situations have been described as *Pneumocystis* colonization or carriage.

PCP patients, immunodeficient carriers, or transiently parasitized immunocompetent persons have been hypothesized to play a role as sources of *Pneumocystis* infection (4). Although some earlier studies failed to detect the organism in postmortem lung samples or bronchoalveolar samples from immunocompetent adults (20,21), a recent report indicates that *Pneumocystis* DNA can be frequently detected in healthy infants (22).

The ability to detect *Pneumocystis* in normal, healthy persons is due to the development of more sensitive methods. *Pneumocystis* can now be detected in respiratory samples obtained by noninvasive methods using immunofluorescence staining and polymerase chain reaction (PCR) (23,24). By using these methods, *Pneumocystis* carriage was found in 10% to 40% of immunocompetent
patients with different chronic lung diseases (25,26). From an epidemiologic point of view, this high prevalence is difficult to determine if PCP or colonized immunosuppressed patients and their close contacts are the only sources of infection, since the persistence of latent organism in lung appears to be time-limited (8,9). We tested the hypothesis that in a normal community environment healthy adults can be transiently colonized by *Pneumocystis*, and these persons play a role in the persistence of the organism in the human ecosystem. Identifying *Pneumocystis* sources is essential to developing proper measures to prevent a disease that still causes substantial illness and death among immunosuppressed patients. This study attempts to determine whether *P. jirovecii* can be detected in the general normal, healthy population.

**Methods**

**Study Population**

This prospective study included persons who 1) had not been exposed to patients in a hospital environment within the year before the study or 2) had not been diagnosed with or were not suspected to have chronic lung disease, neoplasms, or immunosuppression of any cause. The first 50 persons evaluated in the Occupational Health Service of the Virgen del Rocío University Hospital from February to July 2003 who had not been excluded by the above criteria were enrolled in this study.

The mean age of persons in this study group was 33.9 ± 9.45 years. Nineteen (31.6%) were male. Distribution according to professional standing was 28 (56%) newly employed physician residents, 13 (26%) university or common services staff members, and 9 (18%) administrative staff.

Each participant underwent a clinical-epidemiologic examination, and oropharyngeal samples were collected for analysis in the Occupational Health Service, a building located outside the hospital environment. Demographic variables, underlying medical conditions, habits, and antimicrobial therapy were recorded by using a standardized form. Informed consent was obtained from participants. The study protocol was designed and performed according to the Helsinki Declaration and was approved by the ethics committee, Virgen del Rocío University Hospital, Seville, Spain.

For all *Pneumocystis* carriers, a complete clinical and biologic evaluation was performed, including physical examination, chest x-ray, conventional blood work, anti-HIV serologic examination, and peripheral blood lymphocyte subsets analyses. Volunteers who were designated *P. jirovecii* carriers were reexamined after 6 months, when oropharyngeal samples were again obtained.

**Case Definition**

A *Pneumocystis* carrier was defined as a person who met all of the following conditions: 1) no clinical history of PCP, 2) respiratory specimen with detectable *P. jirovecii* DNA by nested PCR in 2 independent analyses, and 3) successful mitochondrial large subunit ribosomal RNA (mtLSU-rRNA) typing of the respiratory specimen by direct sequencing at least once. Persons who did not meet these criteria were considered *Pneumocystis*-negative.

**Sampling and Detecting *P. jirovecii***

Oropharyngeal wash samples were obtained by gargling with 10 mL of sterile physiologic serum (0.9% NaCl) for a period of 1 min. Samples were centrifuged at 2,900 × g for 5 min and kept frozen at –20ºC until DNA was extracted.

After digestion with proteinase K at 56ºC for 2 h, DNA from 2 aliquots of each oropharyngeal wash sample was extracted on 2 days by using a commercial kit from Qiagen (Hilden, Germany). Sham extractions, carried out in parallel with the processing of samples, were also included to control for contamination in the DNA extraction step. The purified DNA was used as a template to amplify the region containing mtLSU-rRNA by nested PCR, as described elsewhere (27,28). The sensitivity of this nested PCR assay is 1 organism/µL. Briefly, in the first amplification round, the external primers pAZ102-E (5′-GAT GGC TGT TTC CAA GCC CA-3’) and pAZ102-H (5′-GTG TAC GTT GCA AAG TAC TC-3’) were used. This amplification yields a 346–base pair (bp) fragment. The second round of amplification used the primers pAZ102-X (5′-GTG AAA TAC AAA TCG GAC TAG G-3’) and pAZ102-Y (5′-TCA CTT AAT ATT AAT TGG GGA GC-3’) and yielded a 260-bp product. Forty cycles of amplification were carried out for both rounds.

The amplification products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide, and the bands were visualized by UV light. To prevent false-positives from contamination, pipettes with filters were used in all manipulations. DNA extraction, preparation of the reaction mixture, PCR amplification, and detection were performed in different areas under a laminar flow hood. To detect any cross-contamination, all PCRs were performed with negative controls and sterile water.

The products from nested PCR amplification were purified by using Sephacryl S-400 columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and reamplified by using ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). Then, for each reaction, 5 µL of PCR product, 4 µL terminator-ready reaction mix, and 3 pmol of primer were added. The extension products were
purified by ethanol precipitation to remove excess dye terminators. Each sample pellet was resuspended in 12.5 µL of template suppression reagent and heated at 95°C for 3 min for denaturation. Electrophoresis was carried out on the ABI prism 310 sequencer (PE Applied Biosystems) according to manufacturer’s recommendations. The sequenced DNA fragments were analyzed by using Sequence Navigator v.1.0.1 (PE Applied Biosystems).

In the specimens of carriers, the *P. jirovecii* dihydropteroate synthase (DHPS) locus was analyzed by PCR restriction fragment length polymorphism (RFLP), as previously described (28). In brief, the single-copy gene of DHPS was amplified by the primers DHPS-3 (5'-GCG CCT ACA CAT ATT ATG GCC ATT TTA AAT C-3') and DHPS-4 (5'-GGA ACT TTC AAC TTG GCA ACC AC-3') by using a touchdown-PCR protocol, yielding a 370-bp fragment. The PCR product was divided into 3 aliquots. One was used to confirm the presence of a 370-bp fragment from the DHPS gene. The second and third aliquots were used to identify wild-type sequences versus mutations in codons 55 and 57 by RFLP with AccI and HaeIII (Roche Diagnostics, Mannheim, Germany), respectively. When the mutation is present, a 370-bp band appears. After RFLP in wild-type samples, bands appear at 229 bp and 141 bp with AccI and at 221 bp and 149 bp with HaeIII.

**Laboratory Studies**

Peripheral blood lymphocyte subsets were determined by using a flow cytometer (Cytorion Absolute, Ortho, Raritan, NJ, USA) after incubation with monoclonal antibodies OKT3, OKT4, and OKT8 (Ortho). Serum anti-HIV antibodies were determined by a commercial enzyme-linked immunosorbent assay (ELISA) (Multispot HIV-1/HIV-2 rapid test, BioRad, Hercules, CA, USA). The results were interpreted according to the manufacturer’s recommendations.

**Statistical Analysis**

The chi-square test was used for assessing differences between proportions. Results were considered significant at p < 0.05. Statistical analyses were performed by using the Statistical Package for Serial Studies for personal computers (SPSS version 12, SPSS Inc., Chicago, IL, USA).

**Results**

*P. jirovecii* DNA in 12 of 50 samples was successfully amplified twice by nested PCR. The mtLSU-rRNA fragment locus was successfully typed in 10 of the 12 samples in which mtLSU-rRNA had been amplified. Thus, *P. jirovecii* carriage was detected in 20% of the participants. To assess the reproducibility and consistency of results, serial samples were obtained with a 2-day interval in 5 participants (1 carrier and 4 noncarriers). In all of them, consistent positive and negative patterns of results were obtained (positive PCR test after a positive result and negative PCR test after a negative result).

The DHPS primer sets amplified a 370-bp band in 7 (70%) of 10 carriers. In all positive specimens with the DHPS-based PCR assay, the RFLP technique identified a wild DHPS genotype (Table).

Results of physical examination were normal for *Pneumocystis* carriers. All were anti-HIV negative and had normal total lymphocyte and CD4+ cell counts. Chest radiographs were normal in 8 participants, and 1 participant had an apical cystic bullae (Table). Only 1 person had taken steroids for a brief period in the 6 months before the study. No differences were detected due to age, sex, profession, alcohol intake, and smoking habit between *P. jirovecii* carriers and noncarriers.

Five known mtLSU-rRNA types are described for this *Pneumocystis* gene locus (15); 4 genotypes were isolated in the current study. Genotypes at this locus were identified on the basis of polymorphisms at nucleotide positions 85 and 248. Genotype 2 (85:A/248:C) was observed in 4 cases, genotype 1 (85:C/248:C) in 3 cases, genotype 3 (85:T/248:C) in 2 cases, and genotype 4 (85:C/248:T) in the remaining case (Table).

During the follow-up, all carriers were asymptomatic for pulmonary disease. *Pneumocystis* DNA was detected by PCR in a second oropharyngeal wash obtained 6 months after the first in 2 (22.2%) of the 9 available carriers. Neither sample was typed because of insufficient quantity of PCR product.

**Discussion**

This study on immunocompetent healthy adults documents that *P. jirovecii* DNA can be detected by sensitive DNA amplification techniques by using noninvasive sampling of the respiratory tract. DNA detection does not establish the existence of infectious intact organisms. However, in animal models, detecting *Pneumocystis* DNA in nasal and oral samples is a good indicator that the organism is in the lung (29). Also, experiments show that *Pneumocystis* organisms can replicate in the lung alveolus of immunocompetent hosts and remain infectious (30). Thus, our results agree with the hypothesis that the general human population could play an important role as a reservoir and source of *P. jirovecii* infection and support the saprophytic nature of this pathogen in humans.

An important finding of this study is that *Pneumocystis* DNA was not detected in >75% of the immunocompetent colonized adults within 6 months, which suggests the possible transience of the carrier state in healthy persons. This observation agrees with previous reports that show that most immunocompetent healthcare workers who
were colonized with the pathogen cleared the infection (15,16).

The number of persons examined in this study sufficiently demonstrated that *P. jirovecii* is an organism frequently found in healthy adults in the normal community. Since participants were all affiliated in some way with the Virgen del Rocío University Hospital in Seville, Spain, a broader group of healthy adults would need to be examined to estimate the prevalence of the carriers in the general population. Carrying out the study in a hospital could have somewhat biased the results, although we excluded persons with prior exposure to patients within the hospital and collected samples in a building outside the hospital.

The accepted current diagnostic standard for *Pneumocystis* infection is the direct demonstration of the stained microorganism in respiratory samples. Techniques based on PCR amplification of specific genome regions that provide high sensitivity are now widely used to diagnose several infectious diseases. These technical advances have allowed us to detect infections with samples obtained by noninvasive methods and in samples with low pathogen load. Different studies involving both sputum and bronchoalveolar lavage specimens have demonstrated the higher sensitivity of these techniques compared to conventional staining and monoclonal antibody immunohistochemical techniques (31,32). The main drawback to PCR is the possibility of false-positives (usually because of contamination) and the absence of rapid culture methods to confirm the PCR amplification results obtained. We avoided potential false-positives by adopting stringent precautionary measures and by examining the PCR signal from 2 different genes of *P. jirovecii*.

The mtLSU-rRNA gene was selected for genotyping because it has a high degree of genetic conservation and is useful for detecting intraspecific differences between populations (33). The allelic frequency distribution patterns at this gene seen in the present study are similar to those reported in AIDS-associated PCP cases in a large study conducted in 5 cities in the United States (33). In that study, *P. jirovecii* genotypes were correlated with the place of diagnosis, rather than the person’s place of birth. Our results support the concept of a community source of the infectious agents. Furthermore, an epidemiologic study was recently performed in patients in Spain with various pulmonary diseases. By also analyzing mtLSU-rRNA types, we found a high prevalence of genotype 1 (45%) and genotype 3 (40%) and lower of genotype 2 (10%) (28). In contrast, in the present study, genotype 2 was the most prevalent, whereas it was low in the pulmonary patient study, and genotype 3 was more frequently found in pulmonary patients than in normal healthy adults. Patients with pulmonary disorders may have a greater susceptibility to genotype 3.

In our study, the rate of carriers identified as having the DHPS gene is 70%. The low amplification rate obtained is perhaps related to a low pathogen load in the samples. However, this rate is similar to that reported for AIDS patients with PCP in previously published studies (33).

In the last decade, PCR technologies have shown that immunocompromised patients without PCP can be subclinically infected with *Pneumocystis* (19). In addition, a high prevalence of *Pneumocystis* is seen among immunocompetent patients with chronic pulmonary disorders (25,26,34), patients with small-cell lung carcinoma (35), or pregnant women (36). The pathogen has been detected in immunocompetent contacts of patients with PCP and in immunocompetent healthcare workers, whether or not they had contact with immunocompromised patients (15,16).

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<th>DHPS genotype (codon position; identity) by PCR-RFLP assay</th>
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<td>2 (42, F)</td>
<td>Administrative</td>
<td>No</td>
<td>No</td>
<td>2,455</td>
<td>1,168</td>
<td>1 (85.C/248.C)</td>
<td>1 (55.Thr/57.Pro)</td>
<td>–</td>
</tr>
<tr>
<td>3 (43, F)</td>
<td>Administrative</td>
<td>No</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
<td>4 (85.C/248.T)</td>
<td>Not amplified</td>
<td>–</td>
</tr>
<tr>
<td>4 (40, M)</td>
<td>Administrative</td>
<td>Yes</td>
<td>No</td>
<td>1,528</td>
<td>520</td>
<td>3 (85.T/248.C)</td>
<td>Not amplified</td>
<td>–</td>
</tr>
<tr>
<td>5 (41, F)</td>
<td>New resident</td>
<td>No</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
<td>2 (85.A/248.C)</td>
<td>1 (55.Thr/57.Pro)</td>
<td>NA</td>
</tr>
<tr>
<td>6 (27, F)</td>
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<td>No</td>
<td>No</td>
<td>1,354</td>
<td>550</td>
<td>2 (85.A/248.A)</td>
<td>1 (55.Thr/57.Pro)</td>
<td>–</td>
</tr>
<tr>
<td>7 (32, F)</td>
<td>New resident</td>
<td>No</td>
<td>No</td>
<td>2,300</td>
<td>604</td>
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<td>1 (55.Thr/57.Pro)</td>
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</tr>
<tr>
<td>8 (40, M)</td>
<td>University staff</td>
<td>Yes</td>
<td>Yes</td>
<td>1,730</td>
<td>924</td>
<td>2 (85.A/248.C)</td>
<td>1 (55.Thr/57.Pro)</td>
<td>+</td>
</tr>
<tr>
<td>9 (26, M)</td>
<td>University staff</td>
<td>Yes</td>
<td>Yes</td>
<td>3,602</td>
<td>924</td>
<td>2 (85.A/248.C)</td>
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<td>–</td>
</tr>
<tr>
<td>10 (42, M)</td>
<td>University staff</td>
<td>No</td>
<td>No</td>
<td>1,518</td>
<td>432</td>
<td>1 (85.C/248.C)</td>
<td>Not amplified</td>
<td>–</td>
</tr>
</tbody>
</table>

*mtLSU-rRNA, mitochondrial large subunit ribosomal RNA; DHPS, dihydropteroate synthase; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; F, female; M, male; +, positive result; –, negative result; NA, not available.*

†Chest radiograph results were normal for all participants, except participant 5, for whom results were not available, and participant 9, who showed an apical cystic bullae.

Table. Epidemiologic, biologic, and microbiologic features of healthy *Pneumocystis* carriers*
Also, immunocompetent animal model hosts are commonly transiently colonized; *Pneumocystis* can replicate actively in their lungs and can be transmitted to another host (30,37). Moreover, postmortem lung screening using conventional staining for *Pneumocystis* showed small numbers of the organism in the lungs of immunocompetent individuals (38).

Thus, substantial evidence exists of *Pneumocystis* colonization of healthy persons, and our findings are consistent with these observations. Most previous studies that failed to find the organism on autopsy (21) or in respiratory samples (14,29,31,36,39) from immunocompetent persons were performed on only a few persons. In some cases, failure was probably related to the use of either single PCR (31) or a less sensitive PCR (39). In others, failure could be related to the use of sputum or nasal secretions (14,29,36) that may have had lower numbers of organisms than in the oropharyngeal wash we analyzed or because of different procedures used for analyses (29). Protocols for acquiring and processing respiratory samples and analytical probes and methods should be standardized to enable better comparisons between studies performed in different laboratories.

In summary, immunocompetent healthy adults might harbor short-lived infections that could be transmitted to other immunocompetent host in whom a transient infection can develop. Similarly, infants can become infected and a primary infection can develop, and immunosuppressed, susceptible people can become infected and clinical PCP can develop. Today, we know that human pneumocystosis is anthropogenic. Our findings may suggest that healthy adults represent a new dynamic reservoir and source of infection for human *Pneumocystis* species. Immunocompetent carriers in community ecosystems might present a public health issue that merits further research.

Acknowledgment

We thank Edna S. Kaneshiro for assistance during the preparation of the manuscript.

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Dr. Medrano is an instructor and fellow in the Department of Internal Medicine, Virgen del Rocio University Hospital, Seville, Spain. His main areas of interest include physiopathologic and epidemiologic research on human pathogens, such as *Leishmania* and *Pneumocystis* spp.

References


Address for correspondence: Francisco J. Medrano, Department of Internal Medicine, Virgen del Rocío University Hospital, Avda Manuel Siurot s/n, 41013 Seville, Spain; fax: 34-95-501-4278; email: medrano@cica.es

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Stella Goh,* Mark Reacher,† David P. Casemore,‡ Neville Q. Verlander,§ André Charlett,§ Rachel M. Chalmers,¶ Margaret Knowles,# Anthony Pennington,* Joy Williams,* Keith Osborn,** and Sarah Richards††

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The incidence of sporadic cryptosporidiosis among 106,000 residents of 2 local government districts in northwest England before and after installation of membrane filtration of public water supplies was compared to that of 59,700 residents whose public water supplies remained unchanged. A national outbreak of foot and mouth disease in livestock during 2001 was associated with a decline in sporadic human cryptosporidiosis in all regions of the United Kingdom. In a Poisson regression model, membrane filtration was associated with an estimated 79% reduction (incidence ratio 0.207, 95% confidence intervals 0.099–0.431, p < 0.0001) after adjustment for the interval of the foot and mouth disease epidemic and the water source. Despite the confounding effect of that epidemic, membrane filtration of the public water supply was effective in reducing the risk for sporadic human Cryptosporidium infection in this population.

Cryptosporidium is a genus of enteric parasites that cause diarrhea in humans and many animal species worldwide; it is the third most common cause of nonviral infectious diarrhea reported in England and Wales (1,2). Oocysts are shed in large numbers in feces of infected humans and animals and contain highly infectious sporozoites when ingested (1,3). Disease may be prolonged and fatal in immunocompromised persons (1). Cryptosporidium hominis (previously designated C. parvum genotype 1) is found in humans but occurs naturally in livestock animals very rarely; C. parvum (previously designated C. parvum genotype 2) infects humans and livestock (4–6).

Cryptosporidium oocysts are a threat to the safety of drinking water supplies because they remain viable in water and damp soils for prolonged periods and are resistant to concentrations of disinfectants, including chlorine, used in conventional water treatment. Removal of these oocysts depends on sedimentation, coagulation, and filtration (1,7,8). We have previously reported a prospective case-control study of risk factors for sporadic cryptosporidiosis in residents of Allerdale and Copeland local government districts in North Cumbria, rural northwest England, from March 1, 1996, to February 29, 2000. That study showed a strong association with the usual daily volume of cold unboiled tap water drunk and with short visits to farms (9). We present extended observation of the population to August 31, 2002, during which time membrane filtration of public drinking water supplies was introduced for two thirds of the study population and a national outbreak of foot and mouth disease (FMD) in livestock occurred.

Materials and Methods

The study area comprises part of the Lake District National Park. The lakes act as natural reservoirs for local public water supplies and have livestock farms and open grazing land abutting them. Approximately one third of the study population receive public water supplies from Ennerdale Lake, one third from Crummock Lake, and one third from a number of smaller sources. From March 1, 1996, to February 29, 2000, water from Ennerdale and Crummock Lakes was disinfected with chlorine, but unfiltered, because the low level of particulate matter in these
sources precluded chemically assisted flocculation. Membrane filtration began on March 1, 2000, at works treating water from Ennerdale and Crummock Lakes and remained active until the end of the study. The remaining third of the population received water from a number of smaller sources undergoing a variety of conventional treatments, including coagulation, filtration and chlorination, and chlorination alone. No changes in the treatment of water from these other sources occurred at any time.

**FMD Epidemic in Livestock**

The first FMD case in livestock was confirmed on February 21, 2001, in southeast England and the last case on September 30, 2001, in northwest England (10). Epidemic controls were enforced throughout the United Kingdom; they included culling livestock, excluding livestock from traditional pastures, limiting livestock movements, and excluding the public from the countryside. The FMD epidemic was associated with marked attenuation of the usual spring peak in human cryptosporidiosis reporting from all regions of England and Wales and with a decline in human cryptosporidiosis reporting for England and Wales compared with other regions of the United Kingdom; they included culling livestock, excluding livestock from traditional pastures, limiting livestock movements, and excluding the public from the countryside. The FMD epidemic was associated with marked attenuation of the usual spring peak in human cryptosporidiosis reporting from all regions of England and Wales and with a decline predominantly of *C. parvum* (livestock and human species). FMD epidemic controls were applied uniformly across the Allerdale and Copeland Districts and ended on January 21, 2002.

Analysis of risk factors and laboratory testing for *Cryptosporidium* were undertaken as previously described; cases with date of onset from March 1, 2000, to August 31, 2002, and associated controls were added (5,9). To determine if introduction of membrane filtration and the FMD epidemic in livestock were associated with a change in risk factors or incidence of human cryptosporidiosis, observations were divided into 5 intervals: before commissioning of membrane filtration (March 1, 1996, to February 29, 2000); membrane filtration commissioning (March 1, 2000, to July 31, 2000); established membrane filtration before the FMD epidemic (August 1, 2000, to February 20, 2001); the FMD livestock epidemic to ending of local FMD epidemic controls (February 21, 2001 to January 20, 2002); and post-FMD epidemic (January 21, 2002, to August 31, 2002).

**Case Definition**

Case-patients were residents of Allerdale or Copeland who had diarrhea (≥3 loose stools in a 24-hour period) with onset from March 1, 1996, to August 31, 2002; were fecal smear positive for *Cryptosporidium* oocysts, but feces negative for other enteric pathogens; and had spent at least 1 night within the study area in the 14 days before onset. Patients were excluded if, within 14 days of onset, they had contact with another household patient with cryptosporidiosis or any diarrhea illness, traveled outside the United Kingdom, or traveled within the United Kingdom and stayed outside the study area during the entire 14-day period before onset of illness; or if they, or a household member, had already been enrolled as a case-patient or control at any time during the study.

**Control Definition**

Controls were residents of Allerdale or Copeland who had no history of diarrhea (defined as ≥3 loose stools in a 24-hour period) and had spent at least 1 night within the study area in the 14 days before interview. Potential controls were excluded if they had traveled outside the United Kingdom in the 14 days before the date of interview or if they had traveled within the United Kingdom and stayed outside the study area during the entire 14 day period before the date of interview. Potential controls were also excluded if they or a household member had already been enrolled as a case-patient or control at any time during the study.

The local water company provided details of the water sources, water supply zones, number of houses and residents served by each water source and water supply zone, and treatment of water to each zone. The study team and water company maintained vigilance for changes in the water supplies to the population at all stages of the study. In a minority of participants, the water source changed; these case-patients and controls were categorized as receiving mixed supplies from >1 source.

**Risk Factor Analysis**

Five sets of contingency tables, 1 for each interval of observation defined by introduction of membrane filtration and the FMD epidemic, were constructed for each exposure variable, and odds ratios were calculated. Main effects variables were defined as those significant at p < 0.05 in any of the 5 sets of contingency tables. These variables—interval, age, sex, and water supply zone—were put into a multivariable model. Stepwise sequential removal of variables with p > 0.05 was undertaken retaining time interval, age, sex, and water supply zone. The significance of interaction terms between time interval and the remaining main effects variables was tested in separate models with stepwise sequential removal of terms with p > 0.05.

**Incidence Rates and Incident Rate Modeling**

Incidence rates were determined for residents by water supply zone and modeled by Poisson regression using the number of cases as the predictor variable and the number of person-years of observation as the offset (11,12). The models had 3 predictor variables: membrane filtration (before and after), FMD epidemic (before, during, and after local FMD controls), and water source (Ennerdale, Crummock, and “other” water supplies). The interaction between these predictors was explored. The models...
provided estimates of the incidence rate ratio (IRR). The goodness-of-fit of the models was assessed. The species of Cryptosporidium isolates before and after membrane filtration were compared.

Results

Population and Water Supplies
Public water supplies derived from Crummock Lake served 58,295 residents; from Ennerdale Lake, 47,780 residents; and from a variety of other smaller water sources, including a few private water supplies, the remaining 59,699 residents of Allerdale and Copeland (Appendix Table 1; available from http://www.cdc.gov/ncidod/EID/vol11no2/04-0274_app.htm). Public drinking water supplies derived from Crummock and Ennerdale Lakes before March 1, 2000, were chlorinated but not filtered (Appendix Table 1). Separate membrane filtration plants at water treatment works at Crummock and Ennerdale Lakes were commissioned from March 1, 2000, to July 31, 2000; full operation was achieved by August 1, 2000. These plants remained fully operational until the end of the study, August 31, 2002. The treatment of water derived from other sources remained unchanged for the study period. These multiple smaller sources received a variety of conventional treatments, including coagulation, filtration and chlorination, and chlorination alone. In addition, a small number of houses had private water supplies, which were untreated (Appendix Table 1).

Recruitment and Exclusion of Patients
A total of 249 patients identified as having sporadic cryptosporidiosis were ascertained during the study period; 74 (30%) were excluded, and 175 (70%) were enrolled (Table 1). Of the 175 primary cases of cryptosporidiosis enrolled, 153 (87%) had onset dates from March 1, 1996, to February 29, 2000, before the commissioning of membrane filtration at Crummock and Ennerdale Lakes; 22 (13%) patients had onset from March 1, 2000, to August 31, 2002, after the membrane filtration plants were introduced (Table 1).

Recruitment and Exclusion of Controls
A total of 929 potential controls were approached during the study; 392 (42%) were excluded, and 537 (58%) were enrolled. Two hundred and twenty one (24%) persons either refused to participate or were repeatedly unavailable for interview (Table 1). The address was not found for 3 (<1%). One hundred twelve (12%) were excluded because they did not meet the study control definition for a variety of reasons (Table 1). Fifty-six (6%) were not enrolled for administrative reasons. The study team cancelled interviews for 44 (5%) because 3 control interviews had been completed for the associated case and 9 (1%) interviews because the potential controls were found to be in the wrong age band; the reason for exclusion was not recorded for 3 potential controls (<1%). Of the 537 controls enrolled, 468 (87%) had interview dates from March 1, 1996, to February 29, 2000; and 69 (12.9%) had interviews from March 1, 2000, to August 31, 2002 (Table 1).

Study Population

Patients
Of the 175 case-patients, 150 (86%) were <16 years of age, and 96 (55%) were <6 years of age. Ninety (51%) were male (Table 2). The proportion of cases <16 years of age and the proportion who were male were lower after membrane filtration was introduced into Crummock and Ennerdale Lake water. The proportion of case-patients served by water from other sources that never received membrane filtration was higher after introduction of membrane filtration (Table 2).

In addition to diarrhea, a substantial proportion of patients had abdominal pain, vomiting, fever, loss of appetite, and weight loss (Appendix Table 2). Forty-two (24%) patients remained symptomatic at interview. Of the 133 (76%) whose symptoms had abated at interview, the median duration of illness was 9 days (range 2–21 days) (Appendix Table 2). In children <6 years of age, 14 (25%) of 55 of boys and 6 (15%) of 41 girls were admitted to hospital because of diarrhea (Appendix Table 3). The admission rates in children 6–15 years of age were 3 (11%) of 28 boys and 3 (12%) of 26 girls. Most of the patients and all male patients <6 years of age had onset dates before the membrane filtration was introduced (Appendix Table 3). Twenty-six (17%) of the 150 patients who were <16 years of age were admitted to hospital, but none over this age.

Species identification was undertaken for 68 fecal specimens from patients with onset from January 1, 1998, to February 29, 2000 (Appendix Table 4). Fifty-seven (84%) were C. parvum. Thirteen (81%) of the 16 smears derived from patients with onset dates from March 1, 2000, to August 31, 2002, were also C. parvum. Overall, 70 (83.3%) of the 84 specimens for which the species was identified were C. parvum.

Controls
The 537 controls had similar age, sex, and drinking water supplies as the 175 patients (Table 2). The time between notification of a case by a microbiology laboratory to the study-coordinating center to enrollment of the patient and his or her associated controls was a median of 2 weeks (range 1–8 weeks) and was similar before and after the introduction of membrane filtration.
Risk Factor Analysis

None of the interaction terms between the main effects variables and time intervals of observation, defined by introduction of membrane filtration and the FMD epidemic in livestock, were significant, including the term for the usual volume of cold unboiled tap water drunk per day (p = 0.12). These interaction terms were therefore excluded from the final multivariable risk factor model (Table 3). The risk for sporadic cryptosporidiosis was independently associated with the usual volume of cold unboiled tap water drunk each day, with contact with cattle farms and noncattle farms, and with feeding pets leftovers. Water supply zones, the time interval of observation, age, and gender were not independently associated with having a case (Table 3).

Incidence and Seasonality

The incidence within the populations served by public water supplies derived from Ennerdale Lake, Crummock Lake, and other water sources was similar before March 2000 at ≈22 cases per 100,000 person years but declined to <10 per 100,000 person years after March 1, 2000.

Table 1. Exclusions and recruitment of case-patients and controls

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>Case-patients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Refused to participate</td>
<td>1 (0.5)</td>
<td>0 (0)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Could not complete adequate interview</td>
<td>1 (0.5)</td>
<td>1 (2.4)</td>
<td>2 (0.8)</td>
</tr>
<tr>
<td>Did not respond to letters or phone calls</td>
<td>2 (1.0)</td>
<td>0 (0)</td>
<td>2 (0.8)</td>
</tr>
<tr>
<td>Did not meet study case definition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No history of diarrhea</td>
<td>1 (0.5)</td>
<td>1 (2.4)</td>
<td>2 (0.8)</td>
</tr>
<tr>
<td>Mixed enteric infection</td>
<td>1 (0.5)</td>
<td>0</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Secondary case</td>
<td>36 (17.4)</td>
<td>10 (23.8)</td>
<td>46 (18.5)</td>
</tr>
<tr>
<td>Travel outside UK in 14 days before onset</td>
<td>8 (3.9)</td>
<td>8 (19.0)</td>
<td>16 (6.4)</td>
</tr>
<tr>
<td>Visitor to study area</td>
<td>1 (0.5)</td>
<td>0 (0)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Residence outside study area</td>
<td>1 (0.5)</td>
<td>0 (0)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Case-patient or household member previously interviewed as case or control</td>
<td>2 (1.0)</td>
<td>0 (0)</td>
<td>2 (0.8)</td>
</tr>
<tr>
<td><strong>Potential case-patients approached</strong></td>
<td>207 (100)</td>
<td>42 (100)</td>
<td>249 (100)</td>
</tr>
<tr>
<td><strong>Potential cases excluded</strong></td>
<td>54 (26.1)</td>
<td>20 (47.6)</td>
<td>74 (29.7)</td>
</tr>
<tr>
<td><strong>Total case-patients enrolled</strong></td>
<td>153 (73.9)</td>
<td>22 (52.4)</td>
<td>175 (70.3)</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Refused or unavailable for interview</td>
<td>23 (3.0)</td>
<td>12 (7.9)</td>
<td>35 (3.8)</td>
</tr>
<tr>
<td>Unavailable at requested interview times</td>
<td>125 (17.1)</td>
<td>23 (15.2)</td>
<td>148 (15.9)</td>
</tr>
<tr>
<td>Said interview times were not convenient</td>
<td>35 (4.5)</td>
<td>3 (2.0)</td>
<td>38 (4.1)</td>
</tr>
<tr>
<td>Address not found</td>
<td>3 (0.4)</td>
<td>0 (0)</td>
<td>3 (0.3)</td>
</tr>
<tr>
<td>Did not meet study control definition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of diarrhea</td>
<td>46 (5.9)</td>
<td>6 (4.0)</td>
<td>52 (5.6)</td>
</tr>
<tr>
<td>Travel outside UK in 14 days before interview</td>
<td>8 (1.0)</td>
<td>4 (2.6)</td>
<td>12 (1.3)</td>
</tr>
<tr>
<td>Not resident in study area in 14 days before interview</td>
<td>3 (0.4)</td>
<td>1 (0.7)</td>
<td>4 (0.4)</td>
</tr>
<tr>
<td>Moved from study area</td>
<td>27 (3.5)</td>
<td>7 (4.6)</td>
<td>34 (3.7)</td>
</tr>
<tr>
<td>Residence outside study area</td>
<td>2 (0.3)</td>
<td>0 (0)</td>
<td>2 (0.2)</td>
</tr>
<tr>
<td>Control or household member already interviewed as a case or control</td>
<td>7 (0.9)</td>
<td>1 (0.7)</td>
<td>8 (0.9)</td>
</tr>
<tr>
<td>Not enrolled for administrative reasons or reason not recorded</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interview cancelled; 3 controls already enrolled for associated case</td>
<td>19 (2.4)</td>
<td>25 (16.6)</td>
<td>44 (4.7)</td>
</tr>
<tr>
<td>Interview cancelled; potential control found to be in wrong age group</td>
<td>9 (1.2)</td>
<td>0 (0)</td>
<td>9 (1.0)</td>
</tr>
<tr>
<td>Reason for exclusion not recorded</td>
<td>3 (0.4)</td>
<td>0 (0)</td>
<td>3 (0.3)</td>
</tr>
<tr>
<td>Potential controls approached</td>
<td>778 (100)</td>
<td>151 (100)</td>
<td>929 (100)</td>
</tr>
<tr>
<td>Potential controls excluded</td>
<td>310 (39.8)</td>
<td>82 (54.3)</td>
<td>392 (42.2)</td>
</tr>
<tr>
<td>Total controls enrolled</td>
<td>468 (60.2)</td>
<td>69 (45.7)</td>
<td>537 (57.8)</td>
</tr>
</tbody>
</table>
The decline was more marked in the populations served by water derived from treatment works at Ennerdale and Crummock Lakes, where membrane filtration plants had been installed, than in the population served by other water sources, where membrane filtration was not installed. A well-defined spring peak in cases was apparent from 1996 to 1999, but not from 2000 to 2002 (Figures 2 and 3).

Table 2. Baseline characteristics of case-patients and controls

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Case-patients, total</strong></td>
<td>153 (100)</td>
<td>22 (100)</td>
<td>175 (100)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>70 (45.8)</td>
<td>15 (68.2)</td>
<td>85 (48.6)</td>
</tr>
<tr>
<td>Male</td>
<td>83 (54.2)</td>
<td>7 (31.8)</td>
<td>90 (51.4)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1–5</td>
<td>87 (56.9)</td>
<td>9 (40.9)</td>
<td>96 (54.9)</td>
</tr>
<tr>
<td>6–15</td>
<td>47 (30.7)</td>
<td>7 (31.8)</td>
<td>54 (30.9)</td>
</tr>
<tr>
<td>16+</td>
<td>19 (12.4)</td>
<td>6 (27.3)</td>
<td>25 (14.3)</td>
</tr>
<tr>
<td><strong>Water sources and water supply zones</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crummock Lake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crummock North</td>
<td>37 (24.2)</td>
<td>2 (9.1)</td>
<td>39 (22.3)</td>
</tr>
<tr>
<td>Crummock South</td>
<td>19 (12.4)</td>
<td>4 (18.2)</td>
<td>23 (13.1)</td>
</tr>
<tr>
<td>Ennerdale Lake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ennerdale North</td>
<td>30 (19.6)</td>
<td>2 (9.1)</td>
<td>32 (18.3)</td>
</tr>
<tr>
<td>Ennerdale South</td>
<td>13 (8.5)</td>
<td>1 (4.5)</td>
<td>14 (8.0)</td>
</tr>
<tr>
<td><strong>Other sources</strong></td>
<td></td>
<td></td>
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<td>22 (12.6)</td>
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<td>3 (1.7)</td>
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<td>1 (4.5)</td>
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<td>1 (4.5)</td>
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<td>69 (100)</td>
<td>537 (100)</td>
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<tr>
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<td>31 (44.9)</td>
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<tr>
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<td>38 (55.1)</td>
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<td>111 (20.7)</td>
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<td>62 (11.5)</td>
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<td>10 (1.9)</td>
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*Mixed: mixed supply derived from Ennerdale and Crummock, or from Ennerdale and another source, or from Crummock and another source.
After membrane filtration was introduced for Crummock and Ennerdale supplies, an estimated reduction in incidence of ≈79% occurred (IRR 0.207, 95% confidence intervals [CI] 0.099–0.431), p < 0.0001, after adjustment for the FMD epidemic interval and water source in a Poisson regression model (Table 5). The decrease attributed to the FMD interval was ≈60% (IRR 0.394, 95% CI 0.167–0.925), with some evidence of a residual effect after the end of local FMD epidemic controls (IRR 0.686, 95% CI 0.292–1.61). No additional effect was contributed by water source (p = 0.6). The data for this model are presented graphically in Figure 1 and detailed in Table 4. Little difference was made by modeling the intervals for commissioning and postcommissioning of membrane.

<table>
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<th>Risk factors</th>
<th>Case-patients</th>
<th>Controls</th>
<th>Adjusted odds ratio*</th>
<th>Lower 95% CI†</th>
<th>Upper 95% CI</th>
<th>p value</th>
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<td>46</td>
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<td>0.138</td>
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</tbody>
</table>

*Adjusted for accidentally touching animal feces, feeding pets biscuits, feeding pets raw vegetables, contact with anyone outside the household with a history of diarrhea, type of sewage system to the house, consumption of mixed salad, and local authority of residence.
†CI, confidence interval.
filtration separately, and no significant difference was seen between these rate estimates when this modeling was done (p = 0.35)

Discussion
Consumption of cold unboiled tap water from public drinking water supplies was shown to be a leading independent risk factor for sporadic cryptosporidiosis with a highly significant increase in risk with the usual volume drunk each day (Table 3). Risk was also increased by contact with cattle farms and noncattle farms and with feeding pets leftovers. Fifty percent of patients were <6 years of age, and 31% were 6–15 years. Infection was predominantly with *C. parvum* (livestock and human species). The results of the risk factor analysis for the entire study period were similar to those obtained for the interval before installation of membrane filtration, when most cases arose (9). Illness was prolonged and almost one fifth of children <6 years of age required hospital admission. The excess in hospitalization in boys <6 years of age may suggest that young boys are more vulnerable to *Cryptosporidium* than young girls, a bias in favor of admitting young boys, or a combination of these factors.

The 2001 FMD epidemic in livestock, which occurred after membrane filtration was introduced in Allerdale and Crummock Lake water, affected all regions of the United Kingdom (10). This livestock epidemic was associated with a highly significant decline in laboratory reports of human cryptosporidiosis from all regions of England and Wales and was more marked in Northwest England. The decline in reports was most marked for *C. parvum* (the species infectious in humans and livestock species) than for *C. hominis* (infectious only in humans) (10). The FMD epidemic control measures of excluding the public from the countryside, extensively culling farm animals, and limiting animal movements probably decreased direct and indirect exposure of the human population to livestock and livestock feces.

The annual agricultural and horticultural census conducted by the U.K. Department for Environment, Food and Rural Affairs and its predecessor, the Ministry of Agriculture, Food and Fisheries showed >600,000 sheep, 300,000 lambs, 100,000 total cattle and calves, and 40,000 calves <1 year of age in each of the years 1996–2000 within the 135,000 hectares of agricultural land in Allerdale and Copeland local government districts (13,14). A substantial decline occurred in 2001 and 2002 associated with the FMD epidemic, but no evidence suggested that the decline in animal densities or change in human contact with livestock and with the countryside differed within

---

**Table 4. Incidence of sporadic cryptosporidiosis by water source, March 1, 1996–August 31, 2002**

<table>
<thead>
<tr>
<th>Water source and time intervals*</th>
<th>Membrane filtration (MF)</th>
<th>Cases (n)</th>
<th>Person-years</th>
<th>Rate per 100,000 person-years</th>
<th>95% CI†</th>
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<td>56</td>
<td>233,623</td>
<td>23.97</td>
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</tbody>
</table>

Allerdale and Copeland, according to the sources or distribution of the public drinking water supplies (13,14). The decline in incidence attributable to the FMD epidemic effect was therefore expected for our entire study population, regardless of its household water supply. We therefore believe that the experience of the population served by other supplies provided a valid measure of the impact of the FMD epidemic in livestock, whereas the population served by water from Ennerdale and Crummock Lakes experienced the effect of both membrane filtration and the FMD epidemic. The results of the Poisson regression model indicated a marked reduction of incidence in sporadic cryptosporidiosis following introduction of membrane filtration after adjustment for the FMD epidemic interval and water source (Table 5). Despite the confounding effect of the FMD epidemic, our study provides convincing evidence that membrane filtration was highly effective in reducing the risk for sporadic cryptosporidiosis in this population; this measure was also associated with a decline in hospital admissions for cryptosporidiosis in children, especially of boys <6 years of age.

The incidence rates associated with other supplies from a number of different sources and treatment works, some using conventional flocculation and filtration, were similar from March 1996 to February 2001 to the rates in the population served by Crummock and Ennerdale Lakes, whose water was unfiltered at this time. This finding supports the notion that conventional sand filtration and flocculation may be insufficient to prevent intermittent low-level Cryptosporidium oocyst contamination of treated water. The local water company has since closed higher risk sources and substituted them with water from lower risk catchments.

Our observations strongly support recent revision of the UK drinking water regulations requiring water companies to undertake risk assessments of water sources, and where judged to be a risk, to implement continuous monitoring of Cryptosporidium oocyst concentrations in treated water (15). A minimum standard of an average of <1 oocyst per 10 L of water in any 24-hour period is required.

Table 5. Poisson regression model of the incidence of sporadic cryptosporidiosis*

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Category</th>
<th>IRR</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane filtration</td>
<td>No</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>0.207</td>
<td>0.099–0.431</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Foot and mouth disease epidemic</td>
<td>Pre</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>During</td>
<td>0.394</td>
<td>0.167–0.925</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>0.686</td>
<td>0.292–1.612</td>
<td>0.05</td>
</tr>
<tr>
<td>Water supply</td>
<td>Crummock</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ennerdale</td>
<td>0.907</td>
<td>0.620–1.329</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>0.820</td>
<td>0.573–1.174</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*IRR, incidence rate ratio; CI, confidence interval. Goodness-of-fit test (chi square 10.64, 9 df, p = 0.3).
The substantial negative impact of waterborne cryptosporidiosis leading to potentially life-threatening diarrhoea and stunting in childhood is well-recognized in developing countries (16,17). Our findings show that Cryptosporidium remains an obstacle in water and sanitation infrastructure and a threat to child health in industrialized countries as well. The scale of this effect will continue to be underestimated if adequate surveillance of cryptosporidiosis by testing diarrheal feces specimens for Cryptosporidium and collation of positive test results, especially in children, is omitted by health services (18). Although the study population was located in an area of livestock farming with high historic rates of cryptosporidiosis in England, the demonstration that public drinking water supplies were a leading independent risk factor for sporadic cryptosporidiosis and that introduction of membrane filtration at water treatment works was effective in substantially lowering this risk, may have relevance to water companies, regulators, policymakers, and consumers in other countries.

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Dr. Goh is an honorary consultant to the Carlisle and District Primary Care Trust and was formerly the consultant in Communicable Disease Control to North Cumbria Health Authority, in northwest England. She specialized in public health medicine and developed an interest in childhood immunization, enteric infection, Cryptosporidium, and water supplies.

The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.

References


Address for correspondence: Mark Reacher, Health Protection Agency, East of England, Institute of Public Health, University Forvie Site, Robinson Way, Cambridge CB2 2SR, United Kingdom; fax: 44 0 1223 331865; email: mark.reacher@hpa.org.uk
Our study was initiated by previous isolation of 30 imipenem-resistant, gram-negative rods from 7 of 16 U.S. rivers sampled from 1999 to 2001. Imipenem hydrolysis was detected in 22 of those isolates identified as *Enterobacter asburiae*. Random amplified polymorphism DNA analysis showed that these *E. asburiae* isolates were genetically indistinguishable. An identical clavulanic acid–inhibited β-lactamase IMI-2 was identified from each isolate that shared 99% and 97% amino acid identity with the chromosome-encoded β-lactamases IMI-1 and NmcA, respectively, from *E. cloacae* clinical isolates. The *bla*IMI-2 gene was located on a self-transferable 66-kb plasmid. Sequence analysis of a cloned 5.5-kb DNA fragment obtained from 1 of the imipenem-resistant *E. asburiae* isolates identified an upstream LysR-type regulator gene that explained inducibility of IMI-2 expression. β-Lactamase IMI-2 is the first inducible and plasmid-encoded carbapenemase. Identification of clonally related *E. asburiae* isolates from distant rivers indicates an environmental and enterobacterial reservoir for carbapenemase genes.

**Materials and Methods**

**Bacterial Isolates**

A previous study identified 30 imipenem-resistant, gram-negative strains out of 1,861 ampicillin-resistant, gram-negative isolates from 7 out of 16 U.S. rivers that were sampled from 1999 to 2001 (22). Identification of these imipenem-resistant isolates was performed by conventional biochemical techniques (API-20E and API-NE systems [bioMérieux, Marcy-l’Etoile, France]), and confirmed by 16S rDNA sequencing (24).

*E. asburiae* CIP 103358 and *E. asburiae* CIP 105006 were used as reference strains (Institut Pasteur strain collection, Paris, France). *E. cloacae* NOR-1 and *E. cloacae* 1413B were used as strains that produce the chromosome-encoded, clavulanate-inhibited carbapenemases NmcA and IMI-1, respectively (5,8). One of the *E. asburiae* isolates recovered from a river (strain MS7) was used for cloning experiments. Streptomycin-resistant *Escherichia coli* DH10B strain was used in cloning and conjugation experiments (Life Technologies, Eragny, France).

**Antimicrobial Agents and Resistance Study**

The antimicrobial agents and their sources were as
follows: amoxicillin, ceftazidime, clavulanic acid, and ticarcillin (GlaxoSmithKline, Nanterre, France); aztreonam (Bristol-Myers Squibb, Paris La Defense, France); cefalothin (Eli Lilly, Saint-Cloud, France); piperacillin and tazobactam (Lederle, Les Oullins, France); ceftaxime (Aventis, Romainville, France); imipenem (without cilastatin) (Merck Sharp and Dohme, Paris, France); meropenem (AstraZeneca, Paris, France); ampicillin and streptomycin (Sigma, Paris, France).

MICs were determined by an agar dilution technique on Mueller-Hinton (MH) agar (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) with an inoculum of $10^4$ CFU per spot (25). Carbenapenemase activity was determined by UV spectrophotometry with culture extracts of each of the imipenem-resistant, gram-negative rods and imipenem (100 µmol) as substrate, as reported previously (26). One unit of enzyme activity corresponded to the hydrolysis of 1 µmol of substrate per min. Inducibility of the β-lactamase expression was determined with imipenem and cefoxitin as β-lactamase inducers, as described (27). Briefly overnight culture of each imipenem-resistant E. asburiae isolate was diluted (1:10) in a prewarmed trypticase soy broth, allowed to culture in an antimicrobial-free medium for 2 h, and further cultured for 6 h with cefoxitin (2–50 mg/L) or imipenem (10–50 mg/L). β-Lactamase culture extracts were obtained after centrifugation and sonication, as detailed (26).

Nucleic Acid Techniques and Conjugation

Genotype comparison of the imipenem-resistant E. asburiae strains was performed by using the random amplified polymorphism detection (RAPD) technique as described with primer 6MW (CCGACTCGAG NNNNNNATGTGG) and primers UBC 245 and UBC 282 (26,28,29). Transfer of the imipenem resistance marker from each imipenem-resistant E. asburiae isolate to E. coli DH10B was attempted by using the immobilization filter mating out technique, as described (26). Briefly, equal volume (0.1 mL) of overnight cultures of each E. asburiae isolate and E. coli DH10B were put onto a paper filter that was placed on an MH agar plate. Twenty-four hours later, the filter was removed, washed with water (0.2 mL), and the bacterial suspension was spread onto MH agar plates containing ampicillin (100 mg/L) and streptomycin (50 mg/L) for selecting transconjugants after 24 h (26).

Plasmid extraction was performed for each E. asburiae strain and their transconjugants and compared to reference plasmid sizes of E. coli NCTC 50192 by using the Kieser technique designed to extract large size plasmids (30,31). Whole-cell DNA of Enterobacter spp. reference strains and of an E. asburiae strain MS7 was extracted as described (26).

Southern hybridization of plasmid DNA (26) of the transconjugants was performed as described by the manufacturer with the ECL nonradioactive kit (Amersham, Les Ulis, France). An 818–bp internal probe for bla\_\text{IMI-1} was obtained by using primers IMI-A (5'-ATAGCCCATCCTTGTGAGCTC-3') and IMI-B (5'-TCTGCAGTACCTATCTGCTCTC-3') and standard polymerase chain reaction (PCR) amplification procedures (5,26).

Primers designed to hybridize to the ends of the bla\_\text{Sme-1}, bla\_\text{Sme-2}, and bla\_\text{IMI-1} genes were used for standard PCR amplification experiments (5,7,8) with plasmid DNA of each imipenem-resistant E. asburiae isolate and of their transconjugants as templates. Cloning experiments were then performed with BamHI restricted whole-cell DNA of E. asburiae MS7 followed by ligation of DNA fragments into the BamHI-site of cloning vector pGB2 (32). Recombinant plasmids were transformed by electroporation into E. coli DH10B electrocompetent cells (26). E. coli DH10B harboring recombinant plasmids was selected on MH agar plates containing ampicillin (100 mg/L) and streptomycin (100 mg/L).

DNA sequencing of both strands of PCR fragments amplified with the primers for bla\_\text{IMI-1} and plasmid DNA of E. asburiae isolates as templates and of the cloned fragment of a recombinant plasmid was determined with an Applied Biosystems sequencer (ABI377). The nucleotide sequences and the deduced protein sequences were analyzed with software available on the Internet from the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/BLAST).

Results

Bacterial Identification

Twenty-nine of the 30 imipenem-resistant isolates substantially hydrolyzed imipenem, i.e., 10.5 ± 1.6 U/mg of protein of culture extracts. These isolates were a single Aeromonas hydrophila isolate, 6 Stenotrophomonas maltophilia isolates known to naturally produce carbapenemases, and 22 Enterobacter spp. isolates identified as E. asburiae that were further analyzed.

As reported in Table 1, E. asburiae isolates were isolated at different times from several rivers in the midwest. Other tested rivers had ampicillin-resistant isolates that were not imipenem-resistant (Figure). These rivers were Arkansas (Little Rock), Canadian (Oklahoma City), Hudson (New York), Chicago (Chicago), Colorado (Glenwood Springs), Missouri (Parkville), Cuyahoga (Cleveland), Mississippi (New Orleans, St. Louis), Ohio (Cincinnati, Louisville, Pittsburgh, Wheeling), Platte (Grand Island), Scioto (Columbus), Wabash (Terre Haute), and White (Indianapolis). RAPD analysis was then
performed to compare all imipenem-resistant *E. asburiae* isolates. Using a series of different primers, this genotyping technique identified clonally indistinguishable *E. asburiae* isolates, although they were from various geographic origins (data not shown).

### β-Lactam Resistance Marker

The imipenem resistance marker was transferred from each imipenem-resistant *E. asburiae* isolate to *E. coli* DH10B by conjugation. Plasmid analysis identified a 66-kb plasmid (pNat) from cultures of each imipenem-resistant *E. asburiae* isolate, whereas this plasmid was not isolated from *E. cloacae* and *E. asburiae* reference strains (data not shown). PCR experiments with primers for the *bla*IMI-1 gene were positive with plasmid DNA of each *E. asburiae* isolate and transconjugants as templates, whereas primers designed to amplify *bla*NmcA and *bla*Sme-1/Sme-2 failed to give PCR product. The Southern blot analysis confirmed that the *bla*IMI-like gene was located on the natural plasmid pNat (data not shown).

Sequencing PCR products with primers hybridizing at the ends of the *bla*IMI-1 gene and plasmid DNA of each imipenem-resistant *E. asburiae* isolate identified the same β-lactamase IMI-2 in all cases. This novel enzyme had 2 amino acid substitutions (tyrosine to histidine at position Ambler 105 and asparagine to aspartic acid at position Ambler 35) compared to the chromosomally encoded carbapenemase IMI-1 (5). β-Lactamase IMI-1 had been isolated from an *E. cloacae* isolate from Minnesota close to locations where IMI-2–producing isolates have been found (5). However, the *bla*IMI-2 gene was not just a point-mutant derivative of the *bla*IMI-1 gene, since these genes differ by 11 nucleotide substitutions. β-Lactamase IMI-2 was also related to NmcA (97% amino acid identity) (8).

Cloning BamHI-restricted DNA of whole-cell DNA of *E. asburiae* MS7 gave recombinant plasmid pIMI-2 that had a 5.5-kb insert that allowed identification of the surrounding sequence of the *bla*IMI-2 gene. A gene encoding a LysR-type regulator named IMIR-2 was found just upstream of *bla*IMI-2. It shared 95% amino acid identity with IMIR-1, which is located upstream of the *bla*IMI-1 gene (5). The surrounding sequences of *bla*IMI-2 shared significant nucleotide identity with transposable elements. Part of an open reading frame that shared 97% nucleotide identity with that of the transposase gene *tnp*4 of the transposon Tn2501 (Tn3 family) was identified downstream of *bla*IMI-2 (33). Upstream of IMIR-2, a 142-bp sequence shared 76% nucleotide identity with part of the insertion sequence IS2.

### Susceptibility Testing and Expression of Resistance

MICs of several β-lactams, including carbapenems for the IMI-2–positive *E. asburiae* MS7 and for *E. coli* DH10B expressing the *bla*IMI-2 gene were high (Table 2). The MICs of β-lactams for all imipenem-resistant clinical isolates were identical (data not shown). Much higher level of resistance to aztreonam than to expanded-spectrum cephalosporins was found for the IMI-2–positive strains, as reported for the other producers of class A carbapenemases (2). The activity of β-lactamase IMI-2 was partially inhibited by clavulanate and tazobactam. Induction studies showed increase of β-lactamase expression from 17- to 30-fold (170 to 300 U/mg of protein) (for each *E. asburiae* isolate when imipenem (50 mg/L) and cefoxitin (50 mg/L) were used as inducers, respectively. These induction results were consistent with location and functionality of a LysR-type regulator gene upstream of the *bla*IMI-2 gene in the imipenem-resistant *E. asburiae* isolates. No other antimicrobial resistance marker was carried by natural plasmid pNat.

### Discussion

This report indicates that several U.S. rivers may be a reservoir for broad-spectrum carbapenemases. Here, we report a novel clavulanic-acid inhibited Ambler class A β-lactamase IMI-2 that has an usual spectrum of hydrolysis for this type of β-lactamase, including penicillins, carbapenems, and aztreonam (2). β-Lactamase IMI-2 is closely related to several Ambler class A carbapenemases whose genes are chromosomally located, including *bla*IMI-1 and *bla*NmcA, and found in several clinical isolates (5,8). While
this work was in progress, a clinical case of an NmcA-producing E. cloacae isolate was reported from Seattle (34). An extended epidemiologic survey identified Sme-1-type-producing Serratia marcescens isolates from the West Coast to the East Coast, which indicates that these isolates may also represent a reservoir for carbapenemases in Enterobacteriaceae (9). Thus, identification of carbapenemase genes in enterobacterial strains from rivers may have clinical importance.

In the present study, the β-lactamase gene was plasmid-encoded and was adjacent to mobile sequences that may play an additional role in gene transfer. The E. asburiae isolates were clonally related and may correspond to a single clone, although they were obtained from distantly related midwestern rivers. The reason for the presence of these antimicrobial-resistant strains in this region is unknown. Taking into account the small number of specimens withdrawn from the rivers and the selection technique for imipenem-resistant isolates (ampicillin- and not imipenem-containing plates), the prevalence of carbapenemase-producing enterobacterial strains may be high in the environment, at least in the Midwest.

Cloning experiments led to identification of a regulatory gene from an E. asburiae strain (found in the other E. asburiae strains as well [data not shown]) that explained inducibility of carbapenemase expression. Whatever the level of imipenem resistance is, failure of an imipenem-containing regimen may occur when treating infections caused by similar carbapenemase-producing strains, as deduced from results obtained with an animal model of pneumonia (35). Finally, this study raises the question of the importance of this reservoir in Enterobacteriaceae as well as the origin of this plasmid-located carbapenemase gene that may be transferred among other enterobacterial pathogens.

Acknowledgement

We thank K. Bush for providing E. cloacae 1413B that produced the chromosome-encoded, clavulanate-inhibited carbapenemase IMI-1.

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Dr. Aubron is studying antimicrobial resistance mechanisms at the Hospital Bicêtre, South-Paris Medical School, University Paris XI, France. She is a resident specializing in infectious diseases.

References


We compared the rickettsial infection status of *Amblyomma cajennense* ticks, humans, dogs, and horses in both Brazilian spotted fever (BSF)-endemic and -nonendemic areas in the state of São Paulo, Brazil. Most of the horses and few dogs from BSF-endemic areas had serologic titers against *Rickettsia rickettsii* antigens. In contrast, no dogs or horses from BSF-nonendemic areas had serologic titers against *R. rickettsii* antigens, although they were continually exposed to *A. cajennense* ticks. All human serum samples and ticks from both areas were negative by serologic assay and polymerase chain reaction, respectively. Our results indicate that surveys of horse serum are a useful method of BSF surveillance in areas where humans are exposed to *A. cajennense* ticks. In addition, we successfully performed experimental infection of *A. cajennense* ticks with *R. parkeri*.

Brazilian spotted fever (BSF) is an acute, febrile, tick-borne disease caused by the bacterium *Rickettsia rickettsii*. The disease is transmitted by *Amblyomma* ticks and has been considered endemic in some areas of the states of São Paulo, Minas Gerais, Rio de Janeiro, and Espírito Santo (1–7). Although the tick species *Amblyomma aureolatum* is the main vector of BSF in few areas of the state of São Paulo (8, A. Pinter, unpub data), *A. cajennense* is the most common tick vector associated with the disease in Brazil (9–11).

*A. cajennense* is a common tick in rural areas of the state of São Paulo, where it is also the main tick species infesting humans (12,13). In contrast, BSF cases have been reported at only a few locations within the geographic range of this tick species (14). Although unreported cases may have occurred in other areas where BSF is not known to be endemic, this possibility is unlikely for such a highly lethal disease. Ecologic differences might be the main factor regulating the occurrence of *R. rickettsii* among ticks and, consequently, the occurrence of the disease.

The infection rate by *R. rickettsii* within a tick population can be diminished or even suppressed when a second *Rickettsia* species infects most of the members of that tick population (15,16). Thus, we hypothesize that the absence of human cases of BSF in some areas of the state of São Paulo (where human parasitism by *A. cajennense* is intense) is related to the presence of other, less pathogenic *Rickettsia* species infecting *A. cajennense* tick populations. In this regard, our study evaluated the rickettsial infection status of *A. cajennense* populations from both BSF-endemic and -nonendemic areas in the state of São Paulo. We also serologically evaluated humans and domestic animals from these BSF-nonendemic areas to compare it to a recent evaluation that we performed in BSF-endemic areas (17).

**Materials and Methods**

**Study Area**

The study was conducted on 6 farms in the state of São Paulo. Three of these farms (farms 1, 2, and 3) were considered endemic for BSF because of the recent occurrence of several laboratory-confirmed human cases of the disease among residents (4,14). These farms were the same ones evaluated in a study of Horta et al. (17). The remaining 3 farms (4, 5, and 6) were considered nonendemic for BSF because they had never had human cases of this disease. However, *A. cajennense* ticks were abundant there, and human infestation by this tick was a normal finding year-round among farm residents. Farms 1 (22°44′19″S, 46°55′27″W), 2 (22°47′03″S, 46°54′10″W) and 3 (22°41′14″S, 46°53′17″W) were located in the Pedreira Municipality whereas farms 4 (23°23′15″S, 47°26′14″W), 5 (23°23′15″S, 47°26′14″W), and 6 (23°23′15″S, 47°26′14″W) were located in the Pedreira Municipality.
5 (23°36′43″S, 46°57′29″W), and 6 (21°57′07″S, 47°27′05″W) were located in Porto Feliz, Cotia, and Pirassununga Municipalities, respectively.

In all 6 farms, human occupations were basically divided between livestock-raising activities for men and household activities for women and children. Nevertheless, children spent substantial time in outdoor activities. All 6 farms had horses grazing on mixed overgrowth pastures, interspersed with remote forest areas. However, the major ecologic difference was large populations of free-living capybaras that inhabited livestock pastures on farms 1, 2, and 3 and the absence of this animal from horse pastures on farms 4, 5, and 6. All farms, except farm 4, had free-roaming dogs with free access to pasture and forest areas. Recent studies on ticks collected on the pastures and on horses and dogs from these 6 farms allowed the tick species A. cajennense and Dermacentor nitens to be identified on the 6 farms. In addition, the capybara tick, A. cooperi, was present on farms 1, 2, and 3 but absent in the pastures of farms 4, 5, and 6 (13,17–19). Human infestation by Amblyomma ticks was frequent on all the farms.

Ticks

From December 2000 to March 2001, free-living A. cajennense adult ticks were collected from horse pastures of the 6 farms by dragging and by using CO2 traps. Totals of ticks collected from the farms are as follows: farm 1 (244), farm 2 (353), farm 3 (213), farm 4 (222), farm 5 (206), and farm 6 (230). All ticks were brought alive to the laboratory, where their surfaces were disinfected by immersion in 70% alcohol for 10 min followed by washing in sterile water; they were then individually tested by the hemolymph test (20). Briefly, a drop of hemolymph of each tick was dried on a glass slide and stained by the Gimenez method (21). Thereafter, ticks were frozen at −80°C until processed for DNA extraction.

DNA Extraction

All ticks were processed individually for DNA extraction. Each tick was cut into 2 symmetric halves through its median axis. One half was returned to the −80°C freezer for further studies, and the other half was used for DNA extraction according to a modification of a previously described protocol (22). For this purpose, each tick half was placed in a 1.5-mL microtube containing 150 µL of TE buffer (Tris HCl 10 mmol/L, EDTA 1 mmol/L, pH 7.4) and homogenized by using a sterile micropestle. Microtubes containing the homogenized, triturated ticks were then vortexed vigorously. Next, 450 µL of guanidine thiocyanate (5 mol/L) were added to the tube, which was vortexed again and incubated for 10 min at room temperature with short vortexing every 2 min. Thereafter, 100 µL of chloroform was added to the tube, which was inverted several times and left resting for 2 min. The tube was centrifuged at 12,000 rpm for 5 min to separate the aqueous phase, which was transferred to a clean 1.5-mL microtube. Next, 600 µL of isopropanol was added to the aqueous phase (400 µL), which was homogenized by inverting the tube several times and then incubated at −20°C for 2 to 18 h. Thereafter, the tube was centrifuged at 12,000 rpm for 15 min; the supernatant was discarded, and the pellet was dried at room temperature and then resuspended with 30 µL of buffer TE. Finally, the microtubes were incubated at 56°C for 15 min to facilitate DNA homogenization and then stored at −20°C until tested by polymerase chain reaction (PCR).

PCR

Five microliters of the extracted DNA from tick specimen was used as template for amplification of fragments of the rickettsial gltA (citrate synthase gene) and 17-kDa protein gene. A 381-bp portion of the Rickettsia gltA gene was targeted from each extracted tick DNA by using primers RpCS.877 and RpCS.1258n (23), and a 434-bp portion of the Rickettsia genus-specific 17-kDa protein gene was targeted as previously described (24). Ten microliters of the PCR product underwent electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and examined with UV transillumination. For the 10 individual ticks that were tested by PCR, a negative control (5 µL of water) and positive control (5 µL of DNA extracted from an A. cajennense tick experimentally infected with R. parkeri) were included. Procedures to obtain R. parkeri experimentally infected ticks are described below. PCR results were statistically analyzed by the program @Risk Software – Risk Analysis Add-in for Microsoft Excel (Palisade Corporation, Newfield, NY, USA), which adopted Monte Carlo techniques to determine the confidence level of the prevalence of ticks infected by Rickettsia in each tick population (farm), considering α = 0.05.

R. parkeri Experimentally Infected Ticks

Purified R. parkeri organisms (Maculatum strain) were obtained by the renografin purification method from infected Vero cells (25). The resultant purified rickettsiae were resuspended in sucrose-phosphate-glutamic acid buffer and stored frozen at −80°C until tick infection. Seventy adult specimens of A. cajennense were obtained from the third generation of our laboratory colony at the University of São Paulo. This colony was established 15 months earlier from engorged females collected on horses on farm 6 of the present study. Adult ticks had their dorsum attached to double-face adhesive tape, which was taped onto petri dishes. Purified stock of R. parkeri was thawed at room temperature, and each tick was injected by using a 28-gauge microfine insulin needle. Under a stereo-
scopic microscope, a small drop (=2 µL) of *R. parkeri* suspension was injected into the coelom of the tick, through the articulation of coxa IV with trochanter IV, in each of 50 adult ticks. A control group of 20 ticks were injected by the same procedure with phosphate-buffered saline (PBS). Ticks were removed from the adhesive tape and held in an incubator at 35°C and relative humidity ≥95% for 5 days. Ticks were tested by the hemolymph test as described above, at days 3 and 5 after infection. Thereafter, ticks were frozen at −80°C. DNA of hemolymph-positive ticks was extracted, as described above, to be used as positive control for our PCR assays. A sample of 10 PBS-injected ticks were also tested by the PCR method described above.

**Domestic Animals and Humans**

During our visit to farms 1 to 6, blood samples were collected from 100% of the dogs and horses on each farm and ~90% of the resident humans. Blood samples were transported at room temperature to the laboratory, where samples were centrifuged (1,500 x g, 10 min), and the sera were aliquoted into labeled microtubes and stored at −20°C until tested by the indirect immunofluorescence assay (IFA) with *R. rickettsii* antigen, as described (17). The serologic results of farms 1 to 3 have been reported by Horta et al. (17) and will be compared with our results for farms 4 to 6. Collection of animal and human samples was previously approved by ethical principles in animal and human research of the University of São Paulo.

**Results**

**Field Ticks**

A total of 1,468 *A. cajennense* adult ticks (810 from disease-endemic and 658 from disease-nonendemic areas) were tested by the hemolymph test. They were all negative. These same ticks were also negative by the PCR protocols targeting the rickettsial genes *gltA* and 17-kDa protein. In all PCR assays, DNA of *A. cajennense* ticks experimentally infected with *R. parkeri* (positive controls) yielded the expected bands whereas no bands were obtained for the negative controls.

Our results, after being analyzed by the Monte Carlo techniques, are that on farm 5, where 206 ticks (our smallest sample) were tested, the prevalence of *A. cajennense* ticks infected by *Rickettsia* was at most 1.43% (upper limit of 95% confidence interval). If the prevalence was higher than this value, infection in at least 1 tick would have been detected by our PCR. Similarly, in farm 2, where 353 ticks were tested (our largest sample), the prevalence of ticks infected by *Rickettsia* was at most 0.8% (upper limit of 95% confidence interval). Overall, these analyses indicated that the prevalence of rickettsial infection on the 6 farms was no more than 0.8%–1.43%. As we used *Rickettsia* genus specific primers in the PCR, this infection could be due to any *Rickettsia* species.

**Ticks Experimentally Infected with *R. parkeri***

Of 50 ticks infected with *R. parkeri*, 10 (20%) showed typical *Rickettsia*-like organisms within the hemocytes 3 days after injection. On day 5, the number of ticks showing typical *Rickettsia*-like organisms in their hemocytes increased to 28 (56%). None of the 20 ticks injected with PBS showed *Rickettsia*-like organisms in their hemolymph 3 or 5 days after injection. All 28 hemolymph-positive ticks yielded expected bands in both PCR protocols (*gltA* and 17-kDa protein) whereas no PBS-injected ticks yielded amplified DNA bands.

**Serologic Assays**

Serum samples were collected from horses, dogs, and humans from the 6 farms, as shown in the Table. From the BSF-nonendemic areas (farms 4–6), no sample from a dog, horse, or human reacted positively with *R. rickettsii* antigens. The serologic results for the BSF-endemic areas (farms 1–3) were reported by Horta et al. (17). The proportion of horses that reacted positively with *R. rickettsii* antigens (titer ≥64) varied from 57.1% to 80%; for dogs, these proportions varied from 0% to 66.7%. Like farms 4–6, no human serum sample from farms 1 to 3 reacted positively with *R. rickettsii* antigens.

**Discussion**

Our study evaluated *A. cajennense* ticks in BSF-endemic and -nonendemic areas in the state of São Paulo. In addition, we serologically evaluated domestic animals and humans from BSF-nonendemic areas and compared the results with a previous serologic evaluation in BSF-endemic areas (17). Our results for the nonendemic areas showed no evidence of a pathogenic *Rickettsia* species circulating in *A. cajennense* ticks in farms 4 to 6, since all animals, humans, and ticks were negative. In contrast, Horta et al. (17) showed serologic evidence of *R. rickettsii* infection by cross-absorption and IFA analyses in most of the horses and some dogs in the 3 BSF-endemic areas (farms 1–3), a finding that is supported by the recent occurrence of human BSF cases in those farms. The serologic reactivity of horses, dogs, and humans to *R. rickettsii* antigen in BSF-endemic areas where *A. cajennense* is the main vector is characterized by a high frequency of serologically positive horses, followed by a lower frequency in dogs, and an even lower frequency or absence of serologically positive humans (17). This pattern has been observed in several BSF-endemic areas in which *A. cajennense* has been incriminated as the vector (3,17,26,27). The absence of serologic reactivity among the human residents whom we tested is supported by their lack of history of the disease;
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Table. Results of indirect immunofluorescence assay for antibodies to Rickettsia rickettsii in humans and domestic animals from 3 BSF-endemic areas (farms 1–3)* and 3 BSF-nonendemic areas (farms 4–6), São Paulo, Brazil†

<table>
<thead>
<tr>
<th>Source</th>
<th>Farm 1</th>
<th>Farm 2</th>
<th>Farm 3</th>
<th>Farm 4</th>
<th>Farm 5</th>
<th>Farm 6</th>
</tr>
</thead>
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<tr>
<td>Humans</td>
<td>0/20 (0)</td>
<td>0/21 (0)</td>
<td>0/9 (0)</td>
<td>0/4 (0)</td>
<td>0/2 (0)</td>
<td>0/10 (0)</td>
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<tr>
<td>Horses</td>
<td>9/10 (90)</td>
<td>4/7 (57.1)</td>
<td>4/5 (80)</td>
<td>0/16 (0)</td>
<td>0/10 (0)</td>
<td>0/21 (0)</td>
</tr>
<tr>
<td>Dogs</td>
<td>1/4 (25)</td>
<td>4/6 (66.7)</td>
<td>0/6 (0)</td>
<td>No dogs</td>
<td>0/4 (0)</td>
<td>0/1 (0)</td>
</tr>
</tbody>
</table>

*Data from Horta et al. (17).
†BSF, Brazilian spotted fever.
‡Sera showing titers >64 for R. rickettsii antigen.

Previous cases reported in this area were lethal or if not, the survivors do not live in the BSF-endemic area anymore.

Horses are one of the most important hosts for A. cajennense in the state of São Paulo; both immature and adult ticks will successfully feed on this animal (18). This fact makes the horse an excellent sentinel for BSF surveillance. Once the A. cajennense population increases in an area, parasitic stages will have a greater chance to successfully feed on other host species, including dogs and humans. As dogs are naturally infested with ticks more frequently than humans, they are also a good sentinel for BSF surveillance. Results of our study support this statement because our serologic survey of horses and dogs from 3 areas, where no BSF case has been reported, indicated that neither R. rickettsii nor a closely related species circulated in the local A. cajennense ticks. Thus, we recommend surveys of horse sera as a useful method for BSF surveillance in areas where humans are exposed to A. cajennense ticks. This procedure would allow potentially BSF-endemic areas to be identified before human cases occur.

We failed to detect any rickettsial DNA in the field-collected A. cajennense ticks. Although this result is supported by the serologic results in the BSF-nonendemic areas, it was not expected for the BSF-endemic areas, where infection by R. rickettsii in horses and dogs has been indirectly proven by serologic cross-absorption methods (17). Finding R. rickettsii-infected ticks in spotted fever–endemic areas can be difficult. In North Carolina, a U.S. state with a high incidence of Rocky Mountain spotted fever (caused by R. rickettsii), only 1 of 2,123 Dermacentor variabilis ticks studied was infected by R. rickettsii (15). Thus, further studies in São Paulo should encompass a much larger number of A. cajennense ticks.

The major ecologic differences between the BSF-endemic and -nonendemic areas of our study were the presence of capybaras and their main tick species (A. cooperi), found solely in the BSF-endemic areas. In a recent survey of rickettsiae in A. cooperi ticks collected on farms 1, 2, and 3 (19), 2 rickettsiae were isolated from these ticks: R. bellii and a Rickettsia species (strain COOPERI) closely related to R. parkeri and R. africae. Similar to the present study, no R. rickettsii was found infecting A. cooperi ticks.

Burgdorfer et al. (16) found that high infection rates (up to 80%) by a less pathogenic rickettsia was the limiting factor for establishing R. rickettsii in the D. andersoni tick population of the east side of the Bitterroot Valley in Montana, USA. On the west side of this valley, where 8%–16% of the ticks were infected by the less pathogenic rickettsia, disease caused by R. rickettsii was endemic. Based on these observations, the results of our study suggest that unknown factors other than the presence of different Rickettsia species are responsible for the absence of a pathogenic spotted fever group rickettsia’s infection of populations of A. cajennense populations in farms 4, 5, and to 6 (BSF-nonendemic areas).

In a recent study performed in our laboratory (A. Pinter and M.B. Labruna, unpub. data) R. rickettsii was detected in 6 (0.89%) of 669 A. aureolatum adult ticks by using the same PCR protocols as the present study. These ticks were collected in a different BSF-endemic area, in which A. aureolatum is the main vector of the disease. As our results showed that the highest predictable infection rate of R. rickettsii in the A. cajennense population of farm 3 (where 353 ticks were tested) was 0.8%, we might have found a R. rickettsii–infected A. cajennense tick if we had tested a larger sample of ticks from that farm. Even though recent studies have failed to detect or isolate R. rickettsii from A. cajennense ticks in Brazil, earlier studies detected it efficiently in the states of São Paulo (28) and Minas Gerais (9,10), as well as in Colombia (29), Mexico (30), and Panama (31).

Our study showed that R. parkeri could experimentally infect A. cajennense ticks. A previous, more extensive, study showed that A. americanum ticks experimentally infected with R. parkeri were able to maintain this infection for 2 generations and were able to transmit it to guinea pigs through tick feeding (32). Natural infection of ticks by this agent has been reported in A. maculatum (33) and A. triste (34). The Rickettsia species (strain COOPERI), found to be infecting A. cooperi ticks in São Paulo state (19), seems to be another strain of R. parkeri or a closely related species. These results show that R. parkeri can infect different Amblyomma species under experimental or natural conditions. The potential role of A. cajennense to transmit R. parkeri in nature requires further investigation, especially since R. parkeri was recently shown to be pathogenic for humans (35).
Acknowledgments

We are grateful to the owners of farms 1–6 for making our study possible and to David H. Walker for providing IFA slides and the R. parkeri–purified stock.

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Dr. Sangioni is a professor of veterinary parasitology at the Veterinary School of the Centro Integrado de Ensino Superior at Campo Mourão, Brazil. His research interests have focused on the ecology of tickborne diseases.

References


Address for correspondence: Marcelo B. Labruna, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil 05508-000; fax: 55-11-3091 7928; email: labruna@usp.br
An obligate intracellular bacterium was isolated from urine samples from 7 (3.5%) of 202 fruit bats (Eonycteris spelaea) in peninsular Malaysia. The bacterium produced large membrane-bound inclusions in human, simian, and rodent cell lines, including epithelial, fibroblastlike, and lymphoid cells. Thin-section electron microscopy showed reticulate bodies dividing by binary fission and elementary bodies in the inclusions; mitochondria surrounded the inclusions. The inclusions were positive for periodic acid-Schiff stain but could not be stained by fluorescein-labeled anti-Chlamydia trachomatis major outer membrane protein monoclonal antibody. The bacterium was resistant to penicillin and streptomycin (MICs >256 mg/L) but susceptible to tetracycline (MIC = 0.25 mg/L) and chloramphenicol (MIC = 0.5 mg/L). Sequence analysis of the 16S rRNA gene indicated that it was most closely related to 2 isolates of Waddlia chondrophila (94% and 96% identity). The 16S and 23S rRNA gene signatures were only 91% identical. We propose this novel bacterium be called Waddlia malaysiensis.

An estimated 1,415 microbes are infectious for humans (1). Of these, 868 (61%), are considered to be zoonotic; overall, zoonotic pathogens are twice as likely to be associated with emerging diseases (1). Wildlife have been increasingly recognized as important reservoirs of potentially zoonotic microorganisms (2,3). In particular, bats have been shown to be both important reservoirs and vectors of pathogens. These pathogens include viruses such as rabies (4), European lyssavirus (5), Hendra (6) and Menangle (7) viruses in Australia, Nipah and Tioman viruses in Malaysia (8,9), hantaviruses in Korea (10), a number of different bunyaviruses, flaviviruses, and alphaviruses. Moreover, solitary microchiropteran bats are prime contenders as reservoirs of Marburg and Ebola viruses. In addition, bats have been identified as reservoirs of fungi such as Histoplasma capsulatum and Coccidioides immitis. However, apart from leptospirosis (11) and some studies on enteric flora and pathogens (12–14), little is known of the bacteria that infect and are excreted by bats.

As part of an investigation into the reservoir of Nipah virus in Malaysia (8,9,15), a novel chlamydialike bacterium was isolated from the urine of Eonycteris spelaea; the Lesser Dawn Bat (16). This bat is a generalist nectivore that travels tens of kilometers from its cave-roosting sites to feed (16). It is found throughout Burma, Indochina, the Philippines, Malaysia, Indonesia, Nepal, and northern India. Little is known of the potential pathogens harbored by E. spelaea, but 1 survey of lyssavirus infection of bats in the Philippines did not detect virus in brain sections or neutralizing antibody to rabies or Australian bat lyssavirus in serum from E. spelaea (17). Neither Nipah nor Tioman viruses have been isolated from E. spelaea, and detecting this chlamydialike bacterium was a chance finding (15). We describe the isolation and characterization of this novel bacterium and propose that it be given the name Waddlia malaysiensis since it was first isolated in Malaysia.

Material and Methods

Collection of Samples and Isolation of the Bacterium

As part of an investigation into the reservoir of Nipah virus (8,9), we made 3 field trips from May to July 1999 to a colony of fruit bats (E. spelaea) roosting in a cave (Gua Tempurong) situated 25 km from the initial Nipah outbreak in Perak, northern peninsular Malaysia. The first visit was to observe the fruit bats’ roosting behavior, in
particular, timing of return to roost, leaving for feeding, and urination and defecation habits. In the second and third visits, clean plastic sheets (1.5 x 3 m) were suspended over areas where the bats had been observed previously to urinate and defecate (15). The sheets were suspended at 0.5 m above the ground and held taut with 4 metal rods. The sheets and rods were put in place 30 min before the bats were expected to return to roost. Sterile cotton swabs were used to collect the urine as soon as it fell onto the plastic sheets. The swabs were then placed into virus transport medium (2 mL: ICN Biomedicals Inc, Irvine, CA, USA), containing 1% bovine albumin hydrolysate, amphotericin B (20 µg/mL), penicillin G (100 U/mL), and streptomycin (50 µg/mL). The samples were transported at 4°C to the laboratory on the day of collection. Each swab, in transport medium, was gently vortexed, and 200 µL of the medium was transferred into individual wells of a 24-well tissue culture plate (Sterlitech, Stone, U.K.) preseeded with 1 x 10⁵ Vero cells in Eagle’s minimal essential medium (Sigma, Basingstoke, U.K.). The plates were sealed and incubated at 37°C. The culture was examined daily for cytopathic effect (CPE) with phase-contrast microscopy. Isolates were stored at −70°C, and 1 strain was chosen at random for further characterization and transported to Liverpool at −20°C.

**Microbiologic Characteristics**

To determine the range of cells susceptible to infection, different cells were cultured in 25-cm² plastic flasks (Becton Dickinson, Basingstoke, U.K.) in 199 medium (Sigma) with 2% (vol/vol) fetal calf serum but no added antimicrobial agents. Because the bacterium replicated so rapidly, including chlorhexidine, normally used in culture plates to prevent overgrowth of Vero cells, was not necessary. Approximately 10⁷ bacterial cells (as determined by electron microscopic count) were added to each flask of cells and incubated at 37°C in air with 5% CO₂ and examined daily for CPE. For each cell line, growth was determined by both phase-contrast microscopy and demonstration of inclusions by thin-section electron microscopy. A variety of human (Hep-2, HEK, MRC-5, A549 and an Epstein Barr virus (EBV)–transformed human B-lymphoblastoid line), simian (Vero, LLC-MK2), and rodent (3T3, BHK) cell lines were used. Attempts were also made to grow the bacteria on 7% horse blood Columbia agar plates in air with 5% CO₂ and anaerobically at 37°C for 72 h.

To determine antimicrobial susceptibility, coverslip cultures of Vero cells were prepared as described previously except that chlorhexidine was omitted from the growth medium (18,19). After 48 h of incubation, the growth medium was removed and ≈10⁵ bacteria (in 0.5 mL medium) were added to each vial containing the coverslip monolayer of Vero cells. After absorption (without centrifugation) for 30 min, fresh 199 medium with 2% fetal calf serum, which incorporated doubling dilutions of antimicrobial agents from 1 mg/L down to 0.06 mg/L and doubling increases in concentration from 1 mg/L to 256 mg/L, was added. The antimicrobial agents used were chloramphenicol, tetracycline, penicillin G, and streptomycin. The coverslip cultures were incubated at 37°C for 72 h; they were then methanol-fixed and Giemsa-stained as described previously (19). The MIC of an antimicrobial agent was defined as the lowest concentration required to inhibit the formation of inclusions.

To determine staining characteristics, coverslip cultures of Vero cells were infected with ≈10⁵ bacteria. After 48 h of culture, the cells were methanol-fixed and stained by Giemsa, periodic acid-Schiff (PAS), or immunofluorescence staining by using a monoclonal antibody directed against the major outer membrane protein of *C. trachomatis* (Microtrak, Trinity Biotech, Bray, Ireland) as described previously (18,19). For thin-section electron microscopy, infected cells were fixed in cacodylate-buffered glutaraldehyde (2%), scraped from the flask, postfixed through increasing concentrations of ethanol (to 100% vol/vol), and then araldite embedded. Thin-sections were stained in uranyl acetate and Reynolds’s lead citrate and examined with a Philips 301 electron microscope. For negative-stain electron microscopy, suspensions were placed on a Formvar-coated grid and stained in phosphotungstic acid.

**Genomic Characteristics**

Total DNA was extracted from a 72-h culture of the bacterium in Vero cells. The infected cells were scraped from a 25-cm² tissue culture flask (Becton Dickinson, Basingstoke, U.K.) into 2 mL 199 medium without fetal calf serum. One millilitre of this mixture was centrifuged at 13,000 x g for 30 min, and the pellet was suspended in 250 µL of 5% wt/vol Chelex-100 resin slurry (BioRad, Hemel Hempstead, U.K.). This suspension was boiled for 15 min, followed by centrifugation at 13,000 x g for 10 min; the supernatant was then removed and stored at −20°C until used.

For analysis of the 16S rRNA gene, a 1,526-bp amplicon was produced by using primers 16S-FOR and 16S-REV (Table 1) as described by Rurangirwa et al. (20). The amplicon was excited from the agarose gel and purified by using a gel purification kit (Qiagen, West Sussex, U.K.). The amplicon was cloned into a cloning vector, pGEM-T (Promega, Southampton, U.K.) and transformed into *Escherichia coli*. Full-length sequencing of the 1,526-bp amplicon within the cloning vector was achieved by using overlapping internal primers (F1-F4 forward and R1-R4 reverse, Table 1). 16S rRNA signature sequence, 16S-23S rRNA intergenic space, and 23S rRNA domain I signature
sequence polymerase chain reaction (PCR) were carried out by using the method of Everett et al. (21) with the primers shown in Table 1. In each case, PCR amplification was performed in 50-µL volumes. All primers were added at 20 pmol per assay; PCR buffer (plus 1.5 mmol/L MgCl₂), Q solution, and Taq polymerase were obtained from Qiagen Ltd (Crawley, U.K.). The presence of the murA protein signature was sought by PCR by using primer murA-for and murA-rev (Table 1), which amplifies a 690-bp fragment of the UDP-N-acetylglucosamine 1-carboxyvinyltransferase gene of Waddlia chondrophila (22). In this case, PCR was attempted by using a range of Mg²⁺ concentrations from 1.5 to 4.0 mmol/L. Primers to amplify a 331-bp segment of the sctN gene were designed by alignment of the sctN genes of C. trachomatis (AE001337), C. pneumoniae (AE002167), and C. muridarum (AE002271). The sctN gene encodes a type III secretion system ATPase, which is highly conserved among these bacteria (23). Sequence determination was performed by using an automated DNA sequencer (ABI PRISM 377; Perkin-Elmer, Warrington, U.K.) and was analyzed by using commercial software (Lasergene: DNASTar Inc., Madison, WI, USA).

For phylogenetic analyses, sequence data on complete 16S rRNA genes for each of the Chlamydiales genera were retrieved from GenBank and aligned with ClustalW (24). The phylogenetic tree was generated from the alignment by using the genetic distance-based neighbor-joining algorithms of the Data Analysis in Molecular Biology software (http://web.hku.hk/~xxia/software/software.htm). Sequence input order was randomized, and 100 datasets were examined by bootstrapping resampling statistics.

### Table 1. Oligonucleotide primers for PCR and sequencing*

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<th>Primer sequence</th>
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<td>PCR</td>
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<tr>
<td>16S rRNA (1,526 bp from ref. 20)</td>
<td>5'AAGTGTGACGCTT3'</td>
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<tr>
<td>16S-FOR</td>
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<tr>
<td>16S-REV</td>
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<td>16S1GF</td>
<td>5'TGACGTCCAGTGG3'</td>
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<td>Tm = 51°C</td>
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<td>16SF2</td>
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<td>Tm = 61°C</td>
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<td>23S rRNA signature sequence (627 bp: domain I from ref. 21)</td>
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<td>murA-rev</td>
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<td>F1 (M13)</td>
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</tr>
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</table>

*PCR, polymerase chain reaction; Tm, melting temperature.
Results

During the second and third field visits to Gua Tempurong, 206 urine samples were obtained (93 in the second and 113 in the third field visit) from individual bats. A total of 7 urine samples (all from the third visit) produced a characteristic CPE on Vero cells after 5 to 7 days of culture. The same CPE was identified for each of the 7 isolates. One (G817) was therefore selected at random for further characterization.

On negative-stain electron microscopy of the supernatant from G817 cultured on Vero cells, small bacterial cells (0.4–0.6 μm) resembling chlamydial elementary bodies were seen (Figure 1A). Inclusions visible by phase-contrast microscopy could be detected within 48 to 72 h postinfection of Vero cells. Similar inclusions could be seen after infection of human lung (MRC-5, A549), kidney (human embryo kidney [HEK]), laryngeal (HEp-2), and B-lymphoblastoid cells lines; and of simian kidney (LLC-MK2) and rodent epithelial (3T3, BHK) cell lines. Figures 1B and 1B show large inclusions in HEK- and the EBV-transformed human B-lymphoblastoid cell lines, respectively. In Figure 1B, mixtures of reticulate and elementary bodies are visible. A thin-section electron micrograph of an earlier stage of infection of HEK cells (48 h postinfection, Figure 1D) shows a collection of reticulate bodies with evidence of replication by binary fission. Mitochondria can be seen in close proximity. The bacterium could not be cultured on blood or chocolate agar, aerobically or anaerobically, when incubated for up to 7 days, nor did it have catalase or oxidase activities.

Inclusions could be stained by both Giemsa and PAS but not by the Mikrotrak immunofluorescence system, which recognizes the C. trachomatis major outer membrane protein. MICs of tetracycline and chloramphenicol were 0.25 mg/L and 0.5 mg/L, respectively, but streptomycin (256 mg/L) and penicillin G (256 mg/L) did not inhibit the formation of inclusions at therapeutically achievable levels.

All of the 16S rRNA gene, the 16S-23S rRNA intergenic spacer region, and the 627-bp domain I of the 23S rRNA gene were sequenced in both directions. This sequence of 2379 bp has been lodged in GenBank with the accession number AY184804. A BLAST search indicated that a 1,552-bp sequence of the bacterium’s 16S rRNA gene had 96% and 94% identity with two 16S rRNA sequences from W. chondrophila (AF346001 and AF042496). The 16S rRNA (298-bp) and 23S rRNA (627-bp) gene signatures had 91% identity with the 2 W. chondrophila sequences deposited in GenBank. The 16S-23S rRNA intergenic space of the bat isolate was 223 bp compared to 213 bp (AF042496) and 217 bp (AF346001) for W. chondrophila. Figure 2 shows a neighbor-joining dendrogram demonstrating the relationships of the novel bat bacterial isolate to other members the Chlamydiales. This indicates that the novel bacterium is most closely related to, but distinct from, W. chondrophila. No PCR amplicons were detected on amplification of either murA or sctN. When DNA from C. trachomatis (lymphogranuloma venereum strain L1) was used as positive control, amplicons of the correct size were detected.

![Figure 1](https://example.com-figure1.png)

Figure 1. A, Negative stain electronmicrograph of Waddlia malaysiensis elementary bodies. B-D, Thin-section electronmicrographs of cells infected with W. malaysiensis. B, large inclusion with elementary(e) and reticulate(r) bodies in HEK cells 72 h postinfection. C, a large inclusion in Epstein Barr virus—transformed human B-lymphocytes. D, dividing reticulate bodies in HEK cells 48 h postinfection in an inclusion with numerous surrounding mitochondria (arrow).
Discussion

Members of the order Chlamydiales are obligate intracellular bacteria. Recently, a suggestion to revise and update their classification has been made (21). This revision was based on comparisons of 16S rRNA and 23S rRNA genes, and it split the Chlamydiales into 4 families, Chlamydiaceae, Simkaniaceae, Parachlamydiaceae, and a family now named Waddliaceae (20), which has W. chondrophila as the prime member (Table 2). This scheme of nomenclature has largely been accepted, although splitting the family Chlamydiaceae into 2 genera, Chlamydia and Chlamydophila, raised some concerns (25). The Chlamydiales are an expanding group of bacteria with new genera and species increasingly being described and detected in a wide array of hosts (26,27). Recent examples include Rhabdochlamydia porcellionis, isolated from terrestial isopods, which is related to but not entirely within the family Simkaniaceae (28), and 2 insect-associated chlamydia, Fritschea bemesia and F. eriococci in the family Simkaniaceae (29). In addition, a number of Chlamydiales endosymbionts have been recovered from human clinical and environmental isolates of Acanthamoeba spp. that are related to the Parachlamydiaceae (30). Indeed for one of these, UWE25, the full genome has been sequenced (31). Analyses of 16S rRNA, 23S rRNA genes, and the 16S-23S intergenic space indicate that the bacterium we have isolated from fruit bats is most closely related to the Waddliaceae (Figure 2). There are, however, some similarities and differences between our isolate and W. chondrophila. W. chondrophila has been isolated twice from cattle, and the bacteria were obtained from a first-trimester bovine abortion in the United States (20) and a septic stillborn calf in Germany (32). The bacterium from the United States was isolated initially by culture on bovine turbinate cells (20), but the German isolate was able to grow in human diploid fibroblasts, simian (Buffalo Green Monkey, and murine [McCoy]) cells lines (32). Our bat isolate was able to grow in a wide range of cell types from different anatomic sites and animal species. Some evidence suggests that W. chondrophila also has a wide host cell range, but not all possibilities have been tested. There is also recent evidence, based on 16S rDNA amplification, of W. chondrophila in an Australian mammal, Gilbert’s Potoroo (33). Like W. chondrophila, our isolate was resistant to penicillin G and streptomycin (MICs >256 mg/L) and could not be stained by immunofluorescence using monoclonal anti-C. tra

![Phylogenetic relationships of Waddlia malaysiensis to other Chlamydiales.](image)

Table 2. Current status of the Chlamydiales

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus and species</th>
<th>Biovars</th>
<th>Host/animal disease*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Chlamydiaceae</td>
<td>Chlamydia trachomatis</td>
<td>Serovars A–K</td>
<td>Humans: trachoma, STI</td>
</tr>
<tr>
<td></td>
<td>C. muridarum</td>
<td>Serovars L&lt;–L3</td>
<td>Humans: STI</td>
</tr>
<tr>
<td></td>
<td>C. suis</td>
<td></td>
<td>Mice: proliferative ileitis</td>
</tr>
<tr>
<td></td>
<td>Chlamydophila psittaci</td>
<td>Serovars A–H</td>
<td>Birds, cattle: pneumonia†</td>
</tr>
<tr>
<td></td>
<td>C. pneumoniae</td>
<td>3 biovars</td>
<td>Humans, koala, equines:</td>
</tr>
<tr>
<td></td>
<td>C. pecorum</td>
<td></td>
<td>pneumonias, conjunctivitis</td>
</tr>
<tr>
<td></td>
<td>C. felis</td>
<td></td>
<td>Wide host range and disease</td>
</tr>
<tr>
<td></td>
<td>C. cavia</td>
<td></td>
<td>manifestation</td>
</tr>
<tr>
<td></td>
<td>C. abortus</td>
<td></td>
<td>Cats: rhinitis†</td>
</tr>
<tr>
<td>II. Parachlamydiace</td>
<td>Parachlamydia acanthamoebae</td>
<td></td>
<td>Guinea pigs: conjunctivitis</td>
</tr>
<tr>
<td></td>
<td>Neochlamydia hartmannellae</td>
<td></td>
<td>Sheep, cattle, goats: abortion†</td>
</tr>
<tr>
<td></td>
<td>Numerous others including UWE25</td>
<td></td>
<td>Amoebae: RTI</td>
</tr>
<tr>
<td>III. Waddliaceae</td>
<td>Waddlia chondrophi</td>
<td></td>
<td>Amoebae</td>
</tr>
<tr>
<td>IV. Simkaniace</td>
<td>Simkania negevensis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>“Candidatus Fritschea bemesiae”</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>“Candidatus F. eriococci”</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V. Clamydia-like organisms</td>
<td>“Candidatus Rhabdochlamydia porcellionis”</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*STI, sexually transmitted infection; RTI, respiratory tract infection; —perhaps (i.e., disputed); ?, may be 2 biovars but not confirmed.
†Indicates zoonotic potential.
pared to those of UWE25, and sufficient differences exist in the nucleotide sequence (96% and 94% identity). The 16S rDNA and 23S rDNA from cattle on the basis of 16S rRNA gene comparisons were closest to but not identical to, C. trachomatis. No evidence for the presence of one of the key genes (sctT) of the pathogenicity island-associated type III secretion system of C. trachomatis was found in W. malaysiensis; however, this does not mean that no such island is present. Three genes (sctT, sctN, and sctV) from a type III secretion system have been described in the Parachlamydia-like endosymbiont UWE25, and sufficient differences exist in the nucleotide and putative amino acid sequences of these, when compared to those of C. trachomatis (31), that our primers would not amplify it.

Negative-stain electron microscopic examination of the bat bacterium released from Vero cells showed small cocci indistinguishable from the elementary bodies of C. trachomatis. On thin-section electron microscopy of infected cells, large numbers of intracellular bacteria could be seen within membrane-bound inclusions. In mature inclusions in all cell types tested, mixtures of elementary and reticulate bodies were found. In less mature inclusions, dividing reticulate bodies were present, and mitochondria could be seen around the inclusion (Figure 1D). The species name of W. chondrophila was derived from the collections of mitochondria around the intracellular inclusions. The bat isolate was closest to the 2 W. chondrophila isolates made from cattle on the basis of 16S rRNA gene comparisons (96% and 94% identity). The 16S rDNA and 23S rDNA gene sequence also placed the bat bacterium closest to W. chondrophila (91%); in addition, the 16S – 23S rRNA intergenic space was slightly longer than for W. chondrophila. Thus, the bat isolate is part of the genus Waddlia, and we propose the name Waddlia malaysiensis for it since it was first detected in Malaysia. The organism appears sufficiently distinct from W. chondrophila to justify a different species assignment. It is PAS positive, does not have the murA signature of W. chondrophila, and has differences in the 16S – 23S rRNA genomic regions. The collection of mitochondria in proximity to inclusions that gave Waddlia its species name was also exhibited by W. malaysiensis and might therefore be a characteristic of the genus Waddlia.

The Chlamydiaceae infect a wide range of animals including humans (27,34). Some pathogens such as C. trachomatis appear to solely affect humans; others affect only animals; and a sizeable number are zoonotic pathogens (Table 2). W. chondrophila has been isolated from aborted cattle fetuses in the United States and Germany (20,32) but has also been detected in an apparently healthy Potoroo in Australia (33). Recent serologic evidence has suggested a strong statistical association between high titers of W. chondrophila antibodies and bovine abortion (35). Members of the genera Parachlamydia and Simkania infect protozoa such as amoebae and can cause disease in humans (30,36,37). In this respect, evidence exists for replication of W. chondrophila in amoebae (38), which suggests that it might fall into the group of environmentally preadapted pathogens, as has been suggested for S. negevensis (39) and C. pneumoniae (40). Whether W. malaysiensis can grow in amoebae and has zoonotic potential remains to be determined.

Dr. Chua is a pediatrician and medical microbiologist. He was the first to isolate Nipah virus in Malaysia.

References


Address for correspondence: C. A. Hart, Department of Medical Microbiology, University of Liverpool, Duncan Building, Daulby St, Liverpool, L69 3GA, United Kingdom; fax: 0151 706 5805; email: cahmm@liv.ac.uk

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Quarantine for SARS, Taiwan

Ying-Hen Hsieh,* Chwan-Chuan King,† Cathy W. S. Chen,‡ Mei-Shang Ho,§ Jen-Yu Lee,* Feng-Chi Liu,* Yi-Chun Wu,¶ and Jiunn-Shyan Julian Wu¶

During the 2003 outbreak of severe acute respiratory syndrome (SARS) in Taiwan, >150,000 persons were quarantined, 24 of whom were later found to have laboratory-confirmed SARS-coronavirus (SARS-CoV) infection. Since no evidence exists that SARS-CoV is infective before the onset of symptoms and the quarantined persons were exposed but not symptomatic, we thought the quarantine’s effectiveness should be investigated. Using the Taiwan quarantine data, we found that the onset-to-diagnosis time of previously quarantined confirmed case-patients was significantly shortened compared to that for those who had not been quarantined. Thus, quarantine for SARS in Taiwan screened potentially infective persons for swift diagnosis and hospitalization after onset, thereby indirectly reducing infections. Full-scale quarantine measures implemented on April 28 led to a significant improvement in onset-to-diagnosis time of all SARS patients, regardless of previous quarantine status. We discuss the temporal effects of quarantine measures and other interventions on detection and isolation as well as the potential usefulness of quarantine in faster identification of persons with SARS and in improving isolation measures.

Methods

Data

During the outbreak of 2003, 346 SARS cases were officially confirmed in Taiwan, among which were 37 direct SARS deaths (cause of death was recorded as SARS) and 36 SARS-related deaths (cause of death was not directly attributed to SARS) as reported by the World Health Organization (WHO) (1). To guard against the potential threat of a large-scale epidemic, the government attempted to place >150,000 people under home quarantine. Two distinct levels of quarantine were implemented in Taiwan. Level A quarantine, aimed at people having close contact with a suspected SARS case-patient, was implemented on March 18, 2003. Level B quarantine, aimed at travelers from affected areas, was implemented on April 28, in the aftermath of the first SARS death on April 26 (10,11). Most of the quarantined persons were confined to their homes for 10–14 days. Public health nurses would bring the quarantined persons 3 meals every day and sometimes helped them with odd jobs such as washing clothes or taking care of pets. Center for Disease Control–Taiwan officially confirmed 346 SARS-CoV–positive cases, of which 17 case-patients had been previously quarantined; 134 additional laboratory-confirmed antibody-positive SARS cases occurred, of which 7 case-patients had previously been quarantined. The total number of confirmed SARS case-patients in Taiwan by the
end of December 2004 was 480, of which 24 had been quarantined previously. Details regarding the persons quarantined during the SARS outbreak are itemized in Table 1.

The 134 patients with laboratory-confirmed SARS either had milder symptoms, and SARS was therefore clinically diagnosed as suspected, ruled out at the time of the outbreak, or considered probable in patients whose specimens had previously tested negative by polymerase chain reaction (PCR) or anti–SARS-CoV antibody, perhaps due to wrong timing, but later were reconfirmed by >2 different laboratory tests in a follow-up epidemiologic study. Seven people in this group had been previously quarantined. Our criterion for a quarantined person was someone who had been placed under official quarantine for >1 day before the onset of symptoms. Thus, persons in whom symptoms developed on the same date or before the notification of quarantine were considered not quarantined and were therefore excluded. Persons who were known to have had a record of close contacts with others during the supposed quarantine period were also excluded. One of the 24 case-patients actually had an imported case but was quarantined before implementation of level B quarantine on April 28 for reasons other than simply being a traveler from an affected area.

Statistical Analyses

We compared the mean time from onset of symptoms to clinical diagnosis (and admission) for the 24 patients with laboratory-confirmed SARS-CoV who had been quarantined before symptom onset to that of the 451 SARS-CoV–positive case-patients who had not. Note that 5 cases were deleted from the data of 480 total cases for our comparison test because of missing information on their relevant dates. (We will use the term “diagnosis” to mean clinical diagnosis hereafter.) For the mean time from diagnosis to classification as probable case, we only used the officially confirmed cases for comparison, since the laboratory confirmed cases were either ruled out or classified as suspected cases only and thus had no classification of probable time. Again, 2 of these cases were deleted from the data for our comparison test because of missing information on their relevant dates; therefore, 344 case-patients (17 quarantined and 327 nonquarantined) were used. Due to the skewed data, we used the nonparametric Mann-Whitney test.

To investigate the effect of large-scale quarantine on the changes in the efficiency of the public health system to identify SARS patients for isolation, we considered the temporal effect of important events for intervention and control of SARS in Taiwan. On April 28, level B quarantine was implemented, which marked the start of large-scale home quarantine (12).

A second important date in SARS prevention and control was May 10, when changes in the review and classification procedures were implemented by the cabinet-level SARS Prevention and Extrication Committee in Taiwan to expedite the review and classification of SARS cases (13). Before May 9, the relevant medical records (including any available laboratory test results) of all reported SARS patients were reviewed by a central SARS Advisory Committee of the Center for Disease Control–Taiwan in Taipei. Due to the rapid increase in the number of reported cases caused by the hospital cluster outbreaks in Taipei in late April, the SARS Advisory Committee in CDC-Taiwan could not handle the rapidly increasing caseload. Consequently, after May 10, 3 regional offices of the Bureau of National Health Insurance in northern, central, and southern Taiwan took over the responsibility of case review. Local SARS expert committees were established in the 3 regions with each committee consisting of specialists similar to the central committee in Taipei. The experiences and the standard operation procedures of case review and case classification used by the central committee were

Table 1. Cumulative numbers of persons under quarantine during the SARS outbreak, Taiwan, 2003, and the quarantined SARS patients classified by their status*

<table>
<thead>
<tr>
<th>Level and reason for quarantine</th>
<th>No. quarantined persons</th>
<th>No. quarantined officially confirmed SARS-CoV case-patients</th>
<th>No. quarantined laboratory confirmed, antibody positive SARS case-patients with</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family members</td>
<td>7,921</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Classmates and teachers</td>
<td>16,564</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Healthcare workers</td>
<td>2,409</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Others†</td>
<td>19,224</td>
<td>6‡</td>
<td>1</td>
</tr>
<tr>
<td>All others‡</td>
<td>9,514</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Subtotal</td>
<td>55,632</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>Level B</td>
<td>95,828</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>151,460</td>
<td>17</td>
<td>7</td>
</tr>
</tbody>
</table>

*Updated December 2004.
†Passengers and drivers of domestic public transportation traveling for >1 hour in the same bus or train cabin with a SARS case-patient, persons who had contacts with someone under quarantine or receiving care in a medical facility where cluster infection had occurred, and homeless persons.
‡Co-workers and friends of SARS case-patients, airplane passengers who sat within 3 rows of or stayed in the same room as SARS patients, and persons with missing information.
§One case-patient had onset of symptoms 2 days after the end of quarantine.
transferred to the 3 regional SARS expert committees in the Bureau of National Health Insurance through several consensus meetings (14).

We used the Mann-Whitney test to compare the time intervals from onset to clinical diagnosis of SARS symptoms of the patients with confirmed SARS-CoV with onset occurring during the 3 periods of February 25–April 27 (period 1), April 28–May 9 (period 2), and May 10–June 15 (period 3). Five patients were deleted from the data for our comparison test due to missing information on their relevant dates, and 2 patients were deleted because their onsets of SARS did not occur during the 3 time periods.

We also compared, using the Mann-Whitney test, the intervals from diagnosis to classification as probable SARS of the 343 officially confirmed SARS-CoV case-patients (by dividing the cases into 3 groups, according to the time period in which the date of diagnosis occurred). Again, the laboratory-confirmed cases had never been classified as probable cases. Moreover, 2 cases were deleted from the data for our comparison test due to missing information on their relevant dates, and 1 case was deleted because classification as a probable case-patient did not occur during the 3 periods from February 25 to June 15.

Results

The mean time from onset to diagnosis for the previously quarantined persons (1.20 days) was significantly shorter than that of those who were not quarantined (2.89 days) (Table 2). However, the respective mean times from diagnosis to classification (6.21 days and 7.34 days) (Table 2), though slightly reduced for the quarantined persons, were not significantly different. For the mean onset-to-diagnosis time, period 1 was significantly longer (3.60 days to 2.49 days) than period 2 (p < 0.0001), while the mean difference before and after May 10 was not significant (p = 0.0722) (Table 3). The mean diagnosis-to-classification time (Table 4) was not significantly different from period 1 to period 2. However, the time was significantly shortened after May 10 (from period 2 to period 3).

Discussion and Conclusions

The experience in the affected areas has shown that the transmission of SARS can be prevented by adherence to basic public health measures, including rapid case detection, isolation of patients with suspected and probable cases, contact tracing, and good infection control (9). The effect of possible delays in effective isolation of probable case-patients has been studied in some modeling work on SARS (15–17). In Taiwan, all patients were supposed to be placed in the isolation room and negative pressure room, if available, as soon as they were reported as having probable or suspected SARS. For most of May, the number of suspected case-patients alone remained well above 1,000, partly because of confusion in diagnosis and the tendency to overdiagnosis because of heightened alertness on the part of physicians and legal punishment for underreporting. At times, however, due to the lack of available isolation rooms or the number of suspected cases pending review, patients with suspected but unconfirmed SARS were kept for days in an observation room or emergency department under crude isolation, where nosocomial infections readily occurred. At other times, patients scheduled to transfer to another hospital with negative pressure isolation rooms were temporarily kept in the observation room in the emergency department where nosocomial infections might occur because of insufficient isolation and protection procedures (18). When full isolation facilities were not available to all patients, those classified as probable SARS case-patients likely received higher priority and were observed more closely during their isolation by healthcare workers than were the suspected case-patients.

For some case-patients, delays occurred because of the patient’s uncertain status or urgent need for intubation without comprehensive information on the patient’s contact and clinical history; these delays led to insufficient protection and isolation. One well-known case-patient was the index patient at Hoping Hospital in Taipei, where the largest cluster infection in Taiwan occurred. Her condition was diagnosed and reported as suspected SARS on April 9. However, because the patient had no apparent contact with another known SARS case-patient, her case was reviewed but not reclassified as probable until April 25, by which time the clustered cases, which included medical staff members and an x-ray technician who had contact with her, had already forced the hospital to shut down on the previous day. More strict infection control would have been in place had the index patient been confirmed as a probable SARS patient. Several other similar cases occurred in Taiwan, some more than 1 month later.

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Table 2. Comparison of mean time intervals by using the Mann-Whitney test for the onset-to-diagnosis and diagnosis-to-classification times for quarantined and nonquarantined SARS patients, Taiwan, 2003

<table>
<thead>
<tr>
<th></th>
<th>Onset-to-diagnosis interval (d)</th>
<th>Diagnosis-to-classification interval (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quarantined persons</td>
<td>1.203 (n = 24)</td>
<td>7.7647 (n = 17)</td>
</tr>
<tr>
<td>Nonquarantined persons</td>
<td>2.8914 (n = 451)*</td>
<td>7.5443 (n = 327)*</td>
</tr>
<tr>
<td>Mean difference</td>
<td>1.6831 (0.0061)</td>
<td>0.2204 (0.7964)</td>
</tr>
</tbody>
</table>

*S cases deleted because of missing information on the relevant dates.
††2 cases deleted because of missing information on the relevant dates.
††Denotes significance at the 1% level; p values of Mann-Whitney test are in parentheses.
Therefore, the importance of rapid classification as probable case-patients cannot be ignored.

Our results show that quarantine reduced the time from onset to diagnosis but did not significantly reduce the time from diagnosis to classification. Thus, a previously quarantined person could expect his or her condition to be diagnosed and to be hospitalized more quickly once clinical symptoms appeared. However, the same person would not receive higher priority in the classification process to determine candidates for effective isolation. Nevertheless, in many hospitals with available isolation rooms, patients with suspected cases were effectively isolated as soon as chest radiographic evidence of infiltrates consistent with pneumonia or acute respiratory distress syndrome became available. Moreover, in the latter stages of the epidemic when a reliable laboratory test for SARS-CoV became more available, many patients were isolated in negative pressure chambers immediately if results of reverse transcription–PCR for SARS-CoV from 2 different laboratories were positive. Therefore, the effect of classification as a probable case-patient might not be as pronounced as it would have been otherwise.

For all laboratory confirmed case-patients, regardless of whether they were quarantined previously, the implementation of full-scale intervention measures, including level B quarantine on April 28, significantly decreased the time from onset to diagnosis, but it only slightly improved the time from diagnosis to classification. However, the small sample size of 24 previously quarantined SARS case-patients did not permit a meaningful test of whether a significant difference existed for the previously quarantined persons during each of the 3 periods.

By comparison, the change in the review and classification procedure initiated on May 10 helped shorten the diagnosis-to-classification time for all SARS patients, indicating that the action by the SARS Prevention and Extrication Committee to expedite the review process had indeed worked. However, by separating the analyses of data into discrete epochs marked by significant events, we have included those cases whose illnesses straddle epochs.

In the future, when facing newly emerging infectious diseases like SARS, in which the patient’s infectivity in the incubation period is unknown, precise clinical diagnosis cannot be made, and modes of transmission are uncertain, quarantine should be used not only to directly prevent possible asymptomatic infections but also to screen out potentially infective persons and thus prevent secondary or even tertiary infections.

The quarantine in Taiwan was indeed useful in helping to identify persons who are likely to develop symptoms and isolate them more quickly if and when they did, although its effect on isolation and infection control could perhaps be improved by quicker classification or confirmation of previously quarantined patients. No conclusion was drawn regarding whether better outbreak control would be achieved by placing fewer persons in quarantine or by concentrating on improving the efficiency of detection and isolation procedures. In fact, each area may be improved in efficiency without jeopardizing the other’s improvement.

**Acknowledgments**

We are grateful to Roy Anderson, John Glasser, and Fred Brauer for constructive discussions that helped formulate some
of the ideas for this work and to the anonymous referees for their many valuable comments. Sincere thanks are also extended to central and local public health personnel and medical staff who devoted all of their efforts to the quarantine and prevention of SARS in Taiwan. Y.H.H. would like to thank Mathematics of Information Technology and Complex Systems (MITACS) for their generous financial support for Y.H.H. to attend MITACS SARS meetings at Banff, Alberta, Canada, where several of the above-mentioned discussions took place.

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Dr. Hsieh is a professor of applied mathematics at National Chung Hsing University. His primary research interests are focused on mathematical and statistical modeling of infectious diseases epidemiology.

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Address for correspondence: Prof. Ying-Hen Hsieh, Department of Applied Mathematics, National Chung Hsing University, 250 Kuo-Kuang Rd., Taichung, Taiwan 402; fax: 886-4-22853949; email: hsieh@amath.nchu.edu.tw

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All human Ebola virus outbreaks during 2001–2003 in the forest zone between Gabon and Republic of Congo resulted from handling infected wild animal carcasses. After the first outbreak, we created an Animal Mortality Monitoring Network in collaboration with the Gabonese and Congolese Ministries of Forestry and Environment and wildlife organizations (Wildlife Conservation Society and Programme de Conservation et Utilisation Rationnelle des Ecosystèmes Forestiers en Afrique Centrale) to predict and possibly prevent human Ebola outbreaks. Since August 2001, 98 wild animal carcasses have been recovered by the network, including 65 great apes. Analysis of 21 carcasses found that 10 gorillas, 3 chimpanzees, and 1 duiker tested positive for Ebola virus. Wild animal outbreaks began before each of the 5 human Ebola outbreaks. Twice we alerted the health authorities to an imminent risk for human outbreaks, weeks before they occurred.

Ebola virus, a member of the Filoviridae family, causes severe hemorrhagic fever in humans and nonhuman primates. The human case-fatality rate ranged from 50% to 89%, according to the viral subtype, from the first outbreaks in Zaire and Sudan in 1976 to the 2003 outbreaks in the Republic of Congo (1–4). No effective therapy or prophylaxis exists, and Ebola is a major public health concern. The first recorded human Ebola outbreaks (Yambuku Zaire 1976; Nzara, Sudan, 1976 and 1979; Tandala, Zaire, 1977) occurred abruptly, from an unidentified source, with subsequent person-to-person spread (1,2,5,6). No trace of the virus was initially found in wild animals close to the outbreaks (7–9). In 1989, for the first time, a nonhuman primate outbreak due to a new subtype of Ebola virus, Ebola subtype Reston, occurred in a colony of Macaca fascicularis in a quarantine facility in Reston, Virginia, USA, after the introduction of monkeys from the Philippines (10). Ebola Reston caused severe hemorrhagic fever in monkeys, but no clinical cases of human infection were identified, even though anti-filovirus antibodies were found in quarantine facility personnel (11). Later, in 1994, Ebola-specific immunohistochemical staining was positive on necropsy specimens from 1 of 12 chimpanzees that died in the Tai forest of Côte d'Ivoire (12). During this outbreak, an ethnologist was infected while performing an autopsy on a chimpanzee carcass; this was the first documented case of human infection transmitted by a nonhuman primate (13). During the 1996 outbreak in Mayibout (Gabon), an epidemiologic survey showed that the index case-patients had been infected by contact with a chimpanzee carcass. Concurrently, many nonhuman primate carcasses were reported in the area close to the outbreak, but none was recovered (14,15). Recently, we showed that all the human Ebola virus outbreaks that occurred in the past 3 years in Gabon and the Republic of Congo resulted from multiple introductions of the virus from different infected animal carcasses (16). We describe the development, testing, and evaluation of an Animal Mortality Monitoring Network (AMMN) in northeastern Gabon and northwestern Republic of Congo designed to alert human and animal health authorities on emerging epidemics.

*Centre International de Recherches Médicales de Franceville, Franceville, Gabon; †European Union Project Cybertracker Monitoring Programme, Libreville, Gabon; ‡Universidad de Barcelona, Barcelona, Spain; §Wildlife Conservation Society, Bronx, New York, USA; ¶Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and #Institut de Recherche pour le Développement, Franceville, Gabon.
Materials and Methods

Epidemiologic Surveillance Network

An alert network was set up by the Ministries of Health in hospitals and clinics in the different regions of Gabon and Republic of Congo, designed to report all human cases of viral hemorrhagic syndromes. Particular attention was paid to the northeastern region of Gabon, which had already been affected by outbreaks, and to its border region with Republic of Congo. Wildlife organizations such as the Wildlife Conservation Society (WCS), Programme de Conservation et Utilisation Rationnelle des Ecosystèmes Forestiers en Afrique Centrale (ECOFAC), and the World Wildlife Fund (WWF) were chosen to form the backbone of AMMN, in close collaboration with the Ministries of Forestry and Environment of the 2 countries. WWF was present in the Minkébé Reserve in Gabon, while ECOFAC was in charge of the Odzala National Park and the Lossi gorilla sanctuary in Republic of Congo (Figure 1).

All information on human cases of viral hemorrhagic syndrome or on the presence of dead animals in affected areas was centralized by a Viral Hemorrhagic Fever Committee (VHFC), composed of representatives of the Ministries of Health, Forestry, and Environment, the World Health Organization (WHO), wildlife agencies, and the Centre International de Recherches Médicales de Franceville (CIRMF). VHFC was also charged with sending specialized CIRMF teams to sample animal carcasses for diagnostic purposes. CIRMF is the regional reference laboratory for viral hemorrhagic fevers, and communicates its results to the ministries of health, forestry, and environment and to WHO.

Ebola Outbreak Investigation: Human Case Data

The Gabonese and Congolese Ministries of Health, in close collaboration with WHO and its partners in the Global Outbreak Alert and Response Network (GOARN), were in charge of human epidemiologic investigations. A case of Ebola hemorrhagic fever was defined as any probable or laboratory-confirmed case, based on internationally recognized criteria (definition available from http://www.who.int/emc/diseases/ebola/ebola7.html).

Ebola Outbreak Investigation: Animal Data

Collection Sites

From August 2001 to June 2003, carcasses were found on both sides of the Gabon–Republic of Congo border in the Ogooué Ivindo (Gabon) and West Basin (Congo) provinces (Figure 1). This entire area is covered by a Marantaceae and Zingiberaceae forest, with both open and closed canopies. The climate is equatorial, with 2 dry seasons (December–February and June–August) and 2 wet seasons (March–May and September–November). Mean rainfall is 1,500 mm per year and mean temperature is 24°C. Relative humidity always exceeds 80% (village of Mboko, Republic of Congo, 1995) (17).

Fauna

The large-animal fauna includes Loxodonta africana (Elephant), Syncerus caffer (Buffalo), Tragelaphus sp. (Situntinga), Cephalophus sp. (Duiker), Hylochoerus meinertzhagim (Giant Forest Hog), Potamochoerus porcus (Red River Hog), Gorilla gorilla, Pan troglodytes (Chimpanzee), Cercopithecus sp. (Guenon), Cercocebus sp. (Mangabey), Colobus sp., Panthera pardus (Leopard), Nandinia (Two-spotted Palm Civet), Civettidis civetta (African Civet), Genetta servalina (Genet), mongoose sp., Orycteropus afer (Antbear), Manis sp. (Pangolin), Atherurus africanus, Thryonomyss swinderianus, and Python sebae (17,18).

Carcass Detection

Local hunters (primarily adult and adolescent men of the Bakota, Bakola, Mboko, Mongom, and Pygmy tribes) were the main sources of information regarding the location of carcasses. Their reported sightings were confirmed by ECOFAC monitoring teams who recorded both the global positioning system (GPS) position on a Cyber Tracker field computer (available from http://www.cybertracker.co.za/) and carcass status before alerting VHFC.

Sampling Team and Methods

When wild animal carcasses were found, VHFC asked CIRMF to send a team to the site for diagnostic purposes. Sampling permits were granted by the Gabonese and Congolese Ministries of Forestry and Environment and Health. Owing to the isolated nature of the outbreak zone
and its distance from CIRMF, a base camp was established nearby. GPS location of the carcasses, and the information provided on their state of decomposition, allowed the autopsy team to sample only the freshest carcasses.

Wild animal Carcass Sampling

Ideally, the carcass sampling teams comprised a minimum of 5 persons (3 porters and 2 persons to perform the autopsy). One of the porters was charged with disinfection procedures. Digital photographs were taken. Necropsy was performed with high-level precautions, including water-tight clothes (Pro-Tech “C,” Tyvek, Contern, Luxembourg) equipped with air filtration equipment and Proflow Automask Litehood face shields (Delta Protection, Lyon, France) (Figure 2), and disposable lancets and forceps. A 2% chlorine spray was used to disinfect reusable equipment (masks and filtration apparatus), as well as the autopsy site and carcass remnants. Hermetic 60-L containers equipped with safety tops were used to transport reusable equipment and waste. Waste was returned to the main camp for incineration.

The nature of the samples taken depended on the state of the carcasses. When the carcasses were in good condition, 0.5-cm³ specimens of liver, spleen, muscle, and skin were taken. Half of the samples were placed in Nunc CryoTube vials (Nalge International, Rochester, New York, USA), which were placed in a small liquid nitrogen dry-shipper container (5.4 L) for cryopreservation (−196°C). The other samples were placed in Nunc CryoTube vials containing 10% formalin, for immunohistochemical testing. Bones were placed in hermetic containers. At the main camp, the dry-shipper contents were transferred into larger dry-shipper containers (20.3 L), which were then forwarded to the CIRMF laboratory at the end of the mission.

Laboratory Studies

Sample Preparation

Potentially infected specimens were collected and manipulated according to WHO guidelines on viral hemorrhagic fever agents in Africa (19). Muscle and skin tissue were fragmented and homogenized in phosphate-buffered saline, and the final supernatant was filtered for antigen detection and RNA amplification. Bones were cut, and internal tissue was scraped. Bone marrow or internal bone tissue was prepared in the same way as muscle and skin.

Testing

Muscle and skin tissue samples were tested by polymerase chain reaction (PCR), antigen detection, and, in some cases, immunohistochemical staining. Bone marrow and internal bone tissue were tested by PCR only.

Antigen Detection

Samples were used for antigen detection as previously described (20). Briefly, Maxisorp (Nalge International) plates were coated with a cocktail of 7 monoclonal antibodies against Ebola virus Zaire antigens; control plates were coated with normal mouse ascitic fluid produced from a parent myeloma cell line. Sample extracts (see above) were then added to the wells, followed by hyper-immune rabbit Ebola polyvalent antiserum and then peroxidase-conjugated goat antibodies against rabbit immunoglobulin G (IgG). The TMB detector system (Dynex Technologies, Issy-les Molineaux, France) was used to measure optical density.

DNA Amplification

For the detection of viral mRNA, total RNA was isolated from sample extracts by using the RNeasy kit (Qiagen, Hilden, Germany), and cDNA was synthesized from mRNA as previously described (21). Two pairs of degenerate primers corresponding to the L-gene of Ebola virus were used for 2 rounds of amplification, yielding a 298-bp fragment (5′-TATMGRAATTTTCTYTTYTCATT -3′ and 5′-ATGTGGTGGGYTATAAWARTCACTRACAT-3′ for primary PCR; 5′-GCWAACGCTTYCCWAGYAAYATGATGG-3′ and 5′-ATAAWTCACTR ACATGCA-TATAACA-3′ for nested PCR).

Immunohistochemical Staining

Formalin-fixed specimens were sent to the Centers for Disease Control and Prevention (Atlanta, Georgia, USA) for immunohistochemical staining as previously described (22).
Results

Human Outbreaks

From October 2001 to December 2003, 5 human Ebola virus outbreaks of the Zaire subtype occurred in the area straddling the border between Gabon (northeast) and Republic of Congo (northwest), with 313 cases and 264 deaths (23,24). The first outbreak occurred from October 2001 to May 2002, with a total of 92 cases and 70 deaths in Gabon and Republic of Congo. Epidemiologic investigations showed that at least 2 duikers, 2 chimpanzees, and 2 gorilla carcasses were involved or suspected of being involved in the infection of 6 human index patients. A second human outbreak began in January 2002 and ended in June 2002 in Entiasia Republic of Congo, with a total of 30 cases and 25 deaths. One gorilla and 1 duiker were suspected of involvement in 2 human index cases. A third outbreak occurred from May to June 2002 in Oloba Republic of Congo, with 13 cases and 12 deaths. A chimpanzee was shown to have infected the human index patient. The fourth outbreak occurred from December 2002 to April 2003 in Mbomo and Kelle, Republic of Congo, with 143 cases and 128 deaths. Gorillas and duikers were suspected of infecting 3 human index patients. The last outbreak occurred from November 2003 to December 2003 in Mbanza and Mbomo, Republic of Congo, with 35 cases and 29 deaths. The source of infection of the human index patient was not clearly identified.

Carcasses

From August 2001 to June 2003, a total of 98 animal carcasses were found in an area of about 20,000 km² (Figure 3). Carcasses of 3 principal species were recovered: 65 great apes (50 gorillas and 15 chimpanzees) and 14 duikers (Figure 3). Only 6% of carcasses sampled were in good condition (entire body); 57% were in poor condition (partial carcasses with muscles or skin); and 38% were in bad condition (bones only). Two peaks of animal deaths were observed (Figure 4). The first occurred in the Ekata region (Gabon) from November to December 2001, with 51 carcasses, including 30 great apes and 8 duikers. The second occurred from December 2002 to February 2003 in the Lossi gorilla sanctuary (Republic of Congo), with 20 carcasses, including 17 great apes, 2 duikers, and 1 Cercopithecus cephus.

Laboratory Findings

An animal carcass was considered infected by Ebola virus if ≥1 of the 3 laboratory tests (antigen detection, DNA amplification, and immunohistochemical staining) was positive. When possible, DNA amplification was confirmed by sequencing the PCR products. Twenty-one gorilla, chimpanzee, and duiker carcasses were sampled in the wild and analyzed in the CIRMF biosafety level 4 (BSL-4) laboratory. Fourteen of these carcasses tested positive for Ebola virus, 6 in 2 or 3 tests and 8 in only 1 test (Table). Eight positive samples were muscles, and 6 were bones or bone marrow. All the muscle and skin tissue samples were tested by both PCR and antigen detection. In total, 10 gorillas, 3 chimpanzees, and 1 duiker tested positive. All the relatively well-preserved gorilla and chimpanzee carcasses tested positive. In contrast, well-preserved samples taken from carcasses of C. cephus, Genetta sp., and Tragelaphus sp. were negative.

Discussion

We describe the successful implementation of a surveillance network of Ebola outbreaks in wild large

![Figure 3. Species distribution of carcasses found in the forest straddling the border between Gabon and Republic of Congo (2001–2003). * = other primates: Cercopithecus sp.; † = other species: Atherurus africanus (1), Genetta sp (3), Loxodonta africana (1), Manis sp. (1), Mongoose sp. (1), Thryonomys swinde- rianus (2), Tragelaphus sp. (1), Python sebae (2), and bird of prey (1).](cid:1)

![Figure 4. Temporal distribution of carcasses found in the forest straddling the border between Gabon and the Republic of Congo (2001–2003). Two peaks of mortality were observed: the first occurred in the Ekata region (Gabon) from November to December 2001 and the second from December 2002 to February 2003 in the Lossi gorilla sanctuary (Republic of Congo).](cid:2)
mammals. We often identified wild animal outbreaks before human Ebola outbreaks. Twice this enabled us to alert the health authorities of Republic of Congo and Gabon to an imminent risk for human outbreaks, after the discovery of carcasses of Ebola virus–infected animals. 

Human Ebola outbreaks in this region have always occurred in remote areas, raising major logistic problems. Roads are often barely passable, and means of communication are frequently nonexistent. The carcass detection and investigation network therefore had to rely on teams already present in these forest zones, and notably those possessing radios or satellite telephones. Conservation organizations such as ECOFAC, WCS, and WWF were thus the ideal partners. ECOFAC monitoring teams played a critical role by exploring remote forest zones, capitalizing on the information provided by villagers and hunters.

Performing an autopsy on high-risk animal carcasses requires heavy equipment, highly qualified personnel, and experienced veterinarians, as illustrated by the case of the Swiss anthropologist who was infected after examining a chimpanzee carcass without adequate protective measures in the Tai forest (13). Carcasses decompose very rapidly in the equatorial forest: an adult male gorilla carcass (≈150 kg) takes only 10 days to decompose entirely, i.e., be reduced to a heap of bones and hair (Figure 5). Carcasses observed 3–4 days after death bear few signs of scavenger activity but are covered with fly eggs and maggots. Maggots consume the entire flesh within 5 to 10 days, while scavengers (mainly mongoose) take pieces and disseminate them around the site. Thus, after ≈3 weeks, only a few bones bearing small-mammal gnaw marks remain.

Although the PCR technique used by CIRMF can detect Ebola virus genetic material in carcasses 3–4 weeks old, the material is often degraded and incomplete. Often, only a small sequence of the L-gene (RNA polymerase) can be analyzed, and this cannot be used for strain identification. Furthermore, degraded samples increase the false-negative rate (25). Rapid sampling is therefore crucial for successful diagnosis, and the availability of a small aeroplane was particularly helpful in certain cases. The presence of the CIRMF BSL4 laboratory relatively close to the outbreak area was a considerable advantage.

Using a combination of 3 laboratory techniques (PCR, immunohistochemical staining, and antigen capture), we...
showed for the first time that wild gorillas and chimpanzees can be decimated by Ebola. Bones of a *Cephalophus dorsalis* carcass also tested positive for Ebola virus by reverse transcription (RT)-PCR, indicating that a third wild species may be naturally susceptible. In Africa, only chimpanzees had previously been diagnosed as positive for Ebola virus, by immunohistochemical testing, in the Tai forest of Côte d’Ivoire, and were considered the cause of the human outbreak in Mayibout (Gabon) (12,14,15). The large number of carcasses found in this region, together with the results of animal population censuses conducted in the Lossi reserve before and after outbreaks, indicates that great apes are affected massively and duikers to a lesser extent (16,26). The lowland gorilla population density in this region (≤6 times as high as the chimpanzee population density) is among the highest in the world (≤10 gorillas/km²) (27), which likely explains why more gorilla carcasses than chimpanzee carcasses were found. High population density can amplify outbreaks but cannot alone explain their severity. Small monkeys, although abundant in this area, do not seem to be affected. Only 1 carcass of *Cercopithecus cephus* was found; it was in good condition but was negative by RT-PCR and antigen capture (Table). Some *Potamochoerus porcus* carcasses were reported by hunters but none could be sampled. Carcasses of large animals are more likely to be found than those of small animals, because the time taken for a carcass to decompose depends on its size.

The source of gorilla infection is unknown, but several lines of evidence point to direct infection by ≥1 natural hosts. First, the detection of different strains of Ebola virus in gorilla carcasses located only a few kilometers apart argues against a major role of gorilla-to-gorilla transmission. Indeed, Ebola virus remains genetically stable during a given outbreak, from the first to the last case (28,29), whereas we obtained 4 different glycoprotein sequences (E.M. Leroy, P. Rouquet, unpub. data) from samples of gorillas and chimps located in the Lossi sanctuary. The large distance separating positive carcasses found during a short period, and the existence of physical barriers such as roads and rivers, also supports direct transmission from a natural host. Finally, the occurrence of simultaneous outbreaks in 2 or 3 different species that display little interspecies contact (30) provides further evidence that gorillas and chimpanzees are directly infected by ≥1 natural hosts. However, cases of gorilla-to-gorilla transmission cannot be ruled out, especially within a given group. Indeed, 5 gorilla carcasses belonging to the same group were found in a close area in the Lossi sanctuary. Ebola outbreaks in gorilla groups may result in their rapid dissolution, especially if the dominant male is rapidly affected, which forces possibly infected females to integrate into another group. However, this type of intergroup transmission appears to be marginal.

Chimpanzees are probably infected by the same mechanisms as gorillas. During the Tai outbreak in Côte d’Ivoire, carnivorous behavior (especially consumption of *Colobus* monkeys) was the suspected source of infection (12), but this notion is challenged by the infection of gorillas, which are almost exclusively herbivorous. However, chimpanzees are considered to be the primate species whose behavior (mainly fighting, social grooming, sexual activities, and predation) carry the highest risk for both intra- and interspecies pathogen transmission (30). This idea is supported by the detection of the infected carcasses of a mother and her 1-year-old offspring. Repeated contact between young individuals and their mothers is known to be a significant risk factor for Ebola virus transmission (2,6).

Duikers represent a special case. Although they are the most common large-mammal species in this region, few carcasses were found. This circumstance may be due to the lack of interactions among individuals, as duikers generally live alone or in pairs. Some duikers, despite being herbivorous, eat the flesh of decomposing carcasses (K. Abernethy, unpub. data). Thus, in addition to being directly infected by the natural host(s), duikers might also become infected by licking or eating fresh carcasses of
Ebola virus–infected animals. This scenario would play a marginal role, however, because carcasses are only infectious for 3 or 4 days after the animal’s death (E.M. Leroy, P. Rollin, unpub. data). Furthermore, we observed little scavenging of carcasses during the first days after the animal’s death.

Serum from a survivor of the human outbreak in Mekambo (Grand Etoumbi, March 2002), who had direct contact with a gorilla carcass, was positive for Ebola virus–specific IgG. Ebola virus L gene sequences were detected in bone marrow samples of this gorilla, conclusively linking the 2 cases. Thus, the last outbreaks in Mekambo (Gabon, 2001) and Lossi (Republic of Congo, 2002–2003) confirm that wild animal mortality can reveal Ebola virus propagation in the forest ecosystem and indicate a role of wild animals as “vectors” in human outbreaks.

No effective medical treatment or vaccine exists for Ebola virus infection. The only way of minimizing human cases is to break the chain of human-human transmission. Humans do not seem to be at a major risk for infection by the unidentified natural host(s). Large outbreaks among wild animals can amplify human outbreaks by increasing the number of index transmission events. Therefore, reducing contacts between humans and dead wildlife can reduce the risks for transmission.

Epidemiologic surveillance of animal mortality rates can thus help prevent the emergence of the disease in human populations (Figure 6). At the time of the Kéllé (Republic of Congo) outbreak, our network detected infected gorilla carcasses (Lossi, December 6, 2002) 3 weeks before the disease emerged in humans (December 25, 2002), showing active Ebola virus propagation in this area. We were thus able to warn health authorities of an imminent human outbreak in the region. Nonetheless, a human outbreak occurred. In June 2003, we issued a new alert on a risk for human outbreaks after the discovery of an infected gorilla carcass near the village of Mbanza (Republic of Congo). An outbreak occurred in this village in November 2003. These failures suggest that human and animal health authorities need to work together more closely. In the future, health authorities need to educate local populations on the risk for infection through contact with carcasses at all times. During expected disease outbreaks, health authorities need to be able to respond immediately by sending teams to affected areas (24). The early successes of the network in this area warrant its extension to all countries with known outbreaks of hemorrhagic fevers. The participation of new frontline partners, such as foresters, would be invaluable to expend logistical existing capacity provided largely by field conservationists. Finally, as the capacity of such a system to react rapidly is crucial for its success, sampling teams should be created to collect material and obtain virologic testing results with a minimum of delay in other countries harboring hemorrhagic viruses. An efficient animal mortality monitoring network backed up by a rapid reaction system would allow public health authorities to predict and possibly prevent human Ebola outbreaks.

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Dr. Rouquet is the head of the Primate Center at CIRMF. He is experienced in the treatment and evaluation of simian immunodeficiency virus and simian/HIV transmission in different primate models of HIV infection (pathogenic and nonpathogenic). Since 1995, he has been involved in hemorrhagic fever research, particularly Ebola.

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Address for correspondence: Pierre Rouquet, Centre International de Recherches Méridicales de Franceville, (CIRMF) BP 769, Franceville, Gabon; fax: (33) 153013602; email: p.rouquet@cirmf.org

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.
We reviewed historical data from 2 smallpox outbreaks in Liverpool and Edinburgh during the early and middle years of the 20th century to assess their contribution to developing modern strategies for response to a deliberate release of smallpox virus. Reports contemporaneous to these outbreaks provide detail on the effectiveness of public health interventions. In both outbreaks, extensive contact tracing, quarantine, and staged vaccination campaigns were initiated, and the outbreaks were controlled within 15 months and 3 months, respectively. In Edinburgh, the number of fatalities associated with vaccination exceeded the number of deaths from the disease. In Liverpool, ambulatory, vaccine-modified cases and misdiagnosis as chickenpox resulted in problems with outbreak control. The relatively slow spread of smallpox, as exemplified by the report from Liverpool, allowed for effective implementation of targeted intervention methods. Targeted surveillance and containment interventions have been successful in the past and should be explored as alternatives to mass vaccination.

Heightened awareness of the potential threat of biologic terrorism has generated debate over the most appropriate modeling strategies to assist in planning public health interventions and the required relevant data and assumptions for model parameterization (1). A fundamental issue for modeling the potential impact of a deliberate release of smallpox virus is the dearth of recent data. For these reasons, the impact of a bioterrorist release upon a modern population and of the subsequent attempts to contain it are difficult to predict with precision. The dynamics of disease outbreaks in the 21st century, and the outcomes of control strategies used to contain them, have been predicted by using models parameterized with contemporary outbreak data (e.g., measles immunization campaigns). However, to obtain a better idea of how an eradicated disease, such as smallpox, might be controlled requires an analysis of historical outbreak data, much as has been done in a number of studies (2–5).

Inherent problems are associated with extrapolating past data to the modern day, such as possible differences in susceptibility to infection between modern and historical populations (e.g., immunity) and also potential differences in risk for disease transmission (e.g., changes in contact patterns) (1). Nonetheless, when these factors can be addressed properly, the advantages of using historical data as a foundation for modern assessments far outweigh the disadvantages. For smallpox particularly, epidemiologic and outbreak data from the past have been largely relied upon to provide insight into, and evaluation of, the efficacy and efficiency of different public health control strategies for a potential bioterrorist attack.

For example, the levels of protection afforded today by smallpox vaccinations carried out many years ago are difficult to calculate, since few relevant recent assessments exist. A recent study reported stable antiviral antibody and slowly declining antiviral T-cell responses to vaccinia virus in volunteers 1–75 years after vaccination (6). How these longer lasting responses correlate with protection from infection itself, from more serious disease, or from death, remains difficult to determine. Natural exposure to the organism is the only way to know whether this response correlates to full (i.e., no disease), or partial (i.e., fewer deaths) protection from smallpox. Since data on natural exposure to smallpox virus are not available for contemporary populations, analysis of historical data is likely to provide the most convincing evidence (3).

Historical data on this and other aspects of disease control were published in the early 1900s after a variola major virus outbreak in Liverpool (1902–1903) (7) and in the mid-1940s after an outbreak in Edinburgh in 1942 (8) (document available from http://www.cdc.gov/ncidod/eid/vol11no2/04-0609.htm_app). These reports form the basis of this article, which discusses the use of historical data in predictive assessments of disease events. The Liverpool smallpox outbreak data are included in a large section specifically on smallpox in the annual Health Department
Report for the city written by the Medical Officer of Health in 1903, at a time when smallpox was still endemic in Liverpool. The report covers all aspects related to health, ranging from typhus and tuberculosis to rainfall, temperature, and demographic statistics. Supplementary information on this outbreak has also been taken from Appendix 10 of the Annual Report of the Medical Officer of The Local Government Board 1904-05, in Report on Smallpox and Smallpox Hospitals at Liverpool, 1902–03, which investigated specific aspects of the outbreak for the local government board (9). A further report has also been used, written in 1913 by the assistant medical officer of health for Liverpool; it reports in greater detail on the effects of the disease in relation to the impact of vaccination and includes a large series of cases from the 1902–1903 outbreak (10).

The report on the 1942 outbreak in Edinburgh also provides data on a range of important aspects of smallpox control, including adverse events to vaccination (8). This large report was written in 1944 by the medical officer of health and his colleagues at a time when smallpox was no longer endemic in the region. The stated purpose of the report was to provide information for medical staff in the event of future outbreaks. The information detailed, therefore, is more descriptive than that in the Liverpool publication but provides more data on the clinical and control aspects used. Again, supplementary articles have been consulted, primarily those concerning the contemporaneous outbreaks in Glasgow and Fife that led up to the Edinburgh outbreak. A close evaluation of the 2 outbreaks illustrates the value of using historical data when considering public health control and containment strategies for potential bioterrorist events.

Outbreaks

Since the 1860s, Liverpool had had cases of smallpox (7). According to the 1904–1905 report, seaports were prone to occurrences of smallpox, and therefore, Liverpool had “abundant opportunities of perfecting its administration in regard of this disease” (9). Although the annual number of cases had declined considerably in the 17 years or so before the outbreak began in 1902, a total of 23 cases were imported by sea and 16 were introduced by “vagrants.” However, according to 1 researcher, an epidemic broke out toward the end of 1902 (10). The outbreak lasted from October 1902 to the end of December 1903 and resulted in 2,032 cases and 161 deaths (case-fatality rate = 8%). The first smallpox case occurred in 1901 and resulted directly from an imported case-patient, a merchant seaman. This importation brought the disease into Liverpool at the end of 1901, a year in which, until that time, practically no smallpox had been reported (9). The administrative actions of the Public Health Department of Liverpool checked the spread of smallpox until November 1902, when an unrecognized case-patient (7), an infant, was medically attended only when the child was dying of the disease. In addition, 6 infected household members were found, and subsequent house-to-house inquiries in the district discovered another 20 clinical case-patients during the next few days, most of whom were friends of the infected family (7). This number of cases is assumed to have resulted from chains of transmission beginning with the infant and spreading through the family and to wider contacts, rather than transmission from the child directly to 26 others. Despite attempts to prevent further spread, the number of cases in the locality reached 99 by the end of January 1903. The disease then continued to spread to the east and south of the city, with the monthly number of cases peaking at 356 in March 1903. The timeline of the outbreak in relation to a number of other key events and control measures is shown in Figure 1.

Similarly, until 1905, Edinburgh had also seldom been free from smallpox. Later, however, smallpox outbreaks became infrequent, with only 4 outbreak years from 1905 to 1920, and then none at all in the 20 years before 1942. The outbreak, therefore, was a relatively new experience for a large section of the population (11). This outbreak was relatively small and lasted 3 months (October 27–December 30, 1942), which resulted in 36 cases including 8 deaths (case-fatality rate = 22%). Smallpox had previously been imported into Scotland on May 29, 1942, by a ship arriving from Bombay into Scotland’s other major city, Glasgow (resulting in 36 cases and 8 deaths) (12). In August, 3 weeks after the last case in Glasgow, an outbreak was reported in Fife (29 cases and 8 deaths). As the outbreak in Fife was being brought under control, the first case of smallpox appeared in Edinburgh Royal Infirmary. The disease then spread to the hospital’s convalescent home and then into the general public. The
means of the spread of disease from Glasgow to Fife and then to Edinburgh, and from hospital settings to the general public, was, however, never identified. Indeed, for 8 of 13 Edinburgh community cases, the source of infection was never discovered. The author of the outbreak report conjectured that subclinical infections, i.e., “mild attacks” or missed cases, might have been the reason for the lost epidemiologic links but adds that these theories were hard to reconcile with the facts (8). A timeline of the Edinburgh outbreak, highlighting the milestone events and control measures employed, is shown in Figure 2.

Vaccination Status of Population

To appreciate the course of the outbreaks and the subsequent effects of the various control measures used, the vaccination history of the populations involved should be put into context. If we assumed that the level of infant vaccination in Liverpool was similar to that for England and Wales as a whole in 1902 and 1903 (≈75%) when solid immunity in the city might have ranged from 9% to 16% (solid immunity, as termed by Dixon [13], is either 5 or 10 years of total protection from attack). However, at that time, considerable support for the antivaccination cause resulted in an infant vaccination rate in the late 19th century that varied from 0% in some districts to nearly 100% in others (13), and as the background rates for Liverpool are not reported, being more specific about the levels of vaccination that existed is difficult.

Vaccination levels also varied from region to region in Scotland. The percentage of vaccinated infants in Scotland was normally ≈30.7% (14); however, the Registrar General for Scotland reported that 55% of infants were being vaccinated in 1941. Whether this report is for Scotland as a whole or for Edinburgh alone is not clear (8). Dixon’s estimate of solid immunity for the whole of England and Wales in 1947, assuming 40% of infants were vaccinated, was 4%–7%. However, this percentage was increased by the vaccination of National Service entrants to ≈20%. For Scotland, with an infant vaccination rate of ≈30%, solid immunity would have been <20% (13). The vaccinal state of the public as a whole was reportedly low in the area around Fife (Methilhill), with only 20%–30% of the population having been previously vaccinated; but in towns nearer Edinburgh (e.g., Cowdenbeath) 40%–50% had been vaccinated (14).

Public Health Response

In both Liverpool and Edinburgh, phased public health responses were implemented (Figures 1 and 2). In Liverpool, at the earliest phase of the outbreak, with the discovery of the first unreported case in Robsart Street (Figures 1 and 3), active case finding in the local area was instituted. One report states that, thereafter, usually within an hour of notification, patients were removed to hospital by ambulance, and the clothing, bedding, and dwellings were immediately disinfected (9). An inspector followed the ambulance and immediately made inquiries about possible sources of infection. Information about the state of vaccination of possible contacts was then sent to vaccination officers; additional medical staff members were employed at this time to assist with vaccination. These vaccination officers in Liverpool first recommended immediate vaccination or revaccination to all close contacts of case-patients, then to related workforces, schools, and the general public. Special arrangements were made for the prompt vaccination of all vagrants coming into the city, who were subsequently paid a small sum for consenting. Offers of vaccination and revaccination to contacts...
and people living close to persons with smallpox were reported in the Health Department Report to have been promptly made and almost universally accepted; these vaccinations were reported to have greatly limited the amount of smallpox in Liverpool. As the number of cases increased as the outbreak developed, hospital isolation accommodations were expanded by committing an increasing number of hospitals to the intake of smallpox patients (Figure 1).

In Edinburgh, after notification of the first Glasgow case on May 29 (Figure 2), the first campaign of vaccination and revaccination for essential personnel (e.g., medical staff, civil defense workers, and police), was agreed to on June 30 and promptly instituted on July 1, 4 months before the disease reached Edinburgh (11). Edinburgh had not had a smallpox case for 20 years, but at this time, the smallpox hospital was reopened, and satellite isolation units in the hospital grounds prepared to receive patients for observation. All contacts of Glasgow case-patients arriving in Edinburgh were, as was routine practice, examined and put under surveillance. The Public Health Department was responsible for the medical supervision of contacts, and medical officers of health were responsible for requesting precautionary behavior in the general public. The second vaccination campaign took place from November to December 1942, when the disease had taken hold in Edinburgh itself. At this time the contacts of patients were vaccinated, and vaccination was extended subsequently to the general public with the opening, on November 8 (Figure 2), of 22 vaccination centers throughout the city.

Despite the previous vaccination of infants and other target groups, levels of immunity contemporaneous with these 2 outbreaks were insufficient on their own to prevent expanding outbreaks. Nevertheless, the spread of infection over both space and time across Liverpool was characteristically slow, taking 3 months to significantly extend out of the district into which it was introduced, to more southeastern districts (Figures 1 and 3).

The first 2 cases in Edinburgh were diagnosed on October 31. On November 1, active case-finding was initiated with house-to-house searches, and a first aid post was opened subsequently, which provided 8,000 vaccinations to people in the area in which these patients lived. Family contacts of patients were sent for observation to a prepared reception house, which was opened on November 2, the day after the first 2 cases had been confirmed (Figure 2). Persons in the reception house were quarantined for 21 days, and all but 1 of their employers agreed to pay their wages during this time (8). The exception to this rule attended work during the day and returned to the reception house at night and was examined both upon leaving and on returning for signs of infection. The Royal Infirmary convalescent home also acted as an additional observation ward.

Contact tracing was an important part of the control methods instituted in both outbreaks. In Edinburgh, the press was used extensively as a means to trace contacts of case-patients and to persuade large numbers of persons to accept vaccination; the use of the press also allowed the authorities to reach possible contacts with a minimum of delay (11). In all, 1,700 contacts of the 36 cases were traced and observed for 18 to 21 days, which represents an average of 47 contacts per case. More than 900 persons were traced as contacts and revaccinated from 3 cases alone. Despite being infected, these ambulatory cases had used public transport or been in contact with large numbers of persons because of their occupation (8). The readiness of the public to cooperate with all the above recommended, routine precautions is noted in the Annual Report, 1942 (11). The press was also used in the Liverpool outbreak. Circulars that detailed the movements of case-patients who had used public transport and the location and availability of public vaccinators were widely distributed. Although the total number of traced contacts is unclear, we know that contacts were visited every day for 14 days after notification, and then every few days for a further 2 weeks. At the peak of the outbreak, when 356 cases existed, as many as 2,000 families were being visited daily, which represents an average of 6 families contacted per case. On the basis of an average household size for England, at that time, we have a rough estimate of 30 contacts traced and vaccinated per case.

In the Liverpool outbreak, the occurrence of a large number of vaccine-modified cases caused particular problems for those attempting to control the outbreak, especially with respect to late or incorrect diagnoses. According to Hanna (10), 72.7% of those vaccinated previously and 16.8% of unvaccinated cases were considered to be “modified discrete and discrete smallpox” (modified here meaning an accelerated clinical course compared with the expected course of ordinary smallpox, usually with fewer lesions, not necessarily modified by vaccination) (13). Chickenpox was a notable misdiagnosis in some instances; 2.6% of chickenpox diagnoses were found subsequently to be smallpox (similarly, in the 1901–1902 London outbreak, the figure for the same misdiagnosis was 2.5%). To help overcome this problem, chickenpox was made a notifiable disease, provisionally in April, and permanently in August 1902. Reporting of smallpox itself, however, was not always straightforward in the Liverpool outbreak. According to 1 author (9), the diagnosis of smallpox was sometimes revoked upon admission to hospital, or vice versa, a nonsmallpox case-patient was often treated as having smallpox in the hospital. On at least 1 occasion, information on patients treated in the hospital did not reach the medical officer of health. In Edinburgh, the first 2 cases
were misdiagnosed as chickenpox and meningococcal septicemia, respectively. Misdiagnosis as chickenpox is a concern that continues to exist today. In Glasgow, in 1942, severe vaccinial reactions, occurring at the end of the possible incubation period of smallpox, also complicated the problem of diagnosis for medical practitioners (12).

As with the 3 ambulatory case-patients in the Edinburgh outbreak discussed above, such patients were also a problematic source of infection in Liverpool (7,10). Some smallpox infections were reported to be so mild (usually vaccine-modified) that doctors were not consulted, and patients and their household contacts continued to visit public areas and shops. For example, 1 unreported smallpox case occurred in a person whose family continued to go to work and socialize, which gave rise to 29 other cases. Twenty prosecutions were instituted against members of this family during the outbreak. The extent to which ambulatory vaccine-modified cases might occur in any modern day U.K. outbreak is not known. However, the proportion of vaccine-modified cases overall would be much less than in Liverpool because of the length of time since the population was last vaccinated. This finding has been discussed in greater detail elsewhere (2).

Previous vaccination status also strongly influenced the relationship between age at time of attack and death (Figure 4). In a study that examined a series of 1,163 case-patients during the 10 years after the Liverpool outbreak (mostly from the epidemic period 1902–1903), 943 (81%) had been vaccinated in infancy, and 220 (18.9%) had not been vaccinated (10). Among those vaccinated in infancy, 28 (2.9%) deaths occurred from smallpox, whereas among the unvaccinated, 60 (27.2%) deaths occurred. The case mortality among the vaccinated rose steadily with age from the 20- to 30-year age group upwards to the ≥60-year group (no deaths occurred in those <20), but never exceeded 10%. However, among the unvaccinated, 58% of patients <2 years of age died, decreasing to 30.6% for those 2 to 5 years of age. The ratio was lower (3.2%) for those 10 to 15 years of age; the case-fatality rate rose (13%) for those 15 year of age, and it was 50% for those ≥40 years of age. The effect of vaccination on protection against death according to age has also been noted by others (15,16). The level of partial immunity to smallpox, i.e., protection from death as opposed to protection from infection, in a modern population may be higher than previously thought (1); spread of infection from ambulant patients with vaccine-modified cases may be an important and problematic means of transmission (10,13,17), as has been pointed out in more recent analyses (2). In the Edinburgh outbreak, 6 of the 8 deaths from smallpox occurred in adults ≥20 years of age who had been vaccinated in infancy.

No data concerning vaccine-related adverse events are available from the Liverpool outbreak, but we know that of the estimated 360,000 vaccinations (based on lymph issue) performed in Edinburgh and adjacent counties (=77% of the local population), 10 vaccine-related deaths occurred; 8 of these were from encephalomyelitis. Compared to vaccination campaigns in England and Wales in 1951 to 1960 (18), the numbers of postvaccinial encephalomyelitis and generalized vaccinia were much higher (Table 1). Indeed, a similarly high incidence of postvaccinial encephalomyelitis was reported during the Fife outbreak (14). Approximately 78% of vaccinees in and around Edinburgh had had a previously successful vaccination; the remainder, ≈22%, had either a previously unsuccessful vaccination or no vaccination at all. In neither of the outbreaks was an intensified national vaccination campaign reported to have been initiated.

**Discussion**

In both the Liverpool and Edinburgh outbreaks, phased public health responses were implemented, and the outbreaks were brought under control within 15 and 3 months, respectively. Because smallpox arrived first in Glasgow, Edinburgh health authorities had time to prepare and implement a 2-phased vaccination campaign along with active surveillance. For Liverpool, the report demonstrates clearly that the spread of infection across the city was slow, which suggests a relatively low transmission rate and a characteristically long generation time, allowing for targeted intervention methods to be effectively implemented. By comparing the incidence of cases in different districts across the panels shown in Figure 3, the outbreak appears to have taken 3 months (November 1902–January 1903) to spread into districts adjacent to the origin of the outbreak and then an additional 3 months (February–April 1903) to spread to more eastern and western districts. The slow spread of smallpox described here is not dissimilar to that described in studies in other countries, for example, Pakistan and Bangladesh in the
Table. Rates of adverse events to smallpox vaccination in Edinburgh, 1942*

<table>
<thead>
<tr>
<th>Adverse event</th>
<th>1st vaccination campaign Jun – Jul†</th>
<th>2nd vaccination campaign Nov – Dec†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonspecific rashes</td>
<td>20</td>
<td>12.5</td>
</tr>
<tr>
<td>Auto-inoculation and generalized vaccinia</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Postvaccinal encephalomyelitis</td>
<td>5.0</td>
<td>4.7</td>
</tr>
</tbody>
</table>

*Source: reference 8.
†Per 100,000.

1960s (17). In Bangladesh, smallpox tended to be more rapidly transmitted within family units but spread more slowly between them (19).

Active surveillance, vaccination of contacts, and prompt hospital isolation of patients were important aspects of disease control measures in both outbreaks. Indeed, the success of the surveillance-containment strategy in Liverpool, the basis of which has been discussed more recently elsewhere (20), was particularly noted by the observers of the time (7,10). Unlike the situation in the United Kingdom today, both Liverpool and Edinburgh had designated smallpox hospitals, either already open or ready to reopen, at the time of these outbreaks. These dedicated facilities must have contributed to infection control efforts. However, control and containment procedures in the 2 cities were hampered in both outbreaks to some extent by reintroduction of the disease from other areas, by patients with ambulant cases of mild infection (probably vaccine-modified), and by missed cases.

These 2 case studies draw attention to issues of current concern, not only to the potential impact of vaccine-modified cases mentioned above, but also to adverse events to vaccination, both of which might have an impact in a modern-day outbreak. However, in contrast to these 2 outbreaks, the fact that routine smallpox vaccination ceased in the West during the 1970s brings complications of its own. Persons <30 years of age have never received the vaccine and are immunologically naïve. This 30-year time gap since vaccination also has implications for the immune status of previous vaccinees and the potential for adverse effect and disease complications and indeed for the spread of disease among this population. If historical events are to be used as sources of evidence, and the data from them extrapolated to modern populations, they must be considered within the ethical and social context of today, by observing societal differences, expedited travel, waning immunity, and increased recognition of contraindications to vaccination. In particular, the number of people who are immunocompromised today continues to rise with the increase of HIV infection, chemotherapy, immunity disorders, and transplantations. So too has the number of people with atopic dermatitis; in the United Kingdom alone, 2.3% of the population is estimated to have this condition (21). However, cardiac adverse events to vaccination, such as myocarditis and pericarditis, were not reported in these 2 case studies, as has been seen in more recent vaccination efforts (22).

The studies also illustrate that the level of background solid immunity in these populations was low and could give rise to expanding outbreaks. The response to these outbreaks was not to implement a national vaccination campaign but rather a targeted approach, expanded when necessary. Although these data, based on the direct experience of infected populations, are not truly predictive for a modern smallpox outbreak (1), they are very instructive.

Analysis of the Edinburgh and Liverpool outbreaks suggests that outbreaks after deliberate release of smallpox virus may evolve over time. Therefore, sufficient opportunity exists for targeted enhanced surveillance measures to be put in place, for additional staff to be mobilized for an effective follow-up, and for a containment strategy to be implemented. The Liverpool outbreak took 15 months to control; the one in Edinburgh 3 months. This time difference probably reflects that reintroductions of smallpox occurred during the 1902–1903 outbreak because the disease was still endemic in the United Kingdom, poorer socioeconomic conditions existed in Liverpool at this time, and crowding was more prevalent, particularly in the dockland areas most heavily affected. By contrast in 1942, smallpox was no longer endemic in the United Kingdom, and socioeconomic conditions in Edinburgh were better. One might hope for at least as swift an end to a similarly sized modern-day outbreak as was seen in Edinburgh.

Modeling of data from other historical outbreaks of smallpox may help to further develop targeted surveillance and containment interventions for smallpox in the present era (3,23). Such interventions warrant further investigation because of clear, accumulating evidence of the substantial disease and death likely to accompany any mass population smallpox vaccination strategy.

Acknowledgments

We thank the archivist of the city of Edinburgh for permission to republish the following: Clark G, Seiter HE, Joe A, Gammie JL, Tait HP, Jack RP. The Edinburgh outbreak of smallpox, 1942. Authority of the Public Health Committee, Edinburgh, Scotland; 1944.

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Miss Kerrod is a risk assessment scientist in the Microbial Risk Assessment Department, Centre for Emergency Preparedness and Response, Health Protection Agency, Porton Down, Salisbury, UK. Her research interests include bioterrorism issues and the epidemiology of infectious diseases.
Smallpox Surveillance and Control Measures

References

Address for correspondence: Emma Kerrod, Centre for Emergency Preparedness and Response, Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG, UK; fax: +44-0-1980-612491; email: emma.kerrod@hpa.org.uk
In Vitro Host-Cell Susceptibility to Usutu Virus

Tamás Bakonyi,*† Helga Lussy,* Herbert Weissenböck,* Ákos Hornyák,†‡ and Norbert Nowotny*§†

We investigated the susceptibility to Usutu virus (Flavivirus) of 13 permanent cell lines, 3 primary cell cultures, and chicken embryos. Vero, PK-15, and goose embryo fibroblast cells developed cytopathic effects; however, viral multiplication was detected in all mammalian cell types by immunohistochemical tests. Chicken embryo fibroblast cells and chicken embryos were resistant.

Until its emergence in Austria in 2001 (1), Usutu virus was regarded as a flavivirus found only in sub-Saharan Africa. The virus was first isolated from Culex naevi in South Africa (2); later it was detected in other mosquito (Cx. perfuscus, Mansonoria africana, Coquillettidia auriis), bird (Turdis libonanus, Bycanistes fascilator), and rodent species (Praomys sp.) (3–5). Also, Usutu virus was isolated once from a man with fever and rash (3).

In Africa, Culex mosquitoes and birds are responsible for transmission and circulation of the virus in nature; however, the infection does not cause overt disease in the local host species. Since its introduction to Europe, Usutu virus has shown substantial pathogenicity for several wild bird species and causes severe die-offs, especially in the Eurasian blackbird (T. merula) populations. Recurring enzootics have been observed from mid-July to the end of September in the affected areas in the eastern part of Austria within the last 4 years (6).

Usutu virus is a member of the Japanese encephalitis virus (JEV) group within the mosquitoborne flaviviruses (7). The most important members of the group, West Nile virus (WNV), Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV), and JEV are able to infect a broad spectrum of animal species. These viruses are transmitted by different mosquito species and frequently cause infections in birds (all virus species), rodents (WNV, SLEV), swine (JEV), and horses (WNV, MVEV, SLEV). WNV, SLEV, JEV, and MVEV are human pathogens as well; they may cause epidemics of encephalitis in humans in certain geographic regions.

The classic manner of flavivirus cultivation is intracerebral inoculation of suckling mice or inoculation of embryonated eggs (8). A variety of primary cells and established cell lines support the replication of flaviviruses: Green monkey (Vero), hamster (BHK-21), human (SW-13, HeLa), porcine (PS), and mosquito cell lines, as well as primary chicken and duck embryo cells have been used for flavivirus isolation and propagation in routine diagnostic applications. The appearance of cytopathic effects (CPEs), plaque formation, and virus yields greatly vary with the different viruses and host cells.

Since Usutu virus was of minor clinical importance until its emergence in central Europe, its biologic features, host spectrum, and pathogenesis had not previously been studied. With the changes in the clinical appearance of Usutu virus infection in the new environment, and the impact of closely related viruses on human and veterinary health care, the detailed characterization of the virus is of high priority.

The Study

We investigated the in vitro susceptibility of various cell cultures and embryonated eggs to Usutu virus infection. Human (HeLa), green monkey (Vero), equine (ED), bovine (MDBK), porcine (PK-15), rabbit (RK-13), canine (MDCK, DK), feline (CR), hamster (BHK-21, BF), rat (C6), and turtle (TH1) permanent cell lines, as well as primary horse kidney (EqK), chicken embryo fibroblast (CEF), and goose embryo fibroblast (GGEF) cell cultures were tested. Cells were propagated in Earle’s minimal essential medium (MEM) (Gibco Invitrogen, Paisley, UK) containing L-glutamine, antimicrobial drugs, and 10% fetal calf serum (FCS). The cells were regularly subcultured by employing standard techniques. To 1-day-old confluent monolayers of the permanent cell lines and primary cell cultures, grown on the surface of chamber slides, the Austrian Usutu virus strain Vienna 2001-blackbird (GenBank accession no. AY453411) was added at a multiplicity of infection (MOI) of 3. The virus was originally isolated in Vero cells in 2001 from the brain homogenate of a blackbird found dead in the area surrounding Vienna. The isolate was propagated twice in Vero cells. The second virus passage was used for the experiments; 50% tissue culture infective dose (TCID50) was determined, and aliquots of the virus were stored frozen at –80°C until used. The virus was added to the cells, which were then incubated at 37°C for 1 h. Thereafter, the inoculum was removed, the cell cultures were washed once with phosphate-buffered saline (PBS), and MEM containing 2% FCS, L-

*University of Veterinary Medicine, Vienna, Austria; †Faculty of Veterinary Science, Budapest, Hungary; ‡Central Veterinary Institute, Budapest, Hungary; §United Arab Emirates University, Al Ain, United Arab Emirates

1This study will be presented at the International Conference on Emerging Infectious Diseases, February 26–March 1, 2005, Al Ain, United Arab Emirates.
glutamine, and antimicrobial drugs were added. For all cell types, controls were cultivated simultaneously and treated in the same way as the infected cultures with the exception that MEM was used for inoculation. All cell cultures were incubated at 37°C for 3 to 5 days; then the medium was removed and the monolayers were fixed with chilled (–20°C) acetone. The cells were stained with hematoxylin-eosin (HE) and examined microscopically. In parallel, immunohistochemical (IHC) testing was carried out on the cell cultures by using the avidin-biotin complex technique, with a polyclonal antiserum raised in mice against WNV antigens, for which cross-reactivity with Usutu virus had been demonstrated previously (1). The number of antigen-positive cells was evaluated microscopically and scored (see Table).

Embryonated chicken eggs (strain LSL White, which was derived from the strain White Leghorn), originating from a specified pathogen free (SPF) herd (VALO eggs, Lohmann, Cuxhaven, Germany), were injected into the allantoic sac with $6 \times 10^5$ TCID$_{50}$ of Usutu virus at the age of 10 days. The eggs were incubated together with mock-infected controls at 37.5°C for further 4 days and were checked daily by transillumination. On day 4 postinfection, the eggs were opened, and the embryos were fixed in 4% buffered formaldehyde solution. Histologic sections were made from paraffin-embedded organs of the embryos, and the slides were analyzed by light microscopy after HE and IHC staining, respectively, as described above.

Three to 4 days after inoculation, pronounced CPEs were observed in Usutu virus–infected Vero and PK-15 cell cultures as well as in GEF cells. The first foci of cell rounding and subsequent shrinkage of the cells were observed on day 2 or day 3 post infection, when groups of 4 to 8 cells, but also single cells, showed rounding and degeneration; within 1 day the affected cells lost their adherence to the bottom of the flask and floated in the medium. Within a further 2 days, 90%–100% of the cells exhibited CPE. Typical Usutu virus CPE is shown in HE-stained Vero cells in Figure 1. The mock-infected Vero, PK-15, and GEF control cell cultures did not show any CPE. The other investigated cell types inoculated with Usutu virus did not develop visible CPE within a period of 5 days, and they were also negative by microscopy after HE staining. However, by IHC with cross-reactive WNV-antiserum, focal virus multiplication was detected in all cell cultures, independent of animal species and tissue type, except chicken embryo fibroblast cells (Figure 2). The percentage of Usutu virus antigen–positive cells varied from $\approx 1\%$ (DK) to 50% (GEF) (Table). In the case of HeLa cells, different clones adapted to the propagation of human rhinoviruses (HeLa Rhino) and herpes simplex viruses (HeLa HSV), respectively, were also tested, but

<table>
<thead>
<tr>
<th>Cell line/culture</th>
<th>IHC result</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa (human)</td>
<td>++</td>
</tr>
<tr>
<td>Vero (simian)</td>
<td>++</td>
</tr>
<tr>
<td>ED (equine)</td>
<td>++</td>
</tr>
<tr>
<td>MDBK (bovine)</td>
<td>+</td>
</tr>
<tr>
<td>PK-15 (porcine)</td>
<td>++</td>
</tr>
<tr>
<td>RK-13 (lapin)</td>
<td>++</td>
</tr>
<tr>
<td>MDCK (canine)</td>
<td>++</td>
</tr>
<tr>
<td>DK (canine)</td>
<td>(+)</td>
</tr>
<tr>
<td>CR (feline)</td>
<td>+</td>
</tr>
<tr>
<td>BHK-21 (hamster)</td>
<td>+</td>
</tr>
<tr>
<td>BF (hamster)</td>
<td>+</td>
</tr>
<tr>
<td>C6 (rat)</td>
<td>+</td>
</tr>
<tr>
<td>TH1 (turtle)</td>
<td>++</td>
</tr>
<tr>
<td>EqK (equine)</td>
<td>++</td>
</tr>
<tr>
<td>CEF (chicken)</td>
<td>–</td>
</tr>
<tr>
<td>GEF (goose)</td>
<td>++</td>
</tr>
</tbody>
</table>

*IHC, immunohistochemical; scoring criteria: (+), 1%–5% positive cells; +, 6%–25% positive cells; ++, 26%–50% positive cells. Primary cell cultures are indicated in italics.*

![Figure 1. Cytopathic effect (CPE) of Vero cells caused by Usutu virus infection, 4 days postinfection (hematoxylin-eosin staining).](image-url)
they gave the same results as the commonly used (ATCC) HeLa cells by HE and IHC staining. The mock-infected control cell cultures were clearly negative in each case.

The Usutu virus–infected chicken embryos did not show any lesions when investigated by gross and histopathologic examination after 4 days of incubation and were negative by IHC as well. To rule out the slight possibility that the Usutu virus strain used for inoculation underwent a change in cell tropism during the 2 passages in Vero cells, CEF, Vero, PK-15, MDCK, and DK cells, as well as embryonated chicken eggs, were reinfected with the original Usutu virus isolate (before passaging); the results were identical to the results obtained with Usutu virus passaged twice before use.

Conclusions

The appearance of CPE in flavivirus-infected cell cultures depends on the virus and host cell type, as well as on MOI levels and incubation time employed (8). In many cases, the presence and multiplication of flaviviruses do not inhibit significantly the host cell macromolecular synthesis, resulting in noncytopathic persistent infections (9,10). Pathogenesis and virulence of flaviviruses are influenced in vivo by several virus- and host-dependent factors, including the role of defective interfering particles, viral receptors, neurovirulence, immune-response (e.g., antibody-dependent enhancement), and host resistance genes (8). Although some of these processes are not yet fully understood, the basic requisite of any pathogenic effect is the host susceptibility to the virus infection. This study demonstrates that Usutu virus can infect cell cultures of various tissue types derived from a wide variety of animal species, including cell lines of human origin. Since only Vero, PK-15, and GEF cells develop CPE after Usutu virus infection, these cell lines and cell culture are the most appropriate ones for diagnostic purposes (e.g., virus isolation, plaque reduction neutralization test). As demonstrated by IHC, considerable differences have been found in the susceptibility of the various cell lines and cultures to Usutu virus infection and in the extent of spread of the infection; even cell lines derived from the same animal species and organ varied significantly in their susceptibility to Usutu virus infection, e.g., MDCK cells strongly support Usutu virus multiplication, while DK cells are far less susceptible. Both of these cell lines, however, have been derived from dog kidneys. On the other hand, the differences between the 2 canine kidney cell lines might also be the consequence of different random mutations (e.g., in genes of the interferon or other innate defense systems) that allowed the cells to immortalize. Since in Austria, Usutu virus infects wild birds and causes high death rates, especially in blackbirds, one would think that birds are most susceptible hosts for the virus. Therefore, the finding that both the chicken embryo fibroblast monolayers and the chicken embryos are apparently resistant to Usutu virus infection was unexpected. Usutu virus, however, is not the only flavivirus with such contradiction in host spectrum. Ilheus virus, a South American mosquito-borne flavivirus

Figure 2. Demonstration of Usutu virus antigen 3 days postinfection. Immunohistochemical (IHC) tests were performed by using a polyclonal antibody to West Nile virus, which cross-reacts with Usutu virus. A) Vero control; B) Vero infected; C) CR (feline) control; D) CR infected; E) goose embryo fibroblast (GEF) control; F) GEF infected; A,B) bar = 50 mm; C–F) bar = 100 μm. IHC staining.
belonging to the Ntaya virus group (7), also naturally affects wild birds and produces plaques in primary rhesus kidney cells and various established cell lines (Vero, PS, BHK-21, and LLC-MK2), but not in avian cells (8). Preliminary results of our chicken experiments with Usutu virus also support that idea that the domestic chicken is resistant to the infection, even when young. Further investigations involving different bird and mammal species will be necessary to show the most important host species, natural reservoirs, and vectors of Usutu virus and to estimate its epidemiologic impact and possible threat to domesticated animals and to the human population.

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Dr. Bakonyi is lecturer of virology at the Faculty of Veterinary Science, Budapest, and also a guest researcher at the University of Veterinary Medicine, Vienna. He is interested in the molecular diagnosis and epidemiology of animal and human viruses.

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Address for correspondence: Norbert Nowotny, Zoonoses and Emerging Infections Group, Clinical Virology, Clinical Department of Diagnostic Imaging, Infectious Diseases and Clinical Pathology, University of Veterinary Medicine, Vienna, A-1210 Vienna, Austria; fax: 43-1-25077-2790; email: Norbert.Nowotny@vu-wien.ac.at
Bat Incidents at Children’s Camps, New York State, 1998–2002

Amy Robbins,* Millicent Eidson,* Mary Keegan,* Douglas Sackett,* and Brian Laniewicz*

From 1998 to 2002, a total of 298 bat incidents were reported at 109 children’s camps in New York; 1,429 campers and staff were involved, and 461 persons received rabies treatment. In 53.8% of the incidents, the bat was captured and samples tested negative for rabies virus, which resulted in 61.3% of persons not receiving rabies treatment.

**R**abies is a neurologic disease with close to a 100% case-fatality rate; once clinical signs appear, it is almost always untreatable (1). After a person is exposed to rabies, death can be prevented only if treatment, commonly referred to as postexposure prophylaxis (PEP), is initiated. PEP includes an initial dose of immune globulin and a series of 5 doses of rabies vaccine in a 1-month period. PEPs are costly in terms of money and time because of the 5 medical visits, particularly if the person must be transported elsewhere for the treatment. The New York State Department of Health (NYSDOH) has a unique program that requires that rabies exposures and treatments be reported. County expenses associated with authorized treatments in accordance with state and federal guidelines are then partially reimbursed (2).

Despite a large number of rabid animals in the United States (7,967 confirmed in 2002), rabies in humans is rare because of the availability of PEP; 31 cases were reported in the United States from 1990 to 2003 (3). Twenty-nine (94%) of the 31 cases were associated with bat rabies variants, and a bat bite could be definitively documented for only 3 of them (3). Four children in the United States (4–8) and 1 child in Quebec, Canada, died of bat-related rabies (9). The families of the children in the United States were unaware of the potential for rabies transmission from bats.

Children’s summer camps share habitats favored by bats and other wildlife; thus, children and camp staff may come into contact with bats that are either roosting in camp buildings or flying among camp facilities while foraging. A camp-related rabies death occurred in Alberta, Canada, in 1985 in a 25-year-old student who had been bitten and scratched by a bat and received no treatment (10).

Of the 3,827 bats tested by the NYSDOH Wadsworth Center’s Rabies Laboratory in 2002, 102 (2.6%) were rabid (11). Although the probability of an individual bat being rabid is relatively low, bats that can expose humans to rabies must be assumed rabid, when a definitive diagnosis of rabies cannot be made. In 1999, the federal Advisory Committee on Immunization Practices (ACIP) updated the national PEP recommendations to include incidents with bats in which there was a “reasonable probability that exposure has occurred” (12). These types of incidents include direct contact with a bat; a bite, scratch, or mucous membrane contact with bat saliva or nervous tissue; a sleeping person awakening to find a bat in the room; or an adult witnessing a bat in the room with a previously unattended child, or a mentally disabled or intoxicated person (12).

The Study

In 1998, the NYSDOH Zoonoses Program began an educational program to address the importance of bats in camp settings. This program was conducted in collaboration with the NYSDOH Center for Environmental Health (CEH), Bureau of Community Environmental Health and Food Protection (BCEHFP). NYSDOH offered training for all local and state health department camp inspectors responsible for inspecting camps before opening each season. Fact sheets on bats and bat-proofing camps and houses, bat capture kits, guidelines for managing bats, risk for rabies transmission (particularly in children’s camp settings), and guidance regarding human exposure to rabies and treatment decisions were provided. Starting in 1999, these materials included rabies awareness refrigerator magnets instructing people to contact health departments and not release bats when they are found in dwellings, and rabies awareness stickers for children to teach them not to touch bats (13). In 2003, ≈700 children’s camps received a videotape about keeping bats out of occupied dwellings and capturing bats for testing in exposure incidents.

Children’s camp operators are required by New York State Public Health Law to obtain a permit, and camps must undergo inspection by the local health department. Associated regulations require camp operators to report certain camper injuries and illnesses within 24 hours of occurrence. Beginning in 1998, bat incidents were reported to the NYSDOH’s Zoonoses Program and to BCEHFP. In 1999, the Children’s Camp Bat Exposure Incident Report form was developed to standardize the reports. Twenty-three different types of incidents could be reported, 13 of which were considered probable rabies exposures requiring consideration of PEP. The form was revised in 2000 to include additional information about the incidents, and in 2001 and 2002 the types of bat incidents reported were limited to the 13 types that require consideration of...
PEP if the bat is not tested and confirmed negative for rabies. These incidents include: bite; scratch; saliva or nervous tissue contact; direct physical contact with live or dead bat; person touched bat without seeing the part of bat touched; bat flew into person and touched person’s bare skin; bat flew into person and touched person’s lightweight clothing, and person reports feeling an unpleasant sensation at the point of contact; person with bare feet stepped on bat; person awakens to find a bat in the room; live bat found in room with an unattended infant, child, or person with sensory or mental impairment; person slept in small, closed-in camp cabin, in which bats were swooping past sleeping person; bat found on ground near an unattended infant, child, or person with mental impairment; unidentified flying object hits person and time of day (dusk or dawn), presence of mark where hit, and place where flying object came from (i.e., good site for roosting bats) all support likelihood that it was a bat. The camps reported the bat incidents to the local health department or NYSDOH district offices, which submitted the incident report forms to BCEHFP; that bureau then forwarded the forms to the Zoonoses Program. Staff from the Zoonoses Program and Wadsworth Center taught local and district camp inspectors how to prevent human contact with bats, bat capture techniques, and methods of evacuating a building during an incident.

Reported incidents and additional information from 3 other reporting sources were added to the children’s camp database for the final analysis. Information included: 1) specimen history forms for camp-associated bats that were tested at the Rabies Laboratory; 2) the Zoonoses Program rabies exposure and PEP database established by a statewide reporting requirement; and 3) CEH’s environmental Health Information and Permitting System (eHIPS).

From 1998 to 2002 during the summer camp season (June through August), 299 bat incidents were reported at 109 of the estimated 2,600 NYS children’s camps, involving 1,429 campers and staff (Table). The average and median ages of persons in bat incidents (based on the reported ages of 963 persons) were 14.8 and 13 years, respectively. During the 5-year period, 461 (32.2%) exposed persons (337 campers, 123 staff, 1 unknown status) received PEP (Figure 1). Forty-six persons refused PEP, and treatment status was unknown for 117. Over the 5-year period, bats were submitted for testing, and rabies was ruled out in 53.8% of the incidents. These test results prevented 805 (61.3%) exposed persons (567 campers, 196 staff, 42 unknown status) from having PEP treatment. Of the 209 bats tested from 1998 to 2002, 4 bats collected in 2000 were rabid, and these incidents did not require any treatment for exposure.

Four types of bat exposure reported most frequently accounted for 1,098 (77%) of persons in bat incidents at children’s camps (Figure 2). Exposure types were unknown for 69 of the incidents from 1998 to 2002. Specific exposure types (more than 1 type could be reported per incident) and numbers of persons exposed were sleeping where a bat was seen (797), sleeping where bats were swooping (205), direct physical contact with a bat (62), and a bat flying into them (36). The proportion of treatments prevented because of bats testing negative for rabies was 63%, 37%, 26%, and 11%, for these 4 types of exposure, respectively.

Conclusions

From 1998 to 2002, almost 300 separate bat incidents involving ≈1,500 children and staff at children’s camps in New York State were reported. Approximately one third of

<table>
<thead>
<tr>
<th>Bat incidents</th>
<th>1998</th>
<th>1999</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>Total</th>
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<tr>
<td>Reported incidents (June–August)</td>
<td>45</td>
<td>34</td>
<td>74</td>
<td>74</td>
<td>72</td>
<td>299</td>
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<tr>
<td>No. of incidents with bat submitted for testing (%)</td>
<td>19 (42.2)</td>
<td>5 (14.7)</td>
<td>44 (59.4)</td>
<td>50 (67.5)</td>
<td>43 (59.7)</td>
<td>161 (53.8)</td>
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<tr>
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<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
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<tr>
<td>No. of camps reporting incidents</td>
<td>16</td>
<td>21</td>
<td>46</td>
<td>42</td>
<td>40</td>
<td>109</td>
</tr>
<tr>
<td>No. of persons in reported incidents</td>
<td>334</td>
<td>145</td>
<td>386</td>
<td>331</td>
<td>233</td>
<td>1,429</td>
</tr>
</tbody>
</table>

*From 1998 to 2000, all bat incidents at children’s camps were requested for reporting. From 2001 to 2002, only bat incidents resulting in concern about potential rabies exposure were requested for reporting.
these persons received PEP because the bats were not captured and tested to rule out rabies. PEP treatment of ≈800 persons was not necessary because the bats were captured and tested negative for rabies virus.

At an estimated cost of $1,136 per PEP (2), this represents healthcare cost savings ≥$900,000. This estimate underestimates the true cost savings of preventing 5 medical visits during a month for each treated person, transportation costs, coordinating and administering the treatments, opportunity and psychological costs of missing camp, and lost wages.

Most of those involved in bat incidents were campers, which is not unexpected, as most camps have a higher number of campers than staff. Gender often depended on which camp was affected, as many camps are single sex. The 4 most common types of bat exposures requiring PEP are ones in which there is a reasonable probability that rabies exposure has occurred. The 2 most common types of incidents in which PEP was required (sleeping where a bat was seen or was swooping) are preventable by properly bat-proofing camp cabins. PEP can also be avoided with proper bat capture technique and cabin evacuation. In 1 camp, after 5 incidents in a short period, PEP treatment was required in 42 cases. Education on bat-proofing and capture did not prevent 25 subsequent incidents in the same season but did result in bat capture and negative rabies test results in 24 of them, preventing 180 campers and staff members from receiving PEP treatment.

Although only a few human rabies cases are diagnosed each year in the United States, inapparent or unreported bat bites appear to account for most of them (14). Equally important, bat exposures strongly affect healthcare costs when rabies cannot be ruled out by capturing and testing bats. Just as it is unacceptable for other wildlife to affect the health and safety of children at camp, keeping bats out of sleeping quarters and other buildings should be part of routine camp safety education, inspection, and certification programs. Although bats are part of the external camp environment, occupied buildings must be bat-proof. If exposures around or in camp buildings do occur, campers and staff must know how to avoid further exposures and how to capture the bat for rabies testing. Systems for reporting camp bat exposures and their consequences will identify this important public health problem and aid public health responses to reduce its impact.

Acknowledgments

We thank Timothy Shay, Felix Mrozek, and staff of the Center for Environmental Health’s Bureau of Community Environmental Health and Food Protection for reporting animal rabies cases and human exposures at children’s camps and for developing systems for surveillance and rabies control; Amy Willsey, Amy Schrom, Yoichiro Hagiwara, and staff of the Zoonoses Program, NYSDOH, for rabies treatment surveillance; Charles Trimarchi, Robert Rudd, Richard Raczkowski, and staff of the Rabies Laboratory, Wadsworth Center, NYSDOH, for laboratory testing; local, district, and regional health and environment departments for rabies surveillance and education; human and animal healthcare providers for rabies and exposure reporting; and staff from the children’s camps for incident reporting.

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Ms. Robbins is an MPH graduate of the University at Albany School of Public Health, currently working for the New Mexico Department of Health. She is the recipient of an applied epidemiology fellowship from the Council of State and Territorial Epidemiologists and the Centers for Disease Control and Prevention. Her research interest is infectious disease epidemiology.

References


Address for correspondence: Millicent Eidson, New York State Department of Health, Corning Tower, Room 621, Albany, NY 12237, USA; fax: 518-473-6590; email: mxe04@health.state.ny.us
West Nile Virus in Morocco, 2003

Isabelle Schuffenecker,* Christophe N. Peyrefitte,† Mohammed el Harrak,‡ Séverine Murri,,* Agnès Leblond,§ and Hervé G. Zeller*

West Nile virus (WNV) reemerged in Morocco in September 2003, causing an equine outbreak. A WNV strain isolated from a brain biopsy was completely sequenced. On the basis of phylogenetic analyses, Moroccan WNV strains isolated during the 1996 and 2003 outbreaks were closely related to other strains responsible for equine outbreaks in the western Mediterranean basin.

In the early 1950s, scientists first recognized that West Nile virus (WNV) reached outbreak levels in humans in Egypt and Israel (1,2). Initially considered a minor arbovirus, WNV has recently emerged as a major public health and veterinary concern in southern Europe, the Mediterranean basin, and the United States and Canada (1–3). Several outbreaks of severe human meningoencephalitis with fatalities have been reported within the last 8 years in Europe and North Africa, specifically in Romania (1996), Russia (1999), Israel (2000), and Tunisia (1997, 2003) (1,3). Several outbreaks of severe human meningoencephalitis with fatalities have been reported within the last 8 years in Europe and North Africa, specifically in Romania (1996), Russia (1999), Israel (2000), and Tunisia (1997, 2003) (1,3). Epizootics in horses have also been documented in Morocco (1996), Italy (1998), France (2000), and Israel (2000) (1,4). WNV was responsible for a cluster of human and equine cases in southern France in 2003 (5,6).

On the basis of phylogenetic analyses, WNV strains isolated since 1996 in southern Europe and the Mediterranean basin belong to the clade 1a of lineage 1 (7,8). Moreover, these strains belong to 2 distinct genotypes (8,9). One cluster includes equine strains isolated in Italy and France, human strains isolated in Russia and Israel, and mosquito strains isolated in Romania and Kenya. The other cluster includes most of the strains isolated from birds and horses in Israel from 1997 to 2001 and the North American isolates. Only 5 strains isolated in the Mediterranean basin have been completely sequenced.

Since the first WNV outbreak in Morocco in 1996, which caused 94 equine cases (including 42 deaths) and 1 human case (10), no WNV infections have been reported. An outbreak of WNV occurred among horses stabled in the Moroccan province of Kenitra in September and October 2003. The complete genome sequence of a WNV strain isolated from a brain biopsy was characterized, as well as the complete genome sequence of a strain isolated during the Morocco 1996 WNV outbreak. We studied phylogenetic relationships of the 2 Moroccan strains with other WNV strains isolated in the Mediterranean basin.

The Study

During the fall of 2003, 9 equine WNV cases were reported to the Moroccan Ministry of Agriculture. All horses had acute neurologic symptoms, fever, paresis of the hindquarters, paralysis, or some combination of these symptoms (Table); 5 horses were euthanized. Clinical cases occurred from September 12 to October 1, 2003. No abnormal bird deaths were observed, and no human cases were reported.

Equine clinical cases were reported from 3 locations –20 to 30 km northeast of Kenitra (34°18’N, 06°30’W), close to the Sebou River delta and the Atlantic Ocean (Figure 1). Irrigation networks are developed in this farming area. In addition, a natural bird reserve, Sidi Boughaba, is located 15 km southeast of Kenitra, along one of the migratory Europe–sub-Saharan routes, where numerous migrating and breeding birds are found.

Virus isolation was performed from a brain biopsy in the BioPharma laboratory in Rabat, Morocco. Brain suspension was injected onto BSR cells. Cytopathic effect was observed 4 days after infection. WNV was identified by immunofluorescence assay and confirmed by reverse transcription–polymerase chain reaction (RT-PCR). The complete WNV genome was sequenced in the National Reference Center for Arboviruses in Lyon, France, after a single passage of the strain (04.05) on Vero E6 cells. Twenty-five overlapping amplicons were amplified and sequenced on both strands (AY701413). The complete sequence of the strain 96-111 isolated during the 1996 Moroccan equine outbreak was also determined (AY701412).

Pairwise alignments of 96-111 and 04.05 sequences using ClustalW1.7 software (11) showed a 98.9% nucleotide identity and a 99.8% amino-acid identity between the 2 Moroccan isolates. Six amino-acid differences were observed between the 2 strains: 1 in the E gene (I732V), 2 in the NS1 gene (V979I and R1079S), 1 in the NS2a gene (H1262Y), and 2 in the NS3 gene (F1551L and A1754T).

Multiple alignments of Moroccan WNV sequences and other WNV sequences available in GenBank database were generated by ClustalW1.7 software. Phylogenetic trees were constructed by using nucleotide alignments, the Jukes Cantor algorithm, and the neighbor-joining method implemented in molecular evolutionary genetics analysis
The robustness of branching patterns was tested by 1,000 bootstrap pseudoreplications.

Comparison of the complete genome sequences showed a high degree of identity between the Moroccan strains and those of the European/Mediterranean/Kenyan cluster. Paired identity at the nucleotide level ranged from 98.2% to 98.9% and from 98.6% to 99% for 04.05 and 96-111 strains, respectively. Paired nucleotide identity with strains of the Israeli/American cluster ranged from 96.2% to 96.3% and 96.5% to 96.6%, respectively. The 5 amino-acid residues characteristic of the European/Mediterranean/Kenyan genotype were conserved in both Moroccan strains, i.e., T416 (E protein), S861 (NS1 protein), I1861 (NS3 protein), V2209 (NS4a protein), and D2522 (NS4b protein).

On the basis of the complete genome sequences, phylogenetic data showed that both Moroccan strains belonged to the clade 1a of the lineage 1 and clustered with the strains of the Israeli/American cluster ranged from 96.2% to 96.3% and 96.5% to 96.6%, respectively. The 5 amino-acid residues characteristic of the European/Mediterranean/Kenyan genotype were conserved in both Moroccan strains, i.e., T416 (E protein), S861 (NS1 protein), I1861 (NS3 protein), V2209 (NS4a protein), and D2522 (NS4b protein).

On the basis of the complete genome sequences, phylogenetic data showed that both Moroccan strains belonged to the clade 1a of the lineage 1 and clustered with the strains of the European/Mediterranean/Kenyan cluster (Figure 2A). On the basis of the envelope sequences, equine WNV strains isolated in the Mediterranean basin from 1996 to 2003 belonged to 2 distinct clusters, i.e., the European/Mediterranean/Kenyan cluster and the American/Israeli cluster (Figure 2B). The Moroccan equine strains clustered with the Italian and French equine strains. They were more distantly related to the 3 equine strains isolated in Israel in 2000.

**Conclusions**

WNV has been circulating in the Mediterranean basin for a long time (1–3); in the western part of the basin, only a few isolates have been obtained and completely sequenced. We report here the isolation and complete genome characterization of 2 WNV strains involved in equine outbreaks in Morocco in 1996 and more recently in 2003.

During the late summer of 2003, an equine outbreak was reported in Morocco. By contrast with the 1996 outbreak (10), the epidemic was restricted geographically and temporally. Climatic and vectorial conditions might have been insufficient to lead to a major transmission of the virus. No young horses were clinically affected, probably because of the structure of the equine population in Kenitra Province, where most horses are bought at the age of 4 or 5 years and the mean age of the equine population is 10 years.

High pairwise nucleotide and amino-acid identity values indicated that the Morocco 1996 and 2003 WNV strains are closely related. Determining if both outbreaks were related to an endemic strain or to distinct introduction events by migratory birds was not possible. Since 1996, WNV-positive serologic results have been found every year in horses with neurologic signs, which suggests endemic circulation of the virus (M. el Harrak, unpub. data.).

On the basis of envelope and complete genome sequences, we demonstrated that both Moroccan strains belonged to the European/Mediterranean/Kenyan cluster previously defined (8). The characterization of 2 new complete WNV genome sequences allowed us to demonstrate the genetic stability of the WNV strains involved in the equine outbreaks reported since 1996 in the western part of the Mediterranean basin. Our data also suggested the
existence of 2 subclusters of WNV strains in the European/Mediterranean/Kenyan cluster. One subcluster includes strains isolated in the western Mediterranean basin (France, Italy, Morocco) that have probably been introduced from West Africa. The other subcluster includes strains isolated in the eastern Mediterranean basin (Israel) and southeastern Europe (Romania, Volgograd) that have probably been introduced from East Africa. The molecular epidemiologic features of the strains in the Mediterranean basin appear to be more complex. Since 1997, at least 2 lineages cocirculate in Israel, i.e., the European/Mediterranean/Kenyan lineage and the more recent Israeli/American lineage in birds, equines, and humans (9,13). Strains of the latter genotype were imported in 1999, probably through infected birds or mosquitos, from the Middle East to North America, causing high rates of avian deaths and high rates of illness and deaths in humans and equines. In Israel, the emergence of the Israeli/American genotype has also been associated with avian deaths. Whether the introduction of this genotype is associated with the high rates of illness and death during the 2000 human outbreak is unclear. Five amino-acid residues are known to distinguish the European/Mediterranean/Kenyan and the Israeli/American genotypes (7,8). In the future, testing the role of those specific residues and comparing the biologic properties of strains of both genotypes will be useful, knowing that only Israeli/American strains are responsible for avian deaths (14) and probable increased neurovirulence (15). No viruses of the Israeli/American genotype have been isolated elsewhere in the Mediterranean basin or in Europe.

During the Morocco 2003 outbreak, WNV reemerged in southern France, causing 7 human and 4 equine cases, and in Tunisia, causing approximately 200 human cases in Monastir province (H. Trikki, pers. comm.). No virus isolation or genome amplification was obtained or reported. Knowing the possibility of transmission through blood donations, surveillance of WNV infections must be enhanced in the Mediterranean basin. For the moment, in contrast with the situation in North America, human and equine outbreaks have been restricted geographically and temporally (3). The mechanisms of WNV reintroduction in Europe and in the Mediterranean basin and the cycle of maintenance in infected areas remain to be elucidated. Further studies should focus on competence of mosquito vectors, identifying bird species involved in the cycle of transmission, and the persistence mechanisms of the virus in WNV-endemic areas.
Acknowledgments

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Dr. Schuffenecker is a biologist working for the French National Reference Center for Arboviruses. She is involved in the diagnosis and epidemiology of vector-borne diseases.

References


Address for correspondence: Isabelle Schuffenecker, Centre de Référence des Arbovirus, Institut Pasteur, 21 Ave Tony Garnier, 69365 Lyon cedex 07, France; fax: 33-4-37-28-24-51; email: schuffenecker@cervi-lyon.inserm.fr
Diagnostic System for Rapid and Sensitive Differential Detection of Pathogens

Thomas Briese,*1 Gustavo Palacios,*1 Mark Kokoris,†1 Omar Jabado,* Zhiqiang Liu,* Neil Renwick,* Vishal Kapoor,* Inmaculada Casas,‡ Francisco Pozo,‡ Ron Limberger,§ Pilar Perez-Brena,‡ Jingyue Ju,* and W. Ian Lipkin*

Naturally emerging and deliberately released pathogens demand new detection strategies to allow early recognition and containment. We describe a diagnostic system for rapid, sensitive, multiplex discrimination of microbial gene sequences and report its application for detecting 22 respiratory pathogens in clinical samples.

Efficient laboratory diagnosis of infectious diseases is increasingly important to clinical management and public health. Methods to directly detect nucleic acids of microbial pathogens in clinical specimens are rapid, sensitive, and may succeed when culturing the organism fails. Clinical syndromes are infrequently specific for single pathogens; thus, assays are needed that allow multiple agents to be simultaneously considered. Current multiplex assays employ gel-based formats in which products are distinguished by size, fluorescent reporter dyes that vary in color, or secondary enzyme hybridization assays. Gel-based assays are reported that detect 2–8 different targets with sensitivities of 2–100 PFU or <1–5 PFU, depending on whether amplification is carried out in a single or nested format, respectively (1–4). Fluorescence reporter systems achieve quantitative detection with sensitivity similar to that of nested amplification; however, their capacity to simultaneously query multiple targets is limited to the number of fluorescent emission peaks that can be unequivocally resolved. At present, up to 4 fluorescent reporter dyes can be detected simultaneously (5,6). Multiplex detection of up to 9 pathogens has been achieved in hybridization enzyme systems; however, the method requires cumbersome postamplification processing (7).

The Study

To address the need for sensitive multiplex assays in diagnostic molecular microbiology, we created a polymerase chain reaction (PCR) platform in which microbial gene targets are coded by a library of 64 distinct Masscode tags (Qiagen Masscode technology, Qiagen, Hilden, Germany). A schematic representation of this approach is shown in Figure 1. Microbial nucleic acids (RNA, DNA, or both) are amplified by multiplex reverse transcription (RT)-PCR using primers labeled by a photocleavable link to molecular tags of different molecular weight. After removing unincorporated primers, tags are released by UV irradiation and analyzed by mass spectrometry. The identity of the microbe in the clinical sample is determined by its cognate tags.

As a first test of this technology, we focused on respiratory disease because differential diagnosis is a common clinical challenge, with implications for outbreak control and individual case management. Multiplex primer sets were designed to identify up to 22 respiratory pathogens in a single Mass Tag PCR reaction; sensitivity was established by using synthetic DNA and RNA standards as well as titered viral stocks; the utility of Mass Tag PCR was determined in blinded analysis of previously diagnosed clinical specimens.

Oligonucleotide primers were designed in conserved genomic regions to detect the broadest number of members for a given pathogen species by efficiently amplifying a 50- to 300-bp product. In some instances, we selected established primer sets; in others, we used a software program designed to cull sequence information from GenBank, perform multiple alignments, and maximize multiplex performance by selecting primers with uniform melting temperatures and minimal cross-hybridization potential (Appendix Table, available at http://www.cdc.gov/ncidod/eid/vol11no02/04-0492_app.htm). Primers, synthesized with a 5′ C6 spacer and aminohexyl modification, were covalently conjugated by a photocleavable link to Masscode tags (Qiagen Masscode technology) (8,9). Masscode tags have a modular structure, including a tetrafluorophenyl ester for tag conjugation to primary amines; an o-nitrobenzyl photolabile linker for photoredox cleavage of the tag from the analyte; a mass spectrometry sensitivity enhancer, which improves the efficiency of atmospheric pressure chemical ionization of the cleaved tag; and a variable mass unit for variation of the cleaved tag mass (8,10–12). A library of 64 different tags has been established. Forward and reverse primers in individual

*Columbia University, New York, New York, USA; †Qiagen Inc., Valencia, California, USA; ‡Instituto de Salud Carlos III, Majadahonda, Madrid, Spain; and §New York State Department of Health, Albany, New York, USA

*These authors contributed equally to this study.
primer sets are labeled with distinct molecular weight tags. Thus, amplification of a microbial gene target produces a dual signal that allows assessment of specificity.

Gene target standards were cloned by PCR into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) by using DNA template (bacterial and DNA viral targets) or cDNA template (RNA viral targets) obtained by reverse transcription of extracts from infected cultured cells or by assembly of overlapping synthetic polynucleotides. Assays were initially established by using plasmid standards diluted in 2.5-µg/mL human placenta DNA (Sigma, St. Louis, MO, USA) and subjected to PCR amplification with a multiplex PCR kit (Qiagen), primers at 0.5 µmol/L each, and the following cycling protocol: an annealing step with a temperature reduction in 1°C increments from 65°C to 51°C during the first 15 cycles and then continuing with a cycling profile of 94°C for 20 s, 50°C for 20 s, and 72°C for 30 s in an MJ PTC200 thermal cycler (MJ Research, Waltham, MA, USA). Amplification products were separated from unused primers by using QIAquick 96 PCR purification cartridges (Qiagen, with modified binding and wash buffers). Masscode tags were decoupled from amplified products through UV light-induced photolysis in a flow cell and analyzed in a single quadrapole mass spectrometer using positive-mode atmospheric pressure chemical ionization (Agilent Technologies, Palo Alto, CA, USA). A detection threshold of 100 DNA copies was determined for 19 of 22 cloned targets by using a 22-plex assay (Table 1). The sensitivity of Mass Tag PCR to detect live virus was tested by using RNA extracted from serial dilutions of titered stocks of coronaviruses (severe acute respiratory syndrome [SARS] and OC43) and parainfluenzaviruses (HPIV 2 and 3). A 100-µL volume of each dilution was analyzed. RNA extracted from a 1-TCID$_{50}$/mL dilution, representing 0.025 TCID$_{50}$ per PCR reaction, was consistently positive in Mass Tag PCR.

Many respiratory pathogens have RNA genomes; thus, where indicated, assay sensitivity was determined by using synthetic RNA standards or RNA extracts of viral stocks. Synthetic RNA standards were generated by using T7 polymerase and linearized plasmid DNA. After quantitation by UV spectrometry, RNA was serially diluted in 2.5-µg/mL yeast tRNA (Sigma), reverse transcribed with random hexamers by using Superscript II (Invitrogen, Carlsbad, CA, USA), and used as template for Mass Tag PCR. As anticipated, sensitivity was reduced by the use of RNA instead of DNA templates (Table 1). The sensitivity of Mass Tag PCR to detect live virus was tested by using RNA extracted from serial dilutions of titered stocks of coronaviruses (severe acute respiratory syndrome [SARS] and OC43) and parainfluenzaviruses (HPIV 2 and 3). A 100-µL volume of each dilution was analyzed. RNA extracted from a 1-TCID$_{50}$/mL dilution, representing 0.025 TCID$_{50}$ per PCR reaction, was consistently positive in Mass Tag PCR.

RNA extracted from banked sputum, nasal swabs, and pulmonary washes of persons with respiratory infection was tested by using an assay panel comprising 30 gene targets that represented 22 respiratory pathogens. Infection in each of these persons had been previously diagnosed through virus isolation, conventional nested RT-PCR, or both. Reverse transcription was performed using random hexamers, and Mass Tag PCR results were consistent in all cases with the established diagnosis. Infections with respiratory syncytial virus, human parainfluenza virus, SARS coronavirus, adenovirus, enterovirus, metapneumovirus, and influenza virus were correctly identified (Table 2 and Figure 2). A panel comprising gene targets representing 17...
Table 2. Multiplex pathogen detection by Mass Tag polymerase chain reaction using Masscode-labeled primers in a 30-plex assay with clinical specimens with previously identified pathogens.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>No. positive/no. tested†</th>
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<tr>
<td>RSV B</td>
<td>3/3</td>
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<tr>
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<td>Adenovirus</td>
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<td>Enterovirus</td>
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</table>

*RSV, respiratory syncytial virus; HPIV, human parainfluenza virus; CoV, coronavirus; SARS, severe acute respiratory syndrome.
†No. positive and consistent with previous diagnosis/number tested (with respective previous diagnosis).

Conclusions

Our results indicate that Mass Tag PCR is a sensitive and specific tool for molecular characterization of microflora. The advantage of Mass Tag PCR is its capacity for multiplex analysis. Although the use of degenerate primers (e.g., enteroviruses and adenoviruses, Appendix Table and Table 1) may reduce sensitivity, the limit of multiplexing to detect specific targets will likely be defined by the maximal primer concentration that can be accommodated in a PCR mix. Analysis requires the purification of product from unincorporated primers and mass spectrometry. Although these steps are now performed manually, and mass spectrometers are not yet widely distributed in clinical laboratories, the increasing popularity of mass spectrometry in biomedical sciences and the advent of smaller, lower-cost instruments could facilitate wider use and integrated instrumentation. In addition to developing

Figure 2. Analysis of clinical specimens. RNA extracts from clinical specimens containing known pathogens were reverse transcribed into cDNA (Superscript RT system, Invitrogen, Carlsbad, CA, 20-µL volume). Five microliters of the reaction were subjected to Mass Tag PCR by using primers coupled to Masscode tags (Qiagen Masscode technology, Qiagen, Hilden, Germany). Detection of (A) influenza virus A (H1N1), (B) respiratory syncytial virus (RSV) group B, (C) human coronavirus SARS (HCoV-SARS), (D) human parainfluenza virus (HPIV) types 1 and (E) 3, and (F) enterovirus (EV) by using a 30-plex assay, including 60 primers targeting influenza A virus matrix gene (FLUAV-M), and for typing N1, N2, H1, H2, H3, and H5 sequences, as well as influenza B virus (FLUBV), RSV groups A and B, HCoV-229E, -OC43, and -SARS, HPIV types 1, 2, 3, and 4 (groups A and B combined; 4 primers), human metapneumovirus (HMPV, 4 primers), measles virus (MEV), EV (degenerate primer pair targeting all serogroups), human adenoviruses (HAdV, degenerate primer pair targeting all serogroups), human herpesvirus 1 (HHV-1, herpes simplex virus), human herpesvirus 3 (HHV-3; varicella-zoster virus), Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophila, Streptococcus pneumoniae, Haemophilus influenzae. Y-axis values indicate signal to noise ratio. The bar indicates an arbitrary cut-off threshold of 2.7 (4 times average background determined with random human DNA).
additional pathogen panels, our continuing work is focused on optimizing multiplexing, sensitivity, and throughput. Potential applications include differential diagnosis of infectious diseases, blood product surveillance, forensic microbiology, and biodefense.

Acknowledgments

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Dr. Briese is associate professor of epidemiology at the Columbia University Mailman School of Public Health and associate director of the Jerome L. and Dawn Greene Infectious Disease Laboratory. His research interests include the molecular epidemiology of emerging viral diseases, virus-host interactions, and novel techniques for pathogen detection and discovery.

References


Address for correspondence: W. Ian Lipkin, Mailman School of Public Health, Columbia University, 722 West 168th St, New York, NY 10032, USA; fax: 212-342-9044; email: wil2001@columbia.edu

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Comparing Aberration Detection Methods with Simulated Data

Lori Hutwagner,* Timothy Browne,* G. Matthew Seeman,* and Aaron T. Fleischauer*

We compared aberration detection methods requiring historical data to those that require little background by using simulated data. Methods that require less historical data are as sensitive and specific as those that require 3–5 years of data. These simulations can determine which method produces appropriate sensitivity and specificity.

The Early Aberration Reporting System (EARS) was developed to allow analysis of public health surveillance data. Several alternative aberration detection methods are available to state and local health departments for syndromic surveillance. Before 2001, most statistical aberration detection methods required at least 5 years of background data (1–6). However, with the release of Bacillus anthracis in the U.S. mail shortly after the September 11, 2001, World Trade Center attacks, substantial interest has emerged in public health tools that could be rapidly implemented without requiring years of background data. Newly developed nonhistorical aberration detection methods can require as little as 1 week of data to begin analysis, although they have not been extensively evaluated against traditional historical methods (7,8).

The objective of our study was to determine the sensitivity, specificity, and time to detection of 3 methods that require <3 years of historical baseline data, C1–MILD (C1), C2–MEDIUM (C2), and C3–ULTRA (C3), and compare the results with those of 2 methods that require 5 years of historical baseline, the historical limits method (2) and the seasonally adjusted cumulative sum (CUSUM) (5), based on simulated data. Simulated data were used to avoid some of the interpretation difficulties that can come from making these comparisons on the basis of empirically observed, natural disease data. All 5 of these methods are components of EARS (7).

The Study

The methods C1, C2, and C3 were named according to their degree of sensitivity, with C1 being the least sensitive and C3 the most sensitive. All 3 methods are based on a positive 1-sided CUSUM calculation. For C1 and C2, the CUSUM threshold reduces to the mean plus 3 standard deviations (SD). The mean and SD for the C1 calculation are based on information from the past 7 days. The mean and SD for the C2 and C3 calculations are based on information from 7 days, ignoring the 2 most recent days. These methods take into consideration daily variation because the mean and SD used by the methods are based on a week’s information. These methods also take seasonality into consideration because the mean and SD are calculated in the same season as the data value in question.

Since 1989, results from the historical limits method have been used to produce Figure 1 in the Morbidity and Mortality Weekly Report. This method compares the number of reported cases in the 4 most recent time periods for a given health outcome with historical incidence data on the same outcome from the preceding 5 years; the method is based on comparing the ratio of current reports with the historical mean and SD. The historical mean and SD are derived from 15 totals of 3 intervals (including the same 4 periods, the preceding 4 periods, and the subsequent 4 periods over the preceding 5 years of historical data).

The seasonally adjusted CUSUM method is based on the positive 1-sided CUSUM where the count of interest is compared to the 5-year mean and the 5-year SD for that period. The seasonally adjusted CUSUM was originally applied to laboratory-based Salmonella serotype data.

To calculate sensitivity, specificity, and time to detection, all 5 detection methods of EARS were used to independently analyze 56,000 sets of artificially generated case-count data based on 56 sets of parameters. These 56 sets of parameters each generated 1,000 iterations of 6 years of daily data, 1994–1999, by using a negative binomial distribution with superimposed outbreaks. Means and standard deviations were based on observed values from national and local public health systems and syndromic surveillance systems. Examples of the data included national and state pneumonia and influenza data and hospital influenzalike illness. Adjustments were made for days of the week, holidays, postholiday periods, seasonality, and trend. Any 6 years could be used, but the years 1994–1999 were used to set day of the week and holiday patterns and to avoid any problems that programs might have with the year 2000. Fifty (89%) of these datasets then had outbreaks superimposed throughout the data. Three types of outbreaks were used, each representing various types of naturally occurring events: log normal, a rapidly increasing outbreak; inverted log normal, a slowly starting outbreak; and a single-day spike. These types of outbreaks were combined with different SDs and incubation times to create 10 different types of outbreaks that had equal probability of being included in the simulated data. A year of final simulated data can be seen in the Figure, with original data and...
outbreaks that were added. As a result of these analyses, the statistically marked aberrations, or flags, produced by the 5 detection methods were evaluated for their specificity, sensitivity, and time to detection. These data can be obtained at http://www.bt.cdc.gov/surveillance/ears/datasets.asp.

In our study, sensitivity was defined as the number of outbreaks in which ≥1 day was flagged, divided by the total number of outbreaks in the data. An outbreak was defined as a period of consecutive days in which varying numbers of aberrant cases were added to the baseline number of cases. An outbreak had days before and after it when no aberrant cases were added to the baseline case counts. Specificity was defined as the total number of days that did not contain aberrant cases (and that were not flagged), divided by the total number of days that did not contain aberrant cases. Based on these definitions, actual values for sensitivity and specificity were calculated.

Time to detection was defined as the number of complete days that occurred between the beginning of an outbreak and the first day the outbreak was flagged. For example, if a method flags an outbreak on the first day, its time to detection is 0. Likewise, if it flags on the second day, its time to detection is 1, and so on. Time to detection is an average of the times to detection for each outbreak and dataset. Only outbreaks that were flagged on at least 1 day were included in the average. Therefore, sensitivity is needed to completely interpret time to detection. We calculated 2-sided 95% confidence values, and they were relatively small and consistent.

Overall, the CUSUM methods (the seasonally adjusted CUSUM, C1, C2, and C3) had similar times to detection, but their sensitivities varied (Table). Specifically, C1, C2, and C3 showed increasing sensitivity from 60% to 71% to 82%, respectively. The seasonally adjusted CUSUM and C3 methods had similar sensitivities, 82.5% and 82.3%, but C3 had a higher specificity, 88.7% and 95.4%. The historical limits and C1 and C2 methods showed varying sensitivities (44%–71%), with C1 and C2 having the highest, but all demonstrated similar specificities (96%–97%).

When results were stratified by outbreak type, 1-day outbreaks (i.e., spikes) exhibited the lowest sensitivities. Analysis was broken down by dataset and outbreak type (online Appendix Tables 1 and 2, available at http://www.cdc.gov/ncidod/EID/vol11no02/04-0587_app1.htm and http://www.cdc.gov/ncidod/EID/vol11no02/04-0587_app2.htm).

For the 6 datasets that contained noise but no outbreaks, no sensitivity or time to detection exist to calculate. The overall specificity for the seasonally adjusted CUSUM, historical limits, C1, C2, and C3 were 88.7%, 98.3%, 97.2%, 97.2%, and 95.2%, respectively. The specificity for these 6 datasets was consistent with general results. The historical limits method showed superior specificity in all but the last dataset.

Conclusions

These simulations demonstrate that the methods for aberration detection that require little baseline data, C1, C2, and C3, are as sensitive and specific as the historical limits and seasonally adjusted CUSUM methods. As expected, C1, C2, and C3 showed increasing sensitivities in accordance with their intended sensitivity levels (C1 being the least sensitive, C3 being the most), but with decreasing specificities as sensitivity increases. Seasonally

### Table. By method, overall sensitivity and specificity and time to detection

<table>
<thead>
<tr>
<th>Type of method</th>
<th>Name</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Time to detection (d)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Historical methods (at least 5 y historical data)</td>
<td>Seasonally adjusted CUSUM†</td>
<td>82.5</td>
<td>88.7</td>
<td>1.272</td>
</tr>
<tr>
<td></td>
<td>Historical limits‡</td>
<td>43.9</td>
<td>96.3</td>
<td>2.942</td>
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<tr>
<td>Nonhistorical methods (&lt;3 y historical data)</td>
<td>C1–MILD§</td>
<td>60.1</td>
<td>97.0</td>
<td>1.122</td>
</tr>
<tr>
<td></td>
<td>C2–MEDIUM¶</td>
<td>71.2</td>
<td>97.0</td>
<td>1.319</td>
</tr>
<tr>
<td></td>
<td>C3–ULTRA‖</td>
<td>82.3</td>
<td>95.4</td>
<td>1.307</td>
</tr>
</tbody>
</table>

*Time to detection must be interpreted with sensitivity because time to detection does not include missed outbreaks.
†The seasonally adjusted CUSUM method sums the positive differences of the current value from the mean for a period similar to the current value over 5 years.
‡The historical limits method compares the current sum of 4 time periods to the mean of the sum of 15 totals of 4 time periods surrounding the current point of interest over 5 years.
§The C1–MILD method is based on CUSUM, but the calculations reduce to the current value being greater than the mean plus 3 standard deviations (SD), with the mean and SD based on the past 7 days.
¶The C2–MEDIUM method is based on CUSUM, but the calculations reduce to the current value being greater than the mean plus 3 SD, with the mean and SD based on the past 7 days shifted by 2 days.
‖The C3–ULTRA method is based on CUSUM, summing the positive difference of the current value from the mean for 3 days, with the mean and SD based on the past 7 days shifted by 2 days.

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adjusted CUSUM and the historical limits method also showed sensitivities and specificities as expected, with the seasonally adjusted CUSUM having the lower specificity and higher sensitivity. These findings emphasize the effectiveness of aberration detection methods without requiring long-term historical data as a baseline.

Since the 10 simulated outbreaks were randomly generated by using consistent rates, the sensitivity, specificity, and time to detection could be stratified by dataset and outbreak type. The results of these analyses were largely congruent with the expected findings, with some variations. The simulated datasets are designed for public health officials to select a dataset that best reflects their data of interest or the type of outbreak they are anticipating to determine which method provides them with the sensitivity and specificity they would find useful. The simulated datasets can also be used to make comparisons with other methods.

The aberration detection methods C1, C2, and C3 are used in several states, counties, and local public health departments. Public health departments are able to apply these methods to data sources that do not have long periods of baseline data. Public health departments are also able to apply 1 set of methods they understand to various types of diseases, covering different frequencies and seasonalities. The C1, C2, and C3 methods have detected outbreaks of public health interest, including West Nile disease and the start of the influenza season.

C1, C2, and C3 demonstrate consistency over the various situations represented in these simulations. Other aberration detection methods exist, as do other simulated datasets. The simulated datasets presented in this paper cover a larger variety of types of data that might be expected in public health. These simulated datasets also include enough past years of data so that methods that require 5 years of historical information can also be used in the comparisons. These simulations provide a method to fairly compare other methods among themselves and to the methods included in EARS.

The simulations were based on means and SDs to help determine which method performs better under which circumstances. When deciding which method to use, the potential user should base the decision on the sensitivity or specificity or the time to detection.

A potential limitation is that the method for calculating average times to detection disregards undetected outbreaks. Therefore, times to detection should not be considered without also taking into account the sensitivity. However, this method was preferred over the alternative of assigning arbitrary numbers of days to detection for outbreaks that were not detected since the alternative method could lead to misinterpretation of the data. Another limitation is that the artificial datasets may not fully reproduce the nuances of natural disease occurrences. While approximations, the simulated data were generated based on naturally observed data and included variations for trend over time, days of the week, seasons, and holidays. Therefore, while these comparisons represent relative sensitivities, specificities, and times to detection, we do not know whether results using naturally occurring data would be consistent.

The results of this study suggest that the EARS historical methods do not have a strong advantage when compared with nonhistorical methods. In fact, the lack of historical data does not impair the EARS outbreak detection methods. This study also demonstrates the effectiveness of artificial outbreak data in comparing and evaluating outbreak detection methods. As aberration detection methods are increasingly being used by state and local health departments to monitor for naturally occurring outbreaks and bioterror events, this study contributes to the quest to determine the most efficient method for analyzing surveillance data.

Ms. Hutwagner works with the Bioterrorism Preparedness and Response Program at the Centers for Disease Control and Prevention on developing aberration detection methods for their national “drop-in surveillance” system and ongoing syndromic surveillance. She has been implementing these methods at various sites in the United States and internationally.

References


Address for correspondence: Lori Hutwagner, Centers for Disease Control and Prevention, 1600 Clifton Rd, Mailstop C18, Atlanta, GA 30333, USA; fax: 404-639-0382; email: lhutwagner@cdc.gov
Malaria Epidemic and Drug Resistance, Djibouti

Christophe Rogier,* Bruno Pradines,* H. Bogreau,* Jean-Louis Koeck,†‡ Mohamed-Ali Kamil,§ and Odile Mercereau-Puijalon¶

Analysis of Plasmodium falciparum isolates collected before, during, and after a 1999 malaria epidemic in Djibouti shows that, despite a high prevalence of resistance to chloroquine, the epidemic cannot be attributed to a sudden increase in drug resistance of local parasite populations.

From March to June 1999, an epidemic of Plasmodium falciparum malaria affecting all age groups spread in the city of Djibouti, Horn of Africa, an area with low and irregular transmission. Since the 1970s, autochthonous cases of malaria have been reported among the local population, but their incidence is usually low (1). Anopheles arabiensis, the main malaria vector in the city (2,3), has been found since the 1970s, possibly from Ethiopia (1,4). The focused distribution and the specificity of the breeding sites allowed a control strategy based on treatment of the larval sites with a larvivorous autochthonous fish, complemented with pinpoint use of bacterial toxins (3). Unfortunately, malaria control activities were progressively decreased so that, since the mid-1990s, vector control activity has been reduced to irregular insecticide indoor or outdoor spraying. Djiboutians frequently travel, and the Djibouti-Ethiopian railway has been suspected to be an effective route for propagating malaria parasites (5).

Although some chloroquine treatment failures were reported in Djibouti in 1990 (6), most persons with P. falciparum were treated by chloroquine or quinine at the beginning of the 2000s, including during the 1999 epidemics. To determine whether this epidemic was associated with temporary changes in environmental conditions or to importation of new (virulent) or resistant P. falciparum strains, we investigated P. falciparum population diversity before, during, and after the outbreak and analyzed in vitro susceptibility profiles to a panel of antimalarials during the epidemics.

The Study
The study was conducted at the Centre Hospitalier des Armées Bouffard, a French military hospital in Djibouti serving military and civilian natives from the entire city, and at other public health facilities of Djibouti. From 1997 to 2002, clinical malaria in the hospital shows the same temporal fluctuations as in dispensaries in the city (Figure). The incidence of patients with P. falciparum malaria admitted to the hospital increased >10-fold from March to May 1999 compared with the same period in 1997, 1998, and 2000–2002. In contrast, the number of admissions, consultations at the outpatient clinic, or blood counts performed for other causes than fever did not vary over the same period. The meteorologic station of the international airport of Djibouti recorded heavy rainfall the month before the epidemic. However, similar rainfall in 1997 or autumn 1999 was not followed by such a dramatic increase in malaria incidence in the ensuing months (Figure). When annual averages were compared, no particular variations in minimal or maximal mean air temperatures were found to occur during the months preceding the epidemic.

Forty-six blood samples were collected from September 14 to December 31, 1998 (period 1), 61 from April 12 to April 30, 1999 (period 2), and 32 from March 15 to May 15, 2002 (period 3), from patients with P. falciparum clinical cases who had not travelled outside the city of Djibouti during the preceding month and declared not having taken any antimalarial drug before the blood sampling. The study was cleared by the Djibouti Ministry of Health. Informed oral consent was obtained from patients before blood collection. Venous blood was collected before treatment administration in Vacutainer EDTA tubes (Becton Dickinson, Rutherford, NJ, USA). Thin blood smears were stained with an RAL kit (Réactifs RAL, Paris, France). Parasitemia was expressed as the proportion of P. falciparum–infected erythrocytes. Aliquots of freshly collected blood were kept at –20°C until DNA extraction.
*P. falciparum* genetic diversity was investigated by using *msp1* and *msp2* encoding highly polymorphic loci from merozoite surface protein genes. *Msp1* and *msp2* were genotyped by using nested polymerase chain reaction (PCR), as described (7), except that family-specific fluorescent primers were used in the nested PCR for assignment to the K1-, Mad20-, or Ro33-type *msp1* family and to the 3D7- or FC27-type *msp2* family. Fragment length was analyzed by the Genescan technology. Approximately 50% of the blood samples contained multiple *msp1* or *msp2* genotypes. The mean multiplicity of infection, i.e., the number of genotypes present in the blood sample, was ≈1.5 concurrent *P. falciparum* infections per person, with a decreasing tendency over the study period (Table 1). For each locus, multi-infection cases were excluded from analysis of genetic diversity. We identified 9 *msp1* alleles in 83 isolates and 17 *msp2* alleles in 108 isolates. The genetic diversity estimated by the unbiased expected

<table>
<thead>
<tr>
<th>Locus</th>
<th>Period 1 1996 (n = 48)</th>
<th>Period 2 1999 (n = 61)</th>
<th>Period 3 2002 (n = 32)</th>
<th>Total (N = 139)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>msp1</em></td>
<td>Mean multiplicity</td>
<td>1.6</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>SD*</td>
<td>0.7</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>No of multiple infections (%)</td>
<td>23 (50)</td>
<td>22 (36)</td>
<td>11 (34)</td>
</tr>
<tr>
<td><em>msp2†</em></td>
<td>Mean multiplicity</td>
<td>1.4</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.7</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>No of multiple infections (%)</td>
<td>14 (31)</td>
<td>12 (20)</td>
<td>2 (6)</td>
</tr>
<tr>
<td><em>msp1</em> and <em>msp2</em></td>
<td>Mean multiplicity</td>
<td>1.8</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.7</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>No of multiple infections (%)</td>
<td>28 (61)</td>
<td>29 (48)</td>
<td>12 (38)</td>
</tr>
</tbody>
</table>

**Table 1. Multiplicity of infections deduced from *msp1* and *msp2* genotyping and frequency (%) of the *Pf dhfr* (codons 51, 59, and 108), *Pf dhps* (codons 436, 437, and 540) and *Pf crt* (codon 76) genotypes**

*SD*, standard deviation; The genotypes at the *Pf dhfr*, *Pf dhps*, and *Pf crt* locus refer to the one-letter symbolized amino acids coded by the codons. A, Alanine; C, Cysteine; F, Phenylalanine; G, Glycine; I, Isoleucine; K, Lysine; N, Asparagine; R, Arginine; S, Serine; T, Threonine.

†*msp2* multiplicity estimated on 45 and 59 samples in 1998 and 1999, respectively.
heterozygocity (8), i.e., the probability that 2 randomly chosen genotypes are different in the sample, before, during, and after the 1999 outbreak was 0.79 (n = 23), 0.37 (n = 39), and 0.64 (n = 21) at the msp1 locus and 0.83 (n = 31), 0.34 (n = 47) and 0.63 (n = 30) at the msp2 locus, respectively. During the epidemic, Ro33-131 accounted for 79% of the msp1 allele and FC27-408 accounted for 81% of the msp2 alleles. Both alleles were present before and after the epidemic but with a much lower prevalence. They accounted for 26% of the msp1 and 35% of the msp2 alleles in 1998 and 14% of the msp1 and 10% of the msp2 alleles in 2002 (Table 2).

To look for resistance-associated point mutations and haplotypes, the complete coding region of Pf dhfr (dihydrofolate reductase) and Pf dhps (dihydropteroate synthase) was amplified and sequenced (ABI 3100 Genetic Analyser, Applied Biosystems, Courtaboeuf, France) as described (9). We focused the analysis on point mutations of Pf dhfr codons 16, 51, 59, 108, and 164 and Pf dhps codons 436, 437, 540, 581, and 613, which have been associated with resistance to pyrimethamine and proguanil metabolite and to sulfadoxine, respectively (10). The prevalences of the Pf dhfr and Pf dhps mutations are shown in Table 1. No mutant was detected for Pf dhfr codons 16 and 164 and Pf dhps codon 581. A single isolate collected in period 2 harbored the Pf dhps A613S mutation. No isolate harbored the quintuple mutant haplotype (Pf dhfr S108N, N51I, and C59R and Pf dhps K540E and A437G) or the Pf dhfr C59R and Pf dhps K540E combination that predicts sulfadoxine-pyrimethamine clinical failure (9). One isolate containing at least 2 P. falciparum populations harbored 3 Pf dhfr mutations (S108N, N51I, and C59R) and the Pf dhps K540E mutation.

From 1998 to 1999, the frequency of isolates with mutated Pf dhfr codons 51, 59, and 108 decreased (not significantly), and Pf dhps allelic frequency did not differ significantly. The prevalence of isolates harboring the Pf dhfr N51I, Pf dhfr S108N, Pf dhps A437G, and Pf dhps K540E mutations increased from 1998–1999 to 2002 (Fisher exact test, p < 0.001 each). Presence of the chloroquine resistance–associated K76T mutation of Pf crt (chloroquine-resistance transporter) (11) was analyzed by nested allele–specific PCR. Over the study period, 93% of the isolates harbored the Pf crt K76T mutation (Table 1), without any significant temporal variation.

Twenty seven P. falciparum isolates collected during the 1999 epidemic with a 0.05%–5.0% parasitemia were transported at 4°C to our laboratory in Marseille, France.

Table 2. Distribution of msp1 and msp2 alleles by allelic families and fragment size (in base pair) among Djibouti isolates with only 1 allele detected by locus

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allelic families</th>
<th>Allele (base pair)</th>
<th>1998 (%)</th>
<th>1999 (%)</th>
<th>2002 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>msp1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1</td>
<td>129</td>
<td>4.3</td>
<td>2.6</td>
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<td></td>
<td>203</td>
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<td>Mad 20</td>
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<td></td>
<td>184</td>
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<td>241</td>
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<td>131</td>
<td>26.1</td>
<td>79.5</td>
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<td>msp2</td>
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<td>3D7</td>
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<td></td>
<td>408</td>
<td>35.5</td>
<td>80.9</td>
<td>10.0</td>
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<td>444</td>
<td>3.2</td>
<td>0.0</td>
<td>0.0</td>
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</tr>
<tr>
<td></td>
<td>468</td>
<td>16.1</td>
<td>0.0</td>
<td>6.7</td>
<td></td>
</tr>
</tbody>
</table>

*Isolates collected in 1998 (msp1: n = 33; msp2: n = 31); 1999 (msp1: n = 39; msp2: n = 47), and 2002 (msp1: n = 21; msp2: n = 30).
and analyzed for in vitro drug sensitivity by using an isotopic microtest (12). Among them, 93% were classified as resistant to chloroquine (Table 3). No isolate was resistant to amodiaquine. In vitro resistance was 4% for both pyrimethamine and cycloguanil.

Conclusions

Before and after the 1999 epidemic, \textit{P. falciparum} genetic diversity in Djibouti was large, with ≈80% and 63% heterozygocity. This finding is somewhat surprising for an area where disease endemicity is low (13) and probably reflects importation of strains from neighboring areas such as Ethiopia or Somalia (1,5). \textit{P. falciparum} genetic diversity was diminished during the epidemic, reflecting the circulation of a restricted number of strains during that period. Most of these strains harbored an \textit{msp1} and \textit{msp2} genotype that was detected before the epidemic. The prevalence of \textit{Pfcr}, \textit{Pfdhfr}, and \textit{Pfdhps} mutant genotypes did not vary significantly from 1998 to 1999. Thus, our data do not support the hypothesis of a sudden increase in the drug resistance of the local \textit{P. falciparum} population as causing the epidemic. Our data are also not consistent with massive invasion by a single strain/genotype but rather suggest expansion during the epidemic of a few strains that were already prevalent. Further genotyping is needed to establish how many strains were circulating and their possible origin. What could have caused this sudden amplification? One possibility is a temporary increase in vector density. Unfortunately, no vectors were captured at that time, and this hypothesis is difficult to explore retrospectively.

The low prevalence of \textit{Pfcdfr} and \textit{Pfcdhs} resistance mutations in 1998 and 1999 and of proguanil or pyrimethamine in vitro resistance in 1999 may explain the very low incidence of clinical malaria among the French soldiers stationed in Djibouti who were taking chloroquine-proguanil chemoprophylaxis. However, the sharp increase of \textit{Pfcdfr} and \textit{Pfcdhs} resistance mutations observed in 2002 threatens sulfadoxine-pyrimethamine efficacy in the near future, even more so since the limited acquired immunity is unlikely to contribute to sustained drug efficacy (14). Molecular and in vitro assays point to a very high prevalence of chloroquine resistance. This finding calls for an urgent in vivo assessment of the antimalarials presently used in Djibouti in order to consider a rapid change in first-line treatment policy.

Acknowledgments

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Dr. Rogier is the chief of the Research Unit in Parasitological Biology and Epidemiology of the Institute for Tropical Medicine of the French Army, Le Pharo, Marseille, France. His main areas of interest include epidemiology and population genetics related to malaria.

References


Address for correspondence: Christophe Rogier, IMTSSA, BP46, Parc du Pharo, 13998 Marseille-Armees, France; fax: 33 4 91 15 01 64; email: christophe.rogier@wanadoo.fr
Late Recognition of SARS in Nosocomial Outbreak, Toronto

Thomas Wong,*† Tamara Wallington,‡ L. Clifford McDonald,§ Zahid Abbas,* Michael Christian,† Donald E. Low,† Denise Gravel,* Marianna Ofner,*‡ Barbara Mederski,¶ Lisa Berger,¶ Lisa Hansen,* Cheryl Harrison,¶ Arlene King,* Barbara Yaffe,‡ and Theresa Tam*

Late recognition of severe acute respiratory syndrome (SARS) was associated with no known SARS contact, hospitalization before the nosocomial outbreak was recognized, symptom onset while hospitalized, wards with SARS clusters, and postoperative status. SARS is difficult to recognize in hospitalized patients with a variety of underlying conditions in the absence of epidemiologic links.

Severe acute respiratory syndrome (SARS) spread globally in 2003, infecting >8,000 people and killing nearly 800. In total, 438 probable or suspected SARS cases and 44 deaths were reported in Canada (1,2). SARS was first recognized retrospectively in Canada in a woman who had returned from Hong Kong on February 23, 2003. This international connection ignited the outbreak in Canada, which affected mainly the Toronto area (1,2).

After enhanced infection control precautions and public health measures were implemented in March 2003, the Canadian outbreak began to subside in April. On May 14, the World Health Organization (WHO) took Toronto off the list as a SARS-affected area in the absence of newly reported cases for at least 2 incubation periods after the last SARS case-patient was isolated. In accordance with public health principles, the enhanced measures were selectively relaxed in low-risk settings in Toronto area hospitals in early May 2003, although full precautions were still recommended for patients with febrile respiratory illnesses. In the third week of May, a cluster of febrile respiratory illness at a Toronto area rehabilitation hospital was reported to the health department. Traceback of these SARS cases identified the index patient as a postoperative patient who was transferred from hospital X to the rehabilitation hospital. This link uncovered clusters of unrecognized SARS infections on a surgical ward and a psychiatry ward at hospital X. Investigation determined that the ventilation system did not contribute to the spread of SARS at that hospital. On May 23, 2003, hospital X was closed to nonobstetric admissions other than newly identified SARS cases, and SARS precautions were reintroduced. As part of an outbreak investigation, we explored potential factors contributing to the late recognition of SARS infections in a cohort of persons with SARS admitted to hospital X.

The Study

Hospital X is a Toronto-area community hospital with 425 beds. During the 2003 outbreak in Toronto, dedicated SARS inpatient units were created at the hospital. All non-healthcare workers with probable or suspected SARS, according to the WHO case definition (3), exposed at and admitted to hospital X with symptom onset from April 17, 2003, to June 8, 2003, were included in this retrospective cohort investigation. Healthcare workers were excluded. If SARS was not recorded as a possible diagnosis in the medical chart, despite SARS-defining manifestations for at least 24 hours of hospitalization, recognition of SARS was classified as late. Otherwise, recognition was classified as prompt. Laboratory diagnosis of SARS was obtained by reverse transcriptase–polymerase chain reaction (RT-PCR) or serologic testing (4,5).

SPSS version 11.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Continuous variables were dichotomized about the median. Factors associated with late recognition were deemed statistically significant if the p value was <0.05 (2-tailed), using chi-square test with Yates correction or Fisher exact test, when appropriate. Relative risks with 95% confidence intervals were listed as undefined when certain cell sizes were 0. The small sample size, small number of patients with the outcome of interest (late SARS recognition), and small cell size for some dichotomous variables precludes multivariate logistic regression analysis.

The SARS outbreak involved 88 case-patients whose exposure setting was hospital X (Figure). SARS occurred in 50 patients, family members, or visitors. Thirty-three of these 50 persons were admitted to hospital X, and all 33 were included in this analysis.

SARS-associated coronavirus (SARS-CoV) laboratory results were available for 29 (88%) of the 33 SARS patients. No samples were available for SARS-CoV testing from 4 deceased patients. Twenty-four (83%) of the 29 SARS patients tested were positive by RT-PCR, serology, or both. The 5 remaining patients had negative acute-phase SARS-CoV serologic results, and their convalescent-phase results were not available (data not shown).
Eleven (33%) patients had late recognition of SARS. Their mean age was 68.8 years, and 8 (73%) were postoperative patients. All were admitted before May 23, 2003, the day when Hospital X reintroduced enhanced SARS precautions. No case-patients had a recognized close contact with another SARS patient initially. None were travel related. (Table 1). Six (55%) patients were admitted to an intensive care unit (ICU), and 3 (27%) required mechanical ventilation (Table 2). All patients had infiltrates on chest radiographs; infiltrates of 9 (81%) were bilateral.

Using univariate analysis, we found that patients with late recognition of SARS were more likely to have no known contact with another SARS patient ($p < 0.001$), to have been a patient on a ward where SARS cluster occurred ($p < 0.01$), to be admitted before the nosocomial outbreak was recognized at the hospital ($p < 0.01$), to have symptom onset while hospitalized ($p < 0.001$), and to be a postoperative patient. ($p = 0.001$) (Tables 1 and 2). Clinical findings and laboratory abnormalities during hospitalization were not associated with late SARS recognition. The small sample size, small number of patients with late SARS recognition, and small cell size for some dichotomous variables precluded multivariate logistic regression analysis.

The hospital reintroduced enhanced SARS precautions on May 23, 2003, under the direction of public health authorities promptly after the nosocomial outbreak was recognized. For patients admitted before that date ($N = 20$), the relative risk for late recognition of SARS for postoperative patients was 2.7 (95% confidence interval 0.99–7.2, $p = 0.07$) and was just short of statistical significance (data not shown). Once SARS transmission was recognized at hospital X and enhanced infection control precautions were reinstated, clinicians were more likely to suspect SARS, and nosocomial transmission was ended abruptly.

**Conclusions**

Our results highlight the difficulty clinicians can have in recognizing locally acquired SARS among patients with other underlying medical conditions but with no apparent epidemiologic linkage. The patients had no known contact with other SARS patients, did not have a travel history to SARS-affected areas outside of Canada when the outbreak was thought to be over in Toronto, and were unaware of a simmering outbreak associated with hospital X. Perhaps because of the nonspecific nature of clinical manifestations, SARS can be especially difficult to recognize among

### Table 1. Proportion of patients with late SARS recognition by demographic and exposure characteristics, Toronto, hospital X, April 17–June 8, 2003*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Late SARS recognition (%)</th>
<th>RR (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;58.5</td>
<td>4/16 (25.0)</td>
<td>0.8 (0.2–1.7)</td>
<td>0.5</td>
</tr>
<tr>
<td>≥58.5</td>
<td>7/17 (41.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>3/15 (20.0)</td>
<td>0.5 (0.1–1.4)</td>
<td>0.3</td>
</tr>
<tr>
<td>M</td>
<td>8/18 (44.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aware of close contact with a SARS patient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0/14 (0)</td>
<td>UD</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>11/19 (57.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposure from being an inpatient on wards at hospital X with SARS clusters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8/13 (61.5)</td>
<td>4.1 (1.3–12.7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>No</td>
<td>3/20 (15.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Admitted to hospital X before May 23, 2003</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11/20 (55.0)</td>
<td>UD</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>No</td>
<td>0/13 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SARS symptom onset while hospitalized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11/15 (73.3)</td>
<td>UD</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>0/18 (0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*SARS, severe acute respiratory syndrome; RR, relative risk; CI, confidence interval; F, female; M, male; UD, undefined.*
patients already hospitalized for other reasons. These symptoms overlap many of the symptoms of hospitalized febrile postoperative patients and patients with other respiratory illnesses (6–12). In addition, at the time of this nosocomial outbreak, the full spectrum of the clinical signs and symptoms of SARS had not yet been well characterized (11).

To detect SARS early, health professionals need to look not only for epidemiologic links but also clusters of unexplained respiratory infection. A cluster of respiratory infec-
tions among families and visitors may not be evident initially because hospitals do not normally track infections in inpatients’ families and visitors. In addition, infected patients may be asymptomatic before they are transferred to another healthcare facility.

Even though our investigation generated interesting hypotheses, it had several limitations, which included reliance on retrospective chart reviews to abstract data. Such information may have been affected by missing data or recall bias. Our analysis did not include hospital work-

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Late SARS recognition/total SARS (%)</th>
<th>RR (95% CI)</th>
<th>p value</th>
</tr>
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<tr>
<td>Postoperative</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8/11 (72.7)</td>
<td>5.3 (1.8–16.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>No</td>
<td>3/22 (13.6)</td>
<td></td>
<td></td>
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<tr>
<td>Maximum temperature during hospitalization (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;38.8</td>
<td>5/15 (33.3)</td>
<td>0.8 (0.3–3.1)</td>
<td>1.0</td>
</tr>
<tr>
<td>≥38.8</td>
<td>6/15 (40.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First symptom includes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10/30 (33.3)</td>
<td>1.0 (0.2–5.4)</td>
<td>1.0</td>
</tr>
<tr>
<td>No</td>
<td>1/3 (33.3)</td>
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<td></td>
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<tr>
<td>Cough</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2/8 (25.0)</td>
<td>0.7 (0.2–2.6)</td>
<td>0.7</td>
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<td>No</td>
<td>9/25 (36.0)</td>
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<td>1/7 (14.3)</td>
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<td>No</td>
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</tr>
<tr>
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<td>1/5 (20.0)</td>
<td>0.6 (0.1–3.5)</td>
<td>0.6</td>
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<tr>
<td>No</td>
<td>10/28 (35.7)</td>
<td></td>
<td></td>
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<tr>
<td>Nausea/vomiting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1/1 (100)</td>
<td>3.2 (1.9–5.4)</td>
<td>0.3</td>
</tr>
<tr>
<td>No</td>
<td>10/32 (31.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Admitted to ICU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6/15 (40.0)</td>
<td>1.4 (0.5–3.8)</td>
<td>0.7</td>
</tr>
<tr>
<td>No</td>
<td>5/19 (27.8)</td>
<td></td>
<td></td>
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<tr>
<td>Supplemental oxygen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10/22 (45.5)</td>
<td>5.0 (0.7–34.2)</td>
<td>0.054</td>
</tr>
<tr>
<td>No</td>
<td>1/11 (9.1)</td>
<td></td>
<td></td>
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<tr>
<td>Mechanical ventilation</td>
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<tr>
<td>Yes</td>
<td>3/11 (27.3)</td>
<td>0.8 (0.3–2.3)</td>
<td>0.7</td>
</tr>
<tr>
<td>No</td>
<td>8/22 (36.4)</td>
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<tr>
<td>Death</td>
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<td></td>
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</tr>
<tr>
<td>Yes</td>
<td>4/10 (40.0)</td>
<td>1.3 (0.5–3.5)</td>
<td>0.7</td>
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<tr>
<td>No</td>
<td>7/23 (30.4)</td>
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<td>Treatment with</td>
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<td>Ribavirin</td>
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<tr>
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<td>0/2 (0)</td>
<td>UD</td>
<td>1.0</td>
</tr>
<tr>
<td>No</td>
<td>10/30 (33.3)</td>
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<tr>
<td>Corticosteroids</td>
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<td>Yes</td>
<td>2/15 (13.3)</td>
<td>0.3 (0.1–1.1)</td>
<td>0.06</td>
</tr>
<tr>
<td>No</td>
<td>8/17 (47.1)</td>
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<td></td>
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<td>Antimicrobial drugs</td>
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<tr>
<td>Yes</td>
<td>8/24 (33.3)</td>
<td>1.0 (0.3–3.0)</td>
<td>1.0</td>
</tr>
<tr>
<td>No</td>
<td>3/9 (33.3)</td>
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</table>

*SARS, severe acute respiratory syndrome; RR, relative risk; CI, confidence interval; UD, undefined; ICU, intensive care unit.*
Late Recognition of SARS in Nosocomial Outbreak

Dr. Wong is an epidemiologist with the Centre for Infectious Disease Prevention and Control at the Public Health Agency of Canada. His interests include infectious disease surveillance and emerging infectious disease response.

Acknowledgments

We acknowledge the efforts of the many clinical and public health professionals whose dedication stopped the spread of SARS in Canada.

Address for correspondence: Thomas Wong, Division of Community Acquired Infections, Public Health Agency of Canada, Room 3444, Building # 6, AL: 0603B, Tunney’s Pasture, Ottawa, Ontario K1A 0L2, Canada; fax: 613-941-9813; email: tom_wong@phac-aspc.gc.ca
Bacteremic Typhoid Fever in Children in an Urban Slum, Bangladesh

W. Abdullah Brooks,* Anowar Hossain,* Doli Goswami,* Amina Tahia Sharmeen,* Kamrun Nahar,* Khorshed Alam,* Noor Ahmed,* Aliya Naheed,* G. Balakrish Nair,* Stephen Luby,* and Robert F. Breiman*

We confirmed a bacteremic typhoid fever incidence of 3.9 episodes/1,000 person-years during fever surveillance in a Dhaka urban slum. The relative risk for preschool children compared with older persons was 8.9. Our regression model showed that these children were clinically ill, which suggests a role for preschool immunization.

Typhoid fever is a major cause of illness; the global incidence in 2000 was an estimated 21,650,974 cases with 216,510 deaths (1). The cause of typhoid fever, Salmonella enterica subspecies enterica serotype Typhi (S. Typhi), is both waterborne and foodborne, with an annual incidence approaching 1% in disease-endemic areas (2–4). Peak incidence is reported to occur in children 5–15 years of age; however, in regions where the disease is highly endemic, children <5 years of age may have among the highest infection rates (1,4–6). Population-based data are limited (1) and would be helpful for refining estimates of the impact of disease and for identifying age groups at highest risk, thereby making it possible to optimize vaccination strategies (7,8).

Data on disease severity and sequelae can contribute to estimating the impact of disease. Most complications—including intestinal perforation and peritonitis, encephalopathy, intestinal hemorrhage, hepatosplenomegaly, vomiting, and diarrhea (4,9)—are late onset. Whether children <5 years of age (preschool children) have silent infection or clinical disease is controversial (4,5,10), which has important implications for both case management and prevention. We report our findings from prospective, population-based active surveillance.

The Study

Since 1998, the ICDDR,B Centre for Health and Population Research has operated a surveillance and intervention site in Kamalapur, an urban slum in Dhaka, Bangladesh. We initiated fever surveillance for dengue fever and dengue hemorrhagic fever in August 2000. To identify treatable causes of fever, we obtained blood cultures from December 6, 2000, to October 8, 2001.

The community comprises 7 geographic strata, representing 379 clusters. We selected the surveillance cohort by using stratified cluster randomization and obtained informed written consent from all households.

Field research assistants screened household members for fever in their homes once weekly with a standardized questionnaire. We defined fever as ≥3 consecutive febrile days (reported) for persons ≥5 years of age, or any duration of fever for preschool children (<5 years of age). This definition facilitated detection of dengue fever. Field research assistants referred febrile participants to our field clinic, where study physicians confirmed fever and collected clinical data by using a standard form. Patients with an axillary temperature of ≥38°C were designated as febrile. After collecting blood for serologic tests of dengue and dengue hemorrhagic fever, we collected an additional 1 mL of blood from preschool children and ≥3 mL from older persons for culture.

Blood cultures were transported within 2 hours to our clinical microbiology laboratory (12 km from the field clinic). Specimens were processed by using standard methods with in-tube lysis centrifugation (Wampole isolator 1.5, Carter-Wallace, Inc., Cranbury, NJ, USA), plated on blood, chocolate, and MacConkey agar and incubated at 37°C for 16 to 18 hours. Colonies were evaluated with biochemical tests and confirmed by serologic identification with commercial antisera (Denka, Sieken, Co., Ltd., Tokyo, Japan). Antimicrobial susceptibility was determined by disk diffusion using standard NCCLS methods (11).

We confirmed typhoid fever if we isolated S. Typhi from blood during a febrile episode. Febrile controls were culture-negative for S. Typhi, Paratyphi, or Salmonella group D during fever.

If S. Typhi was isolated, then we treated the infection with 14 days of standard therapy, adjusting for antimicrobial susceptibility. First-line drugs were amoxicillin (40 mg/kg up to 1,500 mg orally divided 3 times daily) or cotrimoxazole (10 mg/kg trimethoprim divided into 2 daily doses). When patients remained febrile after 72 hours or new danger signs (e.g., lethargy, inability to drink, cyanosis, convulsions), developed, treatment was considered to have failed. We treated treatment failure in persons >12 years of age with ciprofloxacin (500 mg orally twice a day) and referred younger patients to the hospital. We defined recovery as ≥7 consecutive afebrile days after completing therapy.
Statistical analysis was performed by using Stata/SE Release 8.2 (Stata Statistical Software: Release 8.0. 2003, Stata Corporation, College Station, TX, USA). Incidence was determined by dividing the number of cases by person-years of observation, with calculation of exact 95% confidence intervals (CIs). Univariate analysis was performed by using 2-by-2 tables for relative odds (RO) and 95% CIs. We obtained p values by using the Fisher 2-tailed exact test. Multivariate modeling was conducted by stepwise forward logistic regression, using all covariates significantly associated with typhoid fever in univariate analysis. Covariates that were significant when age, sex, and geographic location were controlled for, were retained in the final model. We adjusted models for clustering of repeat patient visits and tested for goodness-of-fit with either Pearson or Hosmer-Lemeshow methods (12). Research Review and Ethical Review Committees of ICDDR,B approved this study.

During the study period, we took blood for culture from 888 (99.9%) of 889 eligible study participants; 54 (6.1%) reported prior medication exposure. All specimens had adequate volume. A microorganism was isolated from 65 (7.3%) cultures. Isolation rates were highest in winter. No positive culture reported >1 organism (Table 1), nor did any culture-positive patient have laboratory-confirmed dengue.

*S. Typhi* was isolated from 26 preschool children (Figure 1) and 23 older study participants (age range 10 months–50 years, median 4.0 years [95% CI 3.0–8.0]). There were 1,393 person-years of observation for preschool children and 11,014 for others. Overall, typhoid fever incidence was 3.9 episodes/1,000 person-years. Typhoid fever incidence among preschool children was 18.7 episodes/1,000 person-years and 2.1 episodes/1,000 person-years among older participants. The incidence rate difference between the 2 age groups was 16.6 cases/1,000 person-years (95% CI 9.4–23.8; p < 0.001). Preschool children’s relative risk for typhoid fever was thus 8.9 (95% CI 4.9–16.4). Typhoid fever among preschool children varied by age, with 4% in the first year of life and 85% occurring in those 2 to 4 years of age (Figure 2).

We investigated surveillance bias resulting from fever definition differences between age groups (4). Preschool children’s mean fever duration (days) prior to visiting the clinic was 4.0 (95% CI 3.2–4.8) and other patients’ mean duration was 4.9 (95% CI 2.9–6.8; p = 0.37). We collected 84.6% of preschool specimens and 78.3% of others’ after 3 febrile days, and 96.2% and 86.7%, respectively, by day 7.

A multivariate model showed that typhoid fever patients were more likely than febrile controls to be preschool age (RO 2.04; 95% CI 1.09–3.82; p = 0.03), have ≥3 days of fever (RO 2.55; 95% CI 1.16–5.63; p = 0.02), have temperature ≥39°C (RO 1.95; CI 1.01–3.80; p = 0.04), and have mental status changes (RO 3.94; CI 1.98–7.81; p < 0.02). Another model indicated preschool typhoid fever patients were significantly more likely than older patients to have fever ≥39°C (RO 1.62; CI 1.21–2.17), mental status changes (RO 3.54; CI 2.25–5.55), and crepitations (rales) on auscultation (RO 4.44; CI 3.11–6.33).

All patients with culture-confirmed typhoid fever recovered, except for 1 child with tuberculosis. Four adults required ciprofloxacin. No hospitalizations, complications, or deaths occurred among confirmed typhoid fever patients.

In vitro antimicrobial susceptibility testing (Table 2) showed a high prevalence of ampicillin, cotrimoxazole, and chloramphenicol resistance, with 27 isolates (55.1%) resistant to all 3; ceftriaxone resistance was found in isolates from 1 preschool child. Routine nalidixic acid testing was not performed, following NCCLS 2000 guidelines.

**Conclusions**

Our data indicate a high infection ratio in this urban population, which is highest among preschool children. These ratios are comparable to recent regional reports (4,6,13) and indicate that typhoid fever in preschool children may be underappreciated. That preschool children have 8.9 times the risk for *S. Typhi* infection as older persons corroborates age-specific rates in highly disease-endemic areas (1). The antimicrobial susceptibility data

**Table 1. Distribution of 65 blood culture isolates**

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. (%)</th>
<th>Cumulative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella Typhi</em></td>
<td>49 (75.4)</td>
<td>75.4</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>2 (3.1)</td>
<td>78.5</td>
</tr>
<tr>
<td><em>Acinetobacter spp.</em></td>
<td>4 (6.2)</td>
<td>84.6</td>
</tr>
<tr>
<td><em>Salmonella group D</em></td>
<td>2 (3.1)</td>
<td>87.7</td>
</tr>
<tr>
<td>Viridans-group <em>Streptococcus</em></td>
<td>2 (3.1)</td>
<td>90.8</td>
</tr>
<tr>
<td><em>Salmonella Paratyphi A</em></td>
<td>3 (4.6)</td>
<td>95.4</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>2 (3.1)</td>
<td>96.5</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>1 (1.5)</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Figure 1. Distribution of typhoid fever by age.
indicate high ratios of in vitro resistance to standard antimicrobial agents, with a high prevalence of multidrug resistance.

The degree of illness of preschool children is controversial; some report benign bacteremia (5,14) and others have found clinical illness (4,13). Our multivariate model shows that preschool children are clinically ill. Coexisting conditions, particularly pneumonia, are not only more common in preschool typhoid fever patients but also may result in misclassification and underreporting, as well as contribute to a worsening cycle of repeated infection and deaths. Future studies should explore these issues in this age group.

Substantial clinical illness among preschool children argues the need for them to be enrolled in vaccination programs. The age-specific infection rates suggest vaccination in the first year of life, integrating with existing Expanded Programme on Immunization (EPI) schedules. This practice would require either a polysaccharide protein-conjugate vaccine to stimulate T-cell–dependent responses (15) or a live attenuated oral vaccine, since T-cell–independent responses do not mature until the child is 18–24 months of age.

The limitations of this study could result in an underestimate of the incidence of typhoid fever. First, this study was not designed to measure typhoid fever incidence or disease impact. The surveillance program was designed to identify dengue. Thus, febrile episodes for young children were defined differently than for older persons. Although we did not find evidence of preferential selection for young children, future studies may adopt a common fever definition. Second, the blood volume examined, though not inadequate, may not have been optimal. Third, blood culture sensitivity is relatively low, estimated at 25%–50% (1). Fourth, the 6.1% estimate of earlier medicine exposure may be an underestimate, as we did not validate these reports. If these agents were antimicrobial, the number of serovar Typhi isolates recovered from peripheral blood would be reduced. Fifth, we had only 10 months of observation and therefore did not attempt an estimate of disease impact, adjustments for blood culture sensitivity, or exposure to antimicrobial agents. Ours is thus a conservative estimate of incidence. Further observation should allow the impact of disease to be estimated.

### Acknowledgments

We gratefully acknowledge Eric Mintz and Pavani Kalluri for their suggestions and assistance with the manuscript preparation.

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Author participation in this article was as follows: W.A. Brooks was the principal investigator and provided the conception, design, execution, and principal data analysis of this study, as well as preparing the manuscript. A. Hossain and K. Alam performed the blood cultures and determined sensitivities. D. Goswami and A. Naheed provided overall supervision of the project operation. A.T. Sharmeen and K. Nahar were responsible for the clinical staff. N. Ahmed supervised field operations. B. Nair, S. Luby, and R. Breiman were senior team members who contributed to the design, interim discussions of the project’s progress, data analysis, and manuscript preparation.

Dr. Brooks is a specialist in pediatrics and preventive medicine. He is on faculty at the Bloomberg School of Public Health at Johns Hopkins University in Baltimore, Maryland, from where he was seconded to ICDDR,B. He established an urban field site in 1998, from which he conducts surveillance and intervention studies on a variety of infectious diseases, primarily but not exclusively in children, including acute respiratory disease, dengue, typhoid fever, and shigellosis.

### Table 2. Antimicrobial resistance patterns of *Salmonella enterica* serovar Typhi, Kamalapur, 2001

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>% resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>55.1</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>57.1</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>57.1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.0</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>2.0</td>
</tr>
</tbody>
</table>

### References


Address for correspondence: W. Abdullah Brooks, ICDDR,B: Centre for Health and Population Research, GPO Box 128, Mohakhali, Dhaka 1000, Bangladesh; fax:503.210.0453; email: abrooks@icddrb.org
Molecular Evidence of Pneumocystis Transmission in Pediatric Transplant Unit

Britta Höcker,* Constanze Wendt,† Aimable Nahimana,‡ Burkhard Tönshoff,* and Philippe M. Hauser‡

We describe an outbreak of *Pneumocystis jirovecii* pneumonia in a pediatric renal transplant unit, likely attributable to patient-to-patient transmission. Single-strand conformation polymorphism molecular typing showed that 3 affected patients had acquired the same 2 strains of *Pneumocystis*, which suggests interhuman infection. An infant with mitochondrialopathy was the probable index patient.

Despite intensive medical treatment, *Pneumocystis jirovecii* pneumonia (PCP) is still a severe disease in immunocompromised patients, with a high death rate of up to 50 % (1). The first report of human *Pneumocystis* infection appeared in 1909; nevertheless, its epidemiology is poorly understood to date. In the 1950s, reports on PCP epidemics in malnourished infants in hospitals and orphanages aroused suspicion of interhuman transmission. In addition, animal studies have demonstrated airborne transmission of *Pneumocystis* (2). A case-control study conducted for a cluster of 5 PCP cases in transplant recipients suggested transmission of *P. jirovecii* from AIDS patients to other immunosuppressed persons (3). However, molecular typing methods for *P. jirovecii* were lacking so that patient-to-patient transmission could not be assessed at the molecular level. When such techniques were developed in the 1990s, 3 analyses showed different *P. jirovecii* genotypes within clusters (4–6). A recent analysis at the molecular level of a cluster of 10 PCP cases strongly suggested that HIV-infected persons with active PCP transmitted *P. jirovecii* to renal transplant recipients (7). The role of interhuman transmission of *P. jirovecii* in the epidemiology of PCP is still unclear.

The Outbreak

Having observed no occurrence of PCP in our pediatric renal transplant unit for the last 20 years and only 1 case in all German pediatric renal transplant units during the last 10 years, we encountered 3 consecutive incidents of PCP during a 5-month period. The first patient was a 13-year-old girl, who had received her second renal graft because of cystic kidney disease; PCP developed 4 months after transplantation. The second patient, a 14-year-old boy, fell ill in the ninth posttransplant month; he had bilateral vesicoureteral reflux as underlying renal disease. The third patient was a 13-year-old girl, who had a transplant 2 years before contracting PCP because of cystic renal dysplasia occurring in the context of Bardet-Biedl syndrome.

All 3 children had been given cyclosporine A (average dose 6.7 mg/kg/day), mycophenolate mofetil (1,060 mg/m²/day), and methylprednisolone (3.2 mg/m²/day), as maintenance immunosuppression. One patient had also received induction therapy with the interleukin-2-receptor-antibody basiliximab. All 3 children had been treated with methylprednisolone pulses for acute rejection episodes 2, 3, and 15 months before PCP was diagnosed.

Clinically, all patients showed nonspecific symptoms, such as mild fever, dyspnea, and dry cough in the absence of auscultatory anomalies. Laboratory tests showed an elevation of lactic dehydrogenase activity, C-reactive protein concentration in blood, and pronounced hypercalcemia (2.7–3.5 mmol/L), which was interpreted as an extrarenal production of 1,25-dihydroxyvitamin D₃ by activated alveolar macrophages. We found a significant reduction of S-adenosylmethionine concentration in plasma (6 nmol/L; normal range 86–128 nmol/L), which appears to be specific to PCP, unlike bacterial or other atypical pneumonias (8). We measured the blood count of CD4⁺ and CD4/DR⁺ T lymphocytes in the third patient to indicate the degree of immunosuppression, since antirejection therapy had been administered 15 months before the occurrence of PCP. The number of CD4⁺T cells was normal at the time of PCP diagnosis (1,100 cells/µL; normal range 505–1,151 cells/µL), while the number of activated T-helper cells was slightly decreased (24 CD4⁺DR⁺ cells/µL; normal range 29–87/µL). Only in the course of PCP did the numbers of CD4⁺ and CD4/DR⁺ T lymphocytes drop significantly (308 CD4⁺T cells/µL and 8 CD4⁺DR⁺ cells/µL). Chest radiographs and thorax computed tomographic scans of the 3 children showed typical signs of interstitial pneumonia, e.g., ground-glass opacity.

Diagnosis of PCP was confirmed by the presence of cysts and vegetative forms in bronchoalveolar lavage fluid, proved by immunofluorescence staining, and through detection of *Pneumocystis* DNA by means of polymerase chain reaction (PCR). In spite of intensive antimicrobial therapy, 2 of our 3 renal transplant patients died, at 10 and 28 days, respectively, after the onset of PCP.

To determine if PCP could have been caused by patient-
to-patient transmission, we closely examined the course of PCP in the infected children (Figure). Patient 1 stayed on the same ward and same floor, but not in the same room (distance between the rooms’ doors =10 m), as an infant with a yet-unclassified mitochondriopathy and pneumonia, which later was diagnosed as PCP. Patient 2’s hospital stay overlapped that of patient 1; the patients were on the same ward and same floor, in rooms with doors separated by 8 m, before the onset of PCP. Patient 3 spent her holiday with patient 2 in a summer camp organized by our Pediatric Nephrology Division.

Proceeding on the assumption that PCP in the 4 children resulted from patient-to-patient transmission, we investigated the genotypes of \( P. jirovecii \) with the multitarget single-strand conformation polymorphism (SSCP) method. This typing procedure is based on the amplification by PCR of 4 variable regions of the genome, followed by the detection of polymorphisms by means of SSCP. These 4 genomic regions are as follows: internal transcribed spacer number 1 of the nuclear rRNA genes operon (ITS1), the intron of the nuclear 26S rRNA gene (26S), the variable region of the mitochondrial 26S rRNA gene (mt26S), and the region surrounding intron number 6 of the \( \beta \)-tubulin gene (\( \beta \)-tub). Typing procedures were carried out as described elsewhere (9). A variable region amplified from a bronchoalveolar lavage fluid specimen can generate either 2 bands (simple pattern) or >2 bands (complex pattern). While a simple pattern corresponds to a single allele of the genomic region, the presence of >2 bands (complex pattern) indicates the existence of several alleles for a given region, most probably attributable to coinfection with multiple \( P. jirovecii \) types (10).

Our analysis showed that all 3 renal transplant patients had acquired the same 2 strains of \( Pneumocystis \), types 1 and 2. The infant with mitochondriopathy had been infected with >2 strains, which possibly included types 1 and 2 (Table). In contrast, 3 unrelated cases in patients (patients 4, 5, and 6) from the same hospital harbored other \( P. jirovecii \) types. The index of discrimination of the method was high (0.93) (10), and the probability that patients 1, 2, and 3 were infected with the same strains by chance is extremely low. We observed that the proportion of coinfecting strains within the clinical specimen was more important than the amount of template DNA to detect or not detect a strain by means of the SSCP typing method. A coinfecting strain has to represent 11% of the population to be detected (11). Coinfecting strains are missed when very low amounts of template DNA are used, sometimes resulting in a negative PCR; however, this was not the case for the specimens analyzed in the present study.

The index patient had more strains than the other patients (Table) but generated smaller amounts of PCR products with the 4 different PCR tests used, which suggests a lower amount of DNA template. Although excluding a common source of \( P. jirovecii \) is difficult, the results strongly suggest that all 3 kidney transplant recipients had infected each other, and the infant could have acted as index patient. This transmission may have occurred directly from 1 patient to another but also indirectly through immunocompetent carriers. Indeed, carriage of \( P. jirovecii \) DNA in the nose of immunocompetent relatives and healthcare workers in close contact with a PCP patient has been described (12).

Conclusions

To our knowledge, this report is the first published on an outbreak of PCP in a pediatric renal transplant unit, probably attributable to patient-to-patient transmission. However, we cannot exclude that the cases described were infected by the same environmental source. The presence of \( P. jirovecii \) in the air of hospital corridors has been described (13), making an environmental reservoir in the hospital possible. Other potential sources of \( P. jirovecii \) could be asymptomatic \( P. jirovecii \) carriers, such as immunosuppressed patients (14,15). Our findings at the molecular level suggest that \( P. jirovecii \) may be transmitted nosocomially and be acquired by immunosuppressed pediatric transplant recipients. The incubation periods of \( P. jirovecii \) infection (17, 15, and 19 weeks for patients 1, 2, and 3, respectively) would be longer than those (2–12 weeks) suggested by the previously described clusters of PCP (3,7,16). This finding may reflect a difference between adults and children.

Until the outbreak of PCP outlined in this article, pediatric renal transplant recipients in our hospital and other pediatric renal transplant units in Germany were not given PCP prophylaxis routinely because of possible side effects, such as a rise of serum creatinine values, myelosuppression,

| Week 2002 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 | 30 | 32 | 34 | 36 | 38 | 40 | 42 | 44 | 46 | 48 | 50 | 52 |
|-----------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Index Pat. | X | D | X | D | X | D | X | D | X | D | X | D | X | D | X | D | X | D | X | D | X | D | X |
| Pat. 1 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Pat. 2 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Pat. 3 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |

Figure. Course of \( Pneumocystis \) pneumonia (PCP) in 3 pediatric renal transplant recipients and 1 infant suffering from a yet-unclassified mitochondriopathy. RTx, acute rejection episode; RTx, renal transplantation; ↓, contact; #, joint holiday; > start of PCP symptoms; X, hospitalization; X, diagnosis of PCP; D, death.
and Lyell syndrome. We had not observed any case of PCP in our transplant recipients for the last 20 years without prophylaxis. In the light of the high death rate for PCP, prophylactic treatment with trimethoprim-sulfamethoxazole is highly recommended for the first 6 posttransplant months and during the 4 months after antirejection therapy, in accordance with the guidelines for adults (1).

According to these guidelines, patient 3, in whom PCP developed 15 months after steroid pulse therapy, would not have been protected by prophylaxis. Whether prophylaxis should be given for a longer period of time remains unknown, particularly since immunosuppression did not appear to be intensive in this patient at the onset of PCP, as indicated by the normal CD4+ T-lymphocyte count in peripheral blood and the only slightly decreased number of activated T-helper cells.

Dr. Höcker is a physician and research fellow at the University Children’s Hospital, Heidelberg, Germany. Her research activities are focused on immunosuppressive therapy and diagnosis and treatment of opportunistic infections in pediatric renal transplant recipients.

References

Address for correspondence: Britta Höcker, University Children’s Hospital, Im Neuenheimer Feld 150, 69120 Heidelberg, Germany; fax: 49-6221-564203; email: Britta_Hoecker@med.uni-heidelberg.de

Table. Pneumocystis jirovecii genotyping by PCR-SSCP of 4 genomic regions*  

<table>
<thead>
<tr>
<th>Patient</th>
<th>Date</th>
<th>ITS1</th>
<th>26S</th>
<th>mt26</th>
<th>β-tub</th>
<th>P. jirovecii type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Index case-patient</td>
<td>3/27/02</td>
<td>A</td>
<td>A, B</td>
<td>A, B, C</td>
<td>A, B</td>
<td>&gt;2 types (nonidentifiable)</td>
</tr>
<tr>
<td>1</td>
<td>7/26/02</td>
<td>A</td>
<td>A, B</td>
<td>A</td>
<td>A</td>
<td>1, 2</td>
</tr>
<tr>
<td>2</td>
<td>10/30/02</td>
<td>A</td>
<td>A, B</td>
<td>A</td>
<td>A</td>
<td>1, 2</td>
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<td>A</td>
<td>1, 2</td>
</tr>
<tr>
<td>4</td>
<td>2/7/03</td>
<td>A, B</td>
<td>A, B, C</td>
<td>B, C</td>
<td>B, C</td>
<td>&gt;2 types (nonidentifiable)</td>
</tr>
<tr>
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<td>A</td>
<td>A, B</td>
<td>C</td>
<td>A</td>
<td>6, 44</td>
</tr>
<tr>
<td>6</td>
<td>2/18/03</td>
<td>A</td>
<td>A, B</td>
<td>A</td>
<td>C</td>
<td>45, 46</td>
</tr>
</tbody>
</table>

*Bold letters signify the most abundant simple pattern with the complex one, as shown by silver staining. Bold numbers signify the most abundant P. jirovecii type. PCR, polymerase chain reaction; SSCP, multigene single-strand conformation polymorphism.
Slowly, purposefully, my mother unbuttons her blouse. It is blue with small white flowers, and the tail is tucked firmly into the elastic waistband of her salmon-pink pants. Beginning at the top and moving down, she works carefully at each of the small plastic buttons.

“Mother,” I plead with her, “you don’t need to do that.”

She smiles at me and continues unfastening buttons. Her padded cotton and elastic bra begins to appear. Her breasts swell pallidly above it.

The room is not well lit. The curtains are drawn, as they always are, against the sun. But I can see more of my mother than I wish to. My father, sitting here with me, says nothing. My wife, Gina, and two other women in the room also sit silently as my mother undresses herself.

I smell her perfume as she works at her blouse, her perfume and the lotion she lathers herself with every morning. I see the wrinkles beneath her arms, the flaps of skin at the elbows. She pulls off the blouse and stands before us with it in her right hand.

Her gray hair sprays in every direction. Her back is littered with small brown moles, her skin like ice over an old pond. And her dark eyes, fallen far back in the sockets of her skull, flutter from face to face like moths.

This is not, of course, my mother. My mother would never have bared this much of herself in front of strangers and certainly never in front of her son. My mother was quiet, shy, prudent. And this is, of course, my mother, her face, her hands, her dried out, fungus-ruined feet. But things have changed.

The nondescript, nappy, brown carpet is just as it always has been. The counters are still lined with the detritus of middle-class life. The cheap fan still chops at the air overhead, and the draining board with its plastic dish rack still drips dishwater into the same stainless steel sink. The clock with its three golden balls spools out the hours like kites, just as it always has. But my mother is mad. And the six of us have gathered today to evaluate her for custodial care. Custodial care! It sounds as though we might turn her over to the janitors at the university where I work. As though they might know what to do with her since we don’t. Appalled or not, though, we have no more time to twist our tales.

The Self

I am an immunologist. I have spent my life studying the intricate paths by which we protect ourselves from this infectious world, studying self, non-self, and why the two should never meet. But as a son watching his mother disintegrate, I am cut adrift.

My mother’s self, the thing that was her for all these years, the thing I had imagined fixed as flint beneath her bones has fractured, shattered like a crystal vase dropped on concrete. It is one thing to watch feathers grow from chicken-skin grafts on nude mice, quite another to watch your mother undress herself in front of total strangers.

Merriam Webster says that self is “the entirety of an individual, the realization or embodiment of an abstraction” (1). I don’t know what that means. Even though it somehow feels right, it seems woefully incomplete and metaphysical. As though no human using ordinary language could truly speak of my mother’s disappearance, no matter how concretely and obviously she is disappearing.

Sir Frank Macfarlane Burnet first described the necessity of biological self after watching an ameba ingest and digest another microorganism: “The fact that one is digested, and the other not, demands that in some way or other the living substance of the ameba can distinguish between the chemical structure characteristics of ‘self’ and any sufficiently different chemical structure as ‘non-self’” (2).

Later in contemplation of an immune response, Burnet added, “The failure of antibody production against autologous cells demands the postulation of an active ability of the reticulo-endothelial cells to recognize ‘self’ patterns from ‘non-self’ patterns in organic material taken into their substance” (3). “Demands” for after all, even the most primitive of us do not regularly eat ourselves. And even the most complicated of us do not regularly mistake our bodies for infectious enemies and destroy the very thing that sustains us. Burnet’s self becomes something substantial, something unique that our appetites and our immune systems ignore while they chew away at the rest of the organic world.
The fact that on the surface these two selves—the self of Merriam Webster and the self of Macfarlane Burnet—seem incommensurate, we probably owe to the Frenchman Rene Descartes.

The Divided Self

Descartes, a mathematician and philosopher, found himself one day deeply concerned with the reality of things. What could he truly trust? What was rationally and irrefutably real? We all know that we make mistakes at times about what is real—the monster under the bed, the shadow in the closet, purpose. Most of us just shrug it off, but Descartes was not so easily mollified. He secluded himself in a darkened room at the back of his chateau and set out to discover what was demonstrably real, trustworthy, certain (4,5).

Descartes considered what we learn through the senses, the stuff we see, hear, taste, touch, and smell—the physical world that apparently surrounds us. Is any of it truly real, unquestionably real? No. Almost immediately, he realized our senses can fool us. Dreams provide hard evidence of that. While we are in a dream, we become completely absorbed with false reality. Dreams do not announce to us that they are not “real.” And many things in the “real” world (mirages, optical illusions, sleights of hand) do not announce to us that they are false.

Descartes, the inventor of analytical geometry, turned to the reality of mathematics, a priori knowledge—knowledge accessible without sensory perception. Because of his deep investment in mathematics, Descartes thought a priori knowledge inviolable, beyond reproach, above suspicion. But as he delved deeper, he realized that some evil genius might have fooled us about mathematics. Mathematics might be nothing more than an elaborate ruse with nothing whatsoever to do with reality (as many of us suspected in grade school). He was forced to abandon mathematics as well as sensory knowledge. Without mathematics and the physical world the only things left to Descartes were his own thoughts. He realized that rationally and philosophically he could not question the reality of the questioner. It simply wouldn’t make sense. So his questions proved his own existence, even if he could not establish the existence of anything else. Cogito ergo sum.

Had Descartes been a microbiologist, things might have ended differently. But for the mathematician, the world devolved to one man’s thoughts. Descartes rested then, in the midst of an absolutely solitary universe. Two types of things existed, the seemingly real but demonstrably untrustworthy physical world (res extans), and the truly real world of the mind (res cogitans). These were two completely separate worlds. The one outside our heads was full of machines and ghosts, including our own bodies. The one in our thoughts was concrete, real, essential.

Reality flourished inside human thought, specifically Descartes’ thought. The rest was doubtful. When he was finished, Descartes had scalpeled the self off the body.

The self, he claimed, was something other than the physical world that surrounds us. Selves did not come from the same stuff as trees, and stones, and arms, and legs, and knuckles, and immune systems. Selves came from somewhere else. Self stuff and body stuff were distinct and immiscible.

But almost 400 years later, as I watch my mother fumble with the tails on her blouse, I am little comforted by Rene Descartes.

The Biological Self

We finally convince my mother to put her blouse back on and button it. It takes her two tries, but she now has each button in its proper hole. I am embarrassed. She seems unabashedly pleased with herself—what remains of it. She smiles again at me. I turn to one of the women seated quietly across the room. I look for forgiveness or some sort of reassurance that my mother’s antics haven’t ruined this for all of us.

“She will do perfectly,” Jennifer says.

“Does she wander at night?” Melissa asks.

Lesch-Nyhan disease was first described in 1964, by two physicians named Michael Lesch and William Nyhan. Two brothers appeared one afternoon in these doctors’ clinic. Both boys were manifesting bizarre but identical symptoms. Much about these original cases turned out to be characteristic of most cases of Lesch-Nyhan disease, which affects boys almost exclusively. And the fact that the disease manifested identically in the twin boys suggested that an altered gene was involved.

Lesch-Nyhan disease is caused by a mutant gene on the X chromosome. Women have two X chromosomes, men only one. So problems on an X chromosome are sometimes hidden in women by the normal allele on the alternative X chromosome. But Y chromosomes have almost nothing in common with X chromosomes. So X-linked mutations are almost always apparent in men.

A single mutation in the gene that encodes an enzyme called hypoxanthine-guanine phosphoribosyl transferase, or HGPRT, is responsible for Lesch-Nyhan disease. In its mutant form, the enzyme does not function. HGPRT catalyzes a biochemical process called purine salvage. Purines are used to make DNA and RNA—the stuff of genes and genetic control and transcription. Because of the importance of DNA synthesis, we have more than one way to make purines. We can synthesize purines from scratch or salvage purines from DNA-breakdown products in our blood (6). People with Lesch-Nyhan disease cannot salvage purines. These people must rely on the purines they
When he stopped her and asked where she was going, she time she wasn’t even wearing the bottoms of her pajamas. In Kanab, Utah, making her way toward town. The first to. “Twice, that I know of, Dad found her outside the house for self. Survivors and reproducers. In these genes is the template and reproduction. Our genes come from a very long line of us with some specific edge in the struggle for survival from necessity. No longer did anyone have to be everything, “me” and “you” were necessary now. While sense of self was perhaps a long way off, self was there, that day, swimming in a thin broth of “other.”

First-self had walked onto the stage. Pronouns became meaningful. “Us” was no longer sufficient to describe everything, “me” and “you” were necessary now. While sense of self was perhaps a long way off, self was there, that day, swimming in a thin broth of “other.”

Bacteria were a major step up from the muck. They were, after all, living, but they suffered from one huge drawback: each of them had only one cell to work with. That meant then, and still means now, that most bacterial cells had to do everything, all the time, all at once. Each cell had to see, hear, touch, taste, and smell. Each cell had to eat and excrete, reproduce and think. Each cell had to make everything that was needed for the survival of the individual. Because of this, bacteria, though remarkable survivors, weren’t and aren’t much good at anything beyond simple survival—poetry probably baffles them.

One day, all of this began to change. A few cells got together, cemented themselves to one another with some new glue. A protoplasmic hand reached into the void and another hand took hold. The door of opportunity swung wide open. For the first time, individual cells were freed from necessity. No longer did anyone have to be everything for everyone. No longer did anyone face everything alone.

Cellular specialization took the world by storm. Some cells stopped eating and became eyes (or something that would one day become eyes), others ears, others nerves, others muscles—there were no limits. Taste buds, antennae, pincers, intestines, hearts, tails, legs, arms, muscles, bones, livers, lungs, hair, nails, claws, blood, hide, and horn were all within reach.

But almost immediately, everyone saw that cellular specialization, alone, led nowhere. Before multicellularity could be had, selfishness was needed. The first few multicellular creatures probably shared everything with everyone. After all, they had no means to distinguish among.
come first. I am not to be eaten by others. I am to eat others. I am to reproduce first. The things I see are for me and me alone.

But “I” had no means yet for such distinction. What or who was me and what or who was not? I could not decide. Before I could reach for the stars, I had to reach within and find some way to know myself from others. Without a sense of self we are little more than bacteria—maybe less.

If I am to keep what I have earned, if I alone am to benefit from my mutations and absorptions, my specializations, my senses, my motility, I must know self from non-self. My eyes must be for my self. My thoughts must be my own. My heart must beat only for me. I must keep all that I can to my self at the expense of non-self, or I have gained nothing.

Selves leave no fossils, so we cannot know for certain how the first colonial (multicellular) organisms came to sense their selves. But biologically, biochemically, basically, they had to know, everything depended on it. And the biology and chemistry of that knowledge were and are the only things between each of us and the rest of us. The evolved self, the self geneticized. A protein marker, perhaps, carried by every cell inside every one of us. A passport to be checked and rechecked at every interaction. A self to be validated over and over.

For a few millennia, that was probably good enough. But life was changing. Microorganisms discovered parasitism. Once inside another’s membrane, food cost nothing, life was simple, and reproduction was almost guaranteed. Now, self discrimination was not enough. Once others learned to hide within self, force was needed to maintain boundaries. Now, we needed immunity to keep us whole. Once again, if our own mutations and adaptations were to serve us, the integrity of self was essential. Infectious diseases posed the first great challenge to the biological preeminence of self. Immune systems quickly found ways to detect and destroy non-self.

In the beginning, biological self was probably nothing more than a simple system for recognition of other and recognition of non-self as food. Now self had teeth. Now self rose like a shield to stand between us and those who would destroy us to further their own journey towards reproduction. We became what we served and protected.

Infection, Immunity, and Self

Over time, the self grew. Like the brain, layers upon layers of self formed inside living things. Like the cerebral cortex, late in evolution, psychological self arose—self-conception, self-perception, self-deception. But still, like the amygdala in the brain, beneath the complicated and sophisticated self beats the heart of a beast, focused only on food, survival, and sex.

Unlike the brain, the layers of self are strewn throughout the body. In between the layers is immunity and infection. And that, it seems, ties it all together. When the psychological self is stressed, the immunologic defense of self falters. Perceived threats—exams, other men and women, public speaking, air travel—stimulate the hypothalamus to produce corticotrophin-releasing hormone (CRH), which stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH induces the adrenal glands to produce cortisol. Cortisol suppresses the immune system (8). The two selves synergized. Furthermore, the consequence of this change in perception of self or environment is, as you might expect, accompanied by considerable increase in susceptibility to infectious diseases (9–13). How we think about ourselves and our surroundings changes our resistance to disease.

Infection and inflammation cross the bridge between selves in the opposite direction. Every organ of the immune system is innervated. Every spot where an immune response takes place is hardwired to the brain. Immunology and neurology are irreversibly intertwined. Interleukins produced by activated macrophages and T cells act on the adrenals, the hypothalamus, the pituitary, and the brain stem. In response, moods change, libido drop, self-perception fogs, appetites trail off, and sleep becomes nearly impossible (8).

Infection, inflammation, and immunity shape self. Self-perception derails immunity. Cytomegalovirus and T. gondii have been implicated in the etiology of schizophrenia (14,15). People with bipolar disorder are more frequently infected with herpesvirus type 1 than people without bipolar disorder (16). Mice born to mothers infected with influenza virus never develop a lust for exploration (17). And of course infection by other parasites, bacteria, and viruses can change animal behavior in unexpected and consequential ways. Infection, at least at times, changes our mental perceptions of ourselves and our surroundings.

Infections change our immune perceptions as well. Among more than 3 million U.S. military personnel followed from 1988 to 2000, the strongest predictors of multiple sclerosis were serum levels of immunoglobulin (Ig) G antibodies to Epstein-Barr virus viral capsid antigen or nuclear antigen (18). Multiple sclerosis is a remarkable autoimmune disease in which the immune system attacks the nervous system—self vs. self. And viral or bacterial infections have been implicated in the etiologies of rheumatoid arthritis, diabetes mellitus, Crohn’s disease, and some types of thyroiditis—all autoimmune diseases. These examples indicate that at least some infections cloud
the immunologic border between self and non-self. As our understanding of infection and immunologic self-perception deepens, more examples will likely surface. Further research will elucidate the interdependence between immunologic and psychological disorders and the link between both and infectious diseases.

Animals protected from birth against infections never develop any functional sense of immunologic self (19). Human autoimmune diseases, such as diabetes mellitus, show a correlation with schizophrenia (20) and other behavioral disorders. Cause and effect relationships remain obscure, but a link may exist between infectious disease and immunologic as well as psychological perception of self. Infection, immunity, and inflammation, like water on old plywood, sometimes split and sometimes cement the layers of self. But together or apart, self is a brick in the bulwark of human biology.

Last Self

All her life my mother preferred things simple. She liked jam better than jelly. She loved cornbread and blackstrap molasses, white gravy, melons, “Amazing Grace.” She hated driving. She grew up poor, truly poor. Maybe poverty burned up all the fuel she was saving for complexity before she ever found any. Regardless, her tastes never changed. She was always most comfortable with ordinary things.

I remember her simplicity. But by the start of the second year of her custodial existence, no matter how hard I tried, I could no longer remember much of anything else about how she once was. I couldn’t recall when her hair might have been brown and combed, her pants not fat with diapers, her smile less vacant.

The craters of her face were gaunt and empty now. Every cold and flu wrecked her lungs and sent mucus cascading from her nose, across her mouth, and onto to the tiled hall to the dining room. She ate Salisbury steak and mashed potatoes, green beans and corn, peach pie with ice cream. And likely she would have eaten even more if anyone had offered it. As she ate, she stared across the top of her fork at the brown plastic tabletop. I watched her chin moving with the food and her eyes as they chewed slowly on nothing. Neither of us spoke. There was really no need.

The last day she spoke to me, she was dressed in red sweat pants and a loose purple jersey top with long sleeves. Her nose ran. When I came into her room, she was lying in bed staring at the ceiling. She rarely spoke complete sentences. She never recognized me. I sat next to her and for several moments said nothing.

The room was split in two by a red blanket hung as a curtain. On the other side of the curtain was another bed. Sometimes Mom had roommates, but for the last week or two, no one had occupied the other bed. Full or empty, the other bed held no interest for Mother. The floor was covered in spattered beige tiles.

I enjoyed these moments, sitting quietly next to her—moments stolen from reality, shielded from certainty. I wasn’t an immunologist here, just an old woman’s son wishing for things that could never be. I reached out and held her hand, thin now with thickened blue veins and knuckles fat from inflammation. She turned to me.

“How are you?” I asked because whether someone is dying, bleeding to death from a severed limb, or just finishing a pastrami sandwich, that question inexplicably comes to my lips.

“Fine,” she said and turned back to the ceiling, smiling. “I’m fine.”

I wiped her nose.

Today, she wore no lipstick, and the aides had taken her bridge from her mouth. Four of her lower front teeth were missing. Her tongue fell through the opening when she spoke and twisted her words.

“What would you like to do today, Mom?” I asked not really expecting anything.

She looked up at me with eyes deep-brown as mahogany. She pursed her lips beneath her small mustache. And for a moment her eyes moved off to one side as though she actually thought about what I’d asked. Finally she looked back into my eyes and said to me:

“Hello,” she said with obvious pleasure.

Then she lifted my hand to her lips and kissed my finger tips.

“How are you?” I asked because whether someone is dying, bleeding to death from a severed limb, or just finishing a pastrami sandwich, that question inexplicably comes to my lips.

“Fine,” she said and turned back to the ceiling, smiling.

“I’m fine.”

I wiped her nose.

Today, she wore no lipstick, and the aides had taken her bridge from her mouth. Four of her lower front teeth were missing. Her tongue fell through the opening when she spoke and twisted her words.

“What would you like to do today, Mom?” I asked not really expecting anything.

She looked up at me with eyes deep-brown as mahogany. She pursed her lips beneath her small mustache. And for a moment her eyes moved off to one side as though she actually thought about what I’d asked. Finally she looked back into my eyes and said to me:

“I’m hungry.”

“Then let’s eat.”

I lifted her into her wheel chair and rolled it down the tiled hall to the dining room. She ate Salisbury steak and mashed potatoes, green beans and corn, peach pie with ice cream. And likely she would have eaten even more if anyone had offered it. As she ate, she stared across the top of her fork at the brown plastic tabletop. I watched her chin moving with the food and her eyes as they chewed slowly on nothing. Neither of us spoke. There was really no need.
Dr. Callahan is associate professor of immunology/public understanding of science and associate professor of English at Colorado State University. His research interests focus on the relationship between infectious and behavioral disease.

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Address for correspondence: Gerald N. Callahan, Department of Microbiology, Immunology, and Pathology, Colorado State University, 1619 Campus Delivery, Fort Collins, CO 80523, USA; fax: 970-491-0603; email: gerald.callahan@colostate.edu
To the Editor: Each year ≈350,000 Americans travel to Africa and ≈500,000 travel to Brazil and the Far East, all schistosomiasis-endemic regions. Data from the European Network on Imported Infectious Diseases Surveillance (TropNetEurop) suggest that most schistosomiasis cases imported to Europe are acquired in Africa; 80% of new cases worldwide occur in sub-Saharan Africa (1,2). Travelers to Africa from the United States are also at high risk for infection. Schistosoma mansoni has the greatest impact on residents of disease-endemic areas who have high-grade infection and progressive hepatosplenic disease with portal hypertension and its manifestations. Most infected, short-term travelers sustain a low-level of fluke infestation with few symptoms, although serious complications can occur.

We report a 38-year-old American man with ectopic S. mansoni fluke migration that led to neural schistosomiasis. His symptoms prompted us to test family members who had accompanied him on a trip to Kenya 5 years earlier. The family members had been unaware of the risk for schistosomiasis that the trip posed. Five years after a Kenyan safari during which the index patient visited northeastern Lake Victoria and swam 1 afternoon, vertigo, nausea, and nystagmus developed. The results of liver function tests were normal and peripheral blood showed no eosinophilia. Biopsy of a large cerebellar lesion noted on magnetic resonance imaging (MRI) was diagnostic, yielding multiple S. mansoni ova within large eosinophilic granulomas, consistent with tumoral neuroschistosomiasis. We tested 24 of 25 family members who had accompanied him to Kenya for schistosomiasis (Figure). All of the accompanying family members, except 3 women, had gone into the water. All members were well, except an 8-year-old boy, in whom granulomatous colitis had developed after the trip.

Eighteen of 25 enzyme-linked immunoassorbent assays (ELISA) were positive for S. mansoni infection, including that of samples from the index patient and the boy (Figure). ELISA was performed on 18 samples at the Centers for Disease Control and Prevention (CDC) and 7 samples at Focus Technologies. Both tests used the same CDC-produced antigen, the microsomal fraction of adult S. mansoni fluke, which has both a sensitivity and specificity for S. mansoni of 99%. Confirmatory immunoblots were performed at CDC on samples from 19 of the 25 ELISA-tested family members, with 1 discordant result, a positive ELISA and negative S. mansoni and hematobium immunoblots. Three of 7 ELISA-negative family members were the nonswimmers. Analyses of single stool specimens from 7 family members, including the index patient, and 1 rectal biopsy sample were negative for ova.

Because of the high positivity rate, praziquantel was prescribed for all 26 travelers. The index patient received 20 mg/kg of praziquantel twice daily for 4 days and high-dose dexamethasone with subsequent 2-month taper; his symptoms resolved over months. An MRI 8 months after treatment demonstrated minimal residual inflammation. All other family members received 20 mg/kg of praziquantel twice in 1 day and tolerated it without adverse events. Ten months after treatment, the boy is growing after years of an inflammatory colitis characterized by hematochezia and growth retardation. He continues to have nonbloody diarrhea and constipation.

We postulate that the mature fluke pair migrated from the mesenteric veins through Batson’s vertebral-venous plexus to the cerebral veins at the cerebellar level. There the female expelled multiple ova into the cerebellum. An ensuing vigorous granulomatous response led to posterior fossa mass effect and compression of medullary nausea centers, which resulted in the patient’s nausea, vertigo, and nystagmus. Ectopic ovum migration more commonly causes neuroschistosomiasis; however, in this case, multiple ova within 1 granulomatous mass suggest fluke-pair migration rather than individual ovum migration. Neuroschistosomiasis is most commonly associated with...
S. japonicum, which has smaller ova. In the literature, we found 16 other case-patients with intracranial tumoral S. mansoni. Eight of the patients demonstrated cerebellar involvement, which suggests a common fluke migratory pathway (3–15). Like our patient, 6 others were not native to disease-endemic regions.

This unsuspected case of neural schistosomiasis illustrates the need for detailed inquiry into every freshwater exposure by persons who have traveled to schistosomiasis-endemic regions. Adult Schistosoma flukes generally survive in venules from 6 to 10 years but can survive <40 years; therefore, remote travel history is relevant. Examination of stool samples for ova has been considered the standard method of diagnosing S. mansoni and S. japonicum infection, and urine examination is used to diagnose S. haematobium. Multiple, fresh morning specimens are ideal. However, stool examination is not likely to be as sensitive as current immunologic assays for detecting low levels of infection. Moreover, in disease-nondeemic regions, operator variability may influence ova detection. Among 13 recorded cases of neurotumoral S. mansoni in which stool specimens were examined, no stool ova were found in 5 cases. In our family cohort, among the 7 ELISA-positive members who submitted stool specimens, no examinations performed at CDC demonstrated eggs (Figure).

The ELISA uses a highly sensitive and specific antigen for S. mansoni. Because the sensitivity is less for S. haematobium and S. japonicum, subsequent species-specific immunoblots are recommended based on travel history that suggests exposure to specific species. Thus, we recommend ELISA, immunoblot if applicable, and stool or urine examination for travelers with possible exposure in disease-endemic regions. ELISA does not have the same utility in persons native to disease-endemic regions because positivity is also consistent with earlier infection. Stool or urine examination is diagnostic in suspected immigrant case-patients.

In all cases, knowing that stool or urine examination shows ova is valuable because repeat examination at 4 to 6 weeks can be used to monitor treatment response. Because praziquantel is well tolerated and effective, empiric therapy among returning travelers after possible exposure is reasonable. However, diagnosing infection when possible and demonstrating cleared infection after therapy are more prudent approaches, particularly as praziquantel resistance emerges (16).

In conclusion, pretravel counseling against freshwater exposure and posttravel screening for schistosomiasis of persons with any freshwater exposure in disease-endemic regions are warranted. As illustrated, the diagnosis of schistosomiasis in a returned traveler should prompt screening for infection in fellow travelers.

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Valeriana Amorosa,*
Daniel Kremens,*
Martin S. Wolfe,††
Timothy Flanigan,§
Kevin M. Cahill,¶**
Kevin Judy,*
Scott Kasner,*
and Emily Blumberg*

*University of Pennsylvania, Philadelphia, Pennsylvania, USA; †George Washington University, Washington, DC, USA; ¶Georgetown University, Washington, DC, USA; §Brown University, Providence, Rhode Island, USA; ¶Royal College of Surgeons, Dublin, Ireland; #New York University, New York, New York, USA; and **Lenox Hill Hospital, New York, New York, USA.

References
Community-associated Methicillin-resistant Staphylococcus aureus, Singapore

To the Editor: Community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA) is an emerging phenomenon that has been reported from almost every continent in the world (1–4). Such strains are usually characterized by multisusceptibility to non-β-lactam antimicrobial drugs, production of Panton-Valentine leukocidin (PVL), and presence of staphylococcal chromosome cassette mec (SCCmec) IVa, a novel smaller variant of the methicillin-resistance locus (5). The genetic backgrounds of CA-MRSA strains from different parts of the world are distinct and specific for each geographic region (1–5).

We conducted a study at our institution, a 1,600-bed adult acute-care, tertiary-level public hospital, to determine evidence and the clinical and molecular profile of CA-MRSA in Singapore. We reviewed the microbiology laboratory records at our institution for multidrug-susceptible MRSA strains isolated from January 1, 2001, to April 15, 2004. S. aureus was identified by colony morphologic features, coagulation of citrated rabbit plasma with EDTA (BBL Becton Dickinson and Co., Cockeysville, MD, USA), and production of clumping factor and protein A (BactiStaph, Remel, Lenexa, KS, USA). Methicillin resistance was determined by susceptibility testing and confirmed by latex agglutination for penicillin binding protein-2a (6). Multidrug-susceptible strains were defined by susceptibility to cotrimoxazole and gentamicin as determined by the Kirby-Bauer disk diffusion method following NCCLS guidelines (7).

The medical records of patients infected by these MRSA were reviewed, and strains were labeled community-associated if they had been isolated within 48 hours of hospitalization from patients who had not been in any hospital for >1 year. Community-associated strains were tested for PVL genes (8), and the SCCmec was typed by following a previously described method (9). Molecular typing was done by pulsed-field gel electrophoresis (PFGE) with restriction endonuclease Smal and multilocus sequence typing (10).

Eight of 266 multidrug-susceptible strains fulfilled the criteria for community acquisition, but only 5 of these strains (corresponding to patients 1, 3, and 6–8) had been archived. The demographic and clinical data of the patients are shown in the Table. Most were young, healthy adults with cutaneous abscesses. Patient 1 had diabetes mellitus but had never been hospitalized; he was the only patient with severe bacteremic pneumonia. Patient 6 had early-stage endometrial cancer resected in 2000 but had not attended her follow-up appointments for >1 year before her hospitalization. Patient 8 had traveled to Taipei,

| Table. Demographic and clinical data of patients with community-associated methicillin-resistant Staphylococcus aureus (MRSA) |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Ethnicity | Indian | Filipino | Chinese | Chinese | Filipino | Chinese | Filipino | Chinese |
| Age | 52 | 20 | 38 | 37 | 31 | 56 | 21 | 33 |
| Sex | M | F | M | M | F | F | F | F |
| Coexisting conditions | Diabetes mellitus | – | – | – | – | Endometrial cancer | – | – |
| Infection type | Pneumonia, bacteremia | Hand abscess | Hand abscess | Hand abscess | Hand abscess | Hand abscess | Hand abscess | Hand abscess |
| Therapy* | IV vancomycin | Yes | No | No | No | No | No | No |
| Appropriate antimicrobial drug usage | I&D | I&D | I&D | I&D | I&D | I&D |

*Therapy: IV, intravenous; –, not applicable; I&D, incision and drainage of abscess.
Taiwan, for a month; the abscess developed 3 days after her return home. Travel history was not documented in the other patients’ records. Patients 2–8 received β-lactam antimicrobial drugs in addition to surgical drainage of their abscesses and recovered without any complications.

All 5 archived strains had different molecular and toxin profiles, and the only consistent feature was the presence of PVL genes. Isolates 3 and 7 possessed SCCmec IV. Isolates 1, 6, and 8 were mecA positive, but their SCCmec belonged to none of the 4 major structural types. Comparisons with published data on CA-MRSA strains showed that isolate 7 was identical to the European strain of CA-MRSA in terms of PFGE pattern, toxin profile, and sequence type (ST 80) (2,5). Isolate 3 had an identical PFGE pattern and sequence type (ST 30) compared to the Oceanian Southwest Pacific strain but differed slightly in toxin profile, as the LukD-LukE leukocidin genes were absent (3,5). Isolate 8 was similar to the Taiwanese strains: it was ST 59 and had non-typable SCCmec (4). It belonged to agr 1 and tested positive for enterotoxin sek, γ2-hemolysin, and β-hemolysin genes.

Isolate 6 had a PFGE pattern that may be distantly related to U.S. strains; the similar sequence type (ST 1) served to emphasize this, although the presence of nontypable SCCmec rather than SCCmec IV implied that methicillin resistance was acquired differently. It belonged to agr 3 and tested positive for LukD-LukE leukocidin, enterotoxins seb and seh, and γ2-hemolysin genes. Isolate 1 is unique to Singapore in that it had a novel sequence type (ST524: 7-6-1-5-71-5-6 and SCCmec). It belonged to agr 1 and tested positive for γ-hemolysin gene as well as for the enterotoxin gene cluster.

Widely diversified CA-MRSA strains exist in Singapore. The demographic profile and clinical symptoms of local patients infected with these strains were consistent with published literature (2–4). The lack of a pediatric unit at our institution prevented a more complete epidemiologic description.

In contrast to previous reports (1–5), our findings are unique in that most of our strains do not have a distinctive molecular profile and may be related to strains from different parts of the world. Epidemiologic and molecular data strongly suggest that isolate 8 was imported from Taiwan. Some of the other strains (especially isolates 3 and 7) may have been imported from other countries too, as Singapore is an international travel hub with >6 million visitors annually.

CA-MRSA has only been isolated sporadically in Singapore, and no dominant clone was seen among our isolates. Singapore may be in an early phase of CA-MRSA emergence, and healthcare workers should remain vigilant for future outbreaks.

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Li-Yang Hsu,* Anne Tristan,† Tse-Hsien Koh,* Michèle Bes,† Jerome Etienne,† Asok Kurup,* Thuan-Tong Tan,* and Ban-Hock Tan*  
*Singapore General Hospital, Singapore; and †Faculté de Médecine Laënnec, Lyon, France

References

Address for correspondence: Li-Yang Hsu, Department of Internal Medicine, Singapore General Hospital, Outram Rd, S169608, Singapore; fax: 65-67322601; email: liyang_hsu@yahoo.com
Mumps Virus–associated Hemophagocytic Syndrome

To the Editor: Virus-associated hemophagocytic syndrome (VAHS) is a fulminant disorder associated with systemic viral infection and is characterized pathologically by the proliferation of hemophagocytic histiocytes in the lymphoreticular tissues. Here we report a case of mumps VAHS following parotitis and pancreatitis.

A 39-year-old, previously healthy woman sought treatment for abdominal pain on June 14, 2002. On physical examination, her bilateral parotid glands were swollen, and her left upper quadrant was tender. Laboratory studies showed a leukocyte count of 4,640/mm³, a hemoglobin concentration of 10.9 g/dL, and a platelet count of 9.1 × 10⁴/mm³. Laboratory studies showed a leukocyte count of 2,350/mm³, a hemoglobin concentration of 10.9 g/dL, and a platelet count of 104/mm³. Laboratory studies showed an interleukin-6 of 12.7 pg/mL (normal 4.0–64.2 ng/mL), and soluble interleukin-2 receptors (1,660 U/mL; normal 145–519 U/mL). Hypercytokinemia was also shown, with an interleukin-6 of 12.7 pg/mL (normal <3.1 pg/mL). Her bone marrow was normocellular, and an increased number of histiocytes with hemophagocytosis was found. Extensive cultures and serologic studies for microbial and viral infections were all negative, whereas tests for immunoglobulin G and immunoglobulin M antibodies against the mumps virus were both positive. Mumps VAHS was diagnosed. Treatment with corticosteroids led to a complete remission of symptoms.

VAHS was initially reported by Risdall et al. in 1979 (1). Although the precise pathogenesis of VAHS remains unknown, current hypotheses focus on the roles played by activating cytokines. VAHS has been reported in connection with a variety of viruses: adenovirus, cytomegalovirus, dengue, Epstein-Barr, hepatitis A, hepatitis B, hepatitis C, herpes simplex, HIV, human herpesvirus 6, human herpesvirus 8, influenza A (antigenic type H1N1), measles, parainfluenza type III, parvovirus B 19, rubella, and varicella-zoster (2). This report is the first of a VAHS case associated with a mumps virus infection. The clinical course of VAHS is highly variable, and in some cases, especially in Epstein-Barr virus infection, VAHS is a dramatic illness with a potentially fatal outcome (2). This case implies that mumps VAHS may have a positive prognosis.

Kunihiho Hiraiwa,* Katsuyuki Obara,† and Atsuhisa Sato†

*Hamamatsu Red Cross Hospital, Hamamatsu, Japan; †Mito Red Cross Hospital, Mito, Japan

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Imported Cutaneous Diphtheria, Germany, 1997–2003

To the Editor: The March 2004 report by de Benoist et al. on the incidence of imported cutaneous diphtheria in the United Kingdom (1) prompted us to describe the situation of cutaneous diphtheria in Germany and to analyze the cases reported to the German Consiliary Laboratory on Diphtheria since its establishment at our institute in 1997. The laboratory provides advisory and diagnostic services mainly to microbiologic laboratories throughout Germany.

From 1997 to 2003, 6 cases of cutaneous infections caused by toxigenic Corynebacterium diphtheriae were documented (Table). None of these was accompanied by secondary diphtheria infection. Toxigenicity was determined by both dtx polymerase chain reaction and Elek test (2). As in the United Kingdom, all cases for which clinical information was available (N = 5) were imported. Three were found in tourists who had traveled to tropical countries: a 20-year-old diver had injured her heel after stepping on coral in Thailand; a 60-year-old tourist had a chronic ulcer develop in the thigh after a trip to Indonesia (no history of an insect bite); and a 39-year-old traveler to Kenya returned with a purulent ear infection with no memory of trauma or insect bite. The remaining imported C. diphtheriae skin infections were reported in 2 Angolan children,

Address for correspondence: Kunihiho Hiraiwa, Hamamatsu Red Cross Hospital, 1-5-30, Takabayashi, Hamamatsu, 430-0907, Japan; fax: 81-53-472-3751; email: hiraiwa9215@hotmail.com
5 and 10 years of age, who were brought to Germany by a humanitarian organization for surgery on severe gun wounds to their lower extremities (foot and thigh with chronic osteomyelitis, respectively). To our knowledge, these reports are the first of cutaneous diphtheria in gunshot wounds in recent years. Moreover, in the patient with the thigh wound, C. diphtheriae was also isolated from a deep fistula, which suggests involvement of C. diphtheriae in the chronic osteomyelitis.

As in the United Kingdom, all cases of diphtheria reported since 1997 were caused by C. diphtheriae mitis. In 4 of 5 cutaneous diphtheria patients who had an available medical history, mixed infections with Staphylococcus aureus and Streptococcus pyogenes were found; 3 of 5 patients were not sufficiently vaccinated against diphtheria as recommended. Systemic symptoms, such as malaise and general weakness, developed in the 20-year-old Thailand tourist, although she had received a booster dose just before her travel. Cutaneous diphtheria must be expected even in vaccinated patients; for instance, among serum samples of 287 healthy German adults with a complete record of basic immunization against diphtheria, only 42.2% showed full serologic protection as indicated by antitoxin levels ≥ 0.1 IU/mL (3).

As de Benoist et al. outline, cutaneous diphtheria might be difficult to diagnose because of its unspecific clinical appearance and the presence of mixed infections in chronic nonhealing skin lesions. Because of the nearly complete disappearance of cutaneous diphtheria in many parts of the western world, microbiologists lack experience in identifying C. diphtheriae grown from specimens. From 1997 to 2003, approximately one fifth of the strains sent to our Consiliary Laboratory on Diphtheria for species identification and toxin testing were either nondiphtheria Corynebacterium spp. or noncoryneform bacteria of different genera (including lactobacilli, Dermabacter hominis, and Propionibacterium acnes).

Clinicians (4) and microbiologists (5) should be aware of the possibility of cutaneous diphtheria in chronically infected skin lesions in patients returning from disease-endemic regions. Medical personnel should include this in civilian as well as military health services, since our cases indicate that toxigenic C. diphtheriae might affect not only travel-related skin injuries caused by leisure or tourist activities but also wounds in patients from war regions in diphtheria-endemic areas.

Andreas Sing* and Jürgen Heesemann*
*Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Munich, Germany

References

Address for correspondence: Andreas Sing, Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, National Consiliary Laboratory on Diphtheria, Pettenkoferstrasse 9a, 80336 Munich, Germany; fax: 49-89-5160-5223; email: sing@m3401.mpk.med.uni-muenchen.de

Antimicrobial Drug Consumption in Companion Animals

To the Editor: During the last decade, use of antimicrobial drugs for growth promotion and therapeutic treatment in food animals has received much attention. The reservoir of resistant bacteria in food animals implies a potential risk for transfer of resistant bacteria, or resistance genes, from food animals to humans. Subsequent emergence of infections in humans, caused by resistant bacteria originating from the animal reservoir, is of great concern. These unintended consequences of antimicrobial drug use in animals led to termination of antimicrobial growth promoters in food animals in countries in the European Union, including Denmark, where the consumption of antimicrobial drugs by production animals was reduced by 50% from 1994 to 2003 (1).

In Denmark, the VetStat program monitors all veterinary use of medicines for animals. VetStat is based on reporting from the pharmacies and from veterinary practitioners and contains detailed information, such as animal species, reason for prescription, and dosage on each prescription. In Denmark, antimicrobial drugs can be obtained only by prescription and only at pharmacies.

So far, use of antimicrobial drugs in companion animals has received little attention; monitoring programs have focused on antimicrobial drug consumption in food animals. According to data generated by the VetStat program in 2003, consumption of fluoroquinolones and cephalosporins in companion animals was substantial when compared to consumption in food animals (1). Fluoroquinolones and cephalosporins are antimicrobial drugs ranked by the U.S. Food and Drug Administration as critically important in human medicine, and for which emergence of...
resistant bacteria is especially undesirable (2). Considering the shared environment of humans and companion animals, transfer of resistant bacteria or mobile resistance determinants from companion animals to humans would be possible, and emergence of resistance to fluoroquinolones and cephalosporins in companion animals should be a matter of concern.

Several scientific publications have reported the occurrence of the same resistance genes in companion animals and in humans (3–6) and the possible transfer of bacteria between companion animals and humans (3–9). Companion animal owners and their families are likely in close contact with their animals daily, which provides the opportunity for transfer of bacteria between companion animals and humans. A large proportion of the human population presumably has daily contact with companion animals, not only in Denmark but also in other countries. In Denmark, 20% of families own dogs and 16% own cats (10).

In 2002, legal restrictions aimed to reduce the usage of fluoroquinolones in food animals were imposed in Denmark. The total annual consumption of fluoroquinolones in animals (companion and food animals) in Denmark was reduced from 183 kg in 2001 to 53 kg in 2003 (1). Of these 53 kg of fluoroquinolones, almost half (24 kg) was used in companion animals (550,000 dogs and 650,000 cats) (10) consume approximately the same amount of fluoroquinolones and cephalosporins as consumed annually in the much larger population of food animals in Denmark (23 million slaughter pigs, 130 million broiler chickens, and 1.2 million cattle and dairy cows) (10). We do not believe that antimicrobial drugs are more generously prescribed for companion animals in Denmark than in other industrialized countries. Rather, the data presented here reflect the apparent contrast between policies of antimicrobial drug use for food animals and policies for companion animals. The use of these antimicrobial drugs is avoided or restricted in food animals to minimize spread of resistance, while in companion animals prescription continues unimpeded. This situation may create undesirable antimicrobial drug resistance in bacteria, which may subsequently spread to humans from the previously neglected reservoir in companion animals.

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Ole E. Heuer,* Vibeke Frøkjær Jensen,* and Anette M. Hammerum†
*Danish Institute for Food and Veterinary Research, Søborg, Denmark; and †National Centre for Antimicrobials and Infection Control, Copenhagen, Denmark

References

Address for correspondence: Ole E. Heuer, Department of Epidemiology and Risk Assessment, Danish Institute for Food and Veterinary Research, Morkhaj Bygade 19, DK-2860 Søborg, Denmark; fax: 45-7234-7028; email: oeh@dfvf.dk

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**Vibrio cholerae**

**SXT Element, Laos**

To the Editor: The SXT element is a *Vibrio cholerae*–derived ICE (integrating and conjugative element), which has also been referred to as a conjugative transposon (1) or a conj-
sstin (2). ICEs excise from the chromosomes of their hosts, transfer to a new host through conjugation, and then integrate into the chromosome again. The SXT element was originally isolated in 1993 from a *V. cholerae* O139 clinical isolate (SXTMO10) (1). The ≈100-kbp SXT element confers resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin (1). Since 1994, *V. cholerae* isolates from Bangladesh, India, and Mozambique have also contained the SXT element (2–4). In SXTMO10, resistance genes are embedded near the 5′ end, in a ≈17.2-kbp transposon-like element that interrupts the SXT-encoded *rumAB* operon. In contrast, in El Tor O1 *V. cholerae* strains isolated in India and Bangladesh, the resistance genes are located in SXTET, which is closely related but not identical to SXTMO10 (2). Comparison of 2 related ICEs, SXT of *V. cholerae* and R391 of *Providencia rettgeri* (5), showed that the conserved backbone apparently contains 3 hot spots for insertions of additional DNA sequences: the first between so43 and *traL*, the second between *traA* and so54, and the third between so73 and *traF*. R391 contains an intact *rumAB* operon and a transposon-associated kanamycin resistance gene located ≈3.5 kb from the *rumAB* operon (6). Mobile genetic elements such as SXT have a crucial role in spreading antimicrobial drug resistance genes among microbial populations, and our understanding of these genetic elements would help to control the emergence of antimicrobial drug resistance.

We have been monitoring the drug sensitivity pattern in the Lao People’s Democratic Republic (Laos) since 1993, and we have found that *V. cholerae* O1 strains isolated after 1997 were resistant to tetracycline, sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin (7). Analysis of the genetic determinants encoding antimicrobial drug resistance showed an SXT element (SXTLAOS), which is different from the previously reported SXTs (8). SXTLAOS contains 2 novel open reading frames (ORFs) in the third hot spot (between so73 and *traF*). SXTET contains a class 9 integron in hot spot so73-*traF* that harbors *dfrA1* as a gene cassette (2). In SXTMO10, the gene encoding trimethoprim resistance (*dfr18*) is encoded in the ≈17.2-kbp composite transposon-like element that interrupts the SXT-encoded *rumAB* operon. SXTLAOS does not encode *dfr18* or *dfrA1*, and the gene encoding trimethoprim resistance has not been identified. In this study, we analyzed hot spot so43-*traL* and hot spot *traA*-so54 to better characterize SXTLAOS.

Two sets of primers were designed to amplify the hot spot regions. Primer HS1-F, which anneals to so43, was 5′ GGC TAT TCC ACC GGT GGT G 3′; primer HS1-R, which anneals to *traL*, was 5′ TGC CGA TCA CTA GCC CCA AC 3′; primer HS2-F, which anneals to *traA*, was 5′ ATG GGT CCT TAC AAT ACG CC 3′; and primer HS2-R, which anneals to so54, was 5′ GGA GAC AGC GCA AGC GCC AG 3′. Polymerase chain reaction (PCR) amplifications on genomic DNA extracted from the *V. cholerae* O1 strain isolated in Laos (strain 00LA1) with primers HS1-F and HS1-R yielded an amplicon of ≈1100 bp, which is slightly different from the amplicon obtained with DNA extracted from *V. cholerae* O139, strain MO10 (≈1,000 bp). PCR amplification using primers HS2-F and HS2-R gave amplicons of similar size (≈2,200 bp) for both strains. The

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within SXT\textsubscript{LAOS}. However, the gene was not found in any of the proposed hot spot regions. The possibility that the trimethoprim resistance determinant is located on the chromosome outside the SXT element and cotransfers with the SXT in an Hfr-like manner cannot be ruled out (9). Therefore, additional hot spot regions may exist in SXT elements for insertion of DNA; otherwise the trimethoprim resistance gene is not encoded within SXT\textsubscript{LAOS}.

The nucleotide sequence data reported in this study will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB185252 for the hot spot sO43-traL and AB186353 for the hot spot traA-sO54.

Claudia Toma,* Noboru Nakasone,* Tianyan Song,* and Masaaki Iwanaga*
*University of the Ryukyus, Okinawa, Japan

References

Address for correspondence: Claudia Toma,
Division of Bacterial Pathogenesis, Department of Microbiology, Graduate School of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-0215, Japan; fax: 81-98-895-1408; email: k950417@med.u-ryukyu.ac.jp

Modeling the Impact of Pandemic Influenza on Pacific Islands

To the Editor: Many Pacific Island countries and areas have been severely impacted in influenza pandemics. The 1918 pandemic killed substantial proportions of the total population: Fiji = 5.2%, Tonga = 4.2% to 8.4%, Guam = 4.5%, Tahiti = 10%, and Western Samoa = 19% to 22% (1, 2). Thirty-one influenza pandemics have occurred since the first pandemic in 1580 (5); another one is likely, if not inevitable (3). The potential use of influenza as a bioweapon is an additional concern (5).

The results indicate that at incidence rates of 15% and 35%, pandemic influenza would cause 650 and 1,530 deaths, respectively, giving crude death rates of 22 to 52 per 100,000, (see the Table in the online Appendix). Most deaths (83%) would occur in the high-risk group, 60% of whom would be 19–64 years of age, and 22% would be ≥65 years of age. Additionally, 3,540 to 8,250 persons would be hospitalized, most of whom (78%) would not have high-risk conditions. Also, 241,000 to 563,000 medical consultations would occur. Most (87%) consultations would be for patients without high-risk conditions (50% birth–18 years of age and 46% 19–64 years of age).

In the peak week of the pandemic (week 4), from 15% to 34% of all hospital beds would be required for patients with influenza (Table). The upper end of impact on hospital beds at >40% would occur for Guam,
Kiribati, Marshall Islands, Northern Mariana Islands, and Tonga. Assuming all consultations required doctors, 42 to 99 influenza consultations per doctor would be required during the peak week (Table). The upper end of impact on consultations for individual Pacific Island countries and areas would vary from 31 (New Caledonia) to 524 (Vanuatu); Fiji, Kiribati, Samoa, Solomon Islands, Tonga, and Vanuatu would have rates >150 consultations per week.

The uncertainties associated with pandemic influenza mean that any modeling of its future impact is relatively crude. For example, the new strain may be particularly infectious, virulent, or both. In contrast, the use of international-level public health interventions as recommended by WHO (9) may prevent pandemic influenza from reaching some Pacific Island countries and areas or particularly remote island groups. These issues and other limitations with the model are detailed in the online Appendix.

Nevertheless, if the death rate is in the range suggested by the model, this outcome would make it the worst internal demographic event since the 1918 influenza pandemic for many Pacific Island countries and areas. The lower death rate (albeit for a single wave) is similar to the U.S. rates for the 1957 influenza pandemic (22 per 100,000) and the 1968 influenza pandemic (14 per 100,000) (10). The upper end is considerably lower than for the 1918 pandemic, which suggests that the range indicated is reasonably plausible. Although relatively high, the death toll from pandemic influenza would still be less than the typical annual impact for some Pacific Island countries and areas from other infectious diseases (including malaria and diarrheal diseases) and from such fundamental determinants of health status such as poor sanitation, poor diet, and tobacco use.

The predicted range of hospitalizations attributable to pandemic influenza would likely overwhelm hospital capacity in many of the Pacific Island countries and areas. Rapid response at the onset of the pandemic could ensure efficacious use of hospital beds and resources, e.g., cancel elective procedures and early discharge to community care. Other contingency plans by hospitals could facilitate lower hospital admission rates (e.g., strengthening the primary care response).

Planning and capacity building could be provided by WHO, the Secretariat of the Pacific Community, and donor nations and agencies with support for improving surveillance and other preventive measures for disease control (see the online Appendix for details). A combination of national capacity building with international support will maximize the capacity to respond to the next influenza pandemic as well as other potential communicable disease threats.

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Mycotic Brain Abscess Caused by Opportunistic Reptile Pathogen

To the Editor: A 38-year-old, HIV-seropositive Nigerian man sought treatment with an 8-month history of severe parietal headache, impaired memory, fatigue, paresthesia of the left arm, and left-sided focal seizures. He had no history of neurologic disorders, including epilepsy. On physical examination, the patient appeared well, alert, and oriented, with slurred speech. Evaluation of the visual fields showed left homonymous hemianopsia. All other neurologic assessments were unremarkable. The patient had a blood pressure of 120/80, a pulse of 88 beats per minute, and a body temperature of 37.3°C. Leukocyte count was 8,600/µL, total lymphocyte count was 1,981/µL, CD4+ cell count was 8,600/µL, total lymphocyte count was 1,981/µL, CD4+ cell count was 102/µL, and CD4/CD8 ratio was 0.07. HIV RNA-load was <50 copies/mL; all other laboratory parameters were normal. The patient had received antiretroviral therapy ( stavudine, lamivudine, nevirapine) for 5 months before admission, but no prophylaxis for opportunistic infections. Magnetic resonance imaging (MRI) of the brain disclosed 2 masses, 3.3 and 4.8 cm in diameter, respectively (Figure A), and signs of chronic sinusitis. A computed tomographic chest scan showed infiltration of both lower segments with multiple, small nodules (Figure B). Blood cultures were repeatedly negative. A computer-guided needle-aspiration of the brain lesions yielded yellow-brown, creamy fluid in which abundant septated fungal hyphae were detected microscopically (Figure C). Cytologic investigation was consistent with a necrotic abscess. The cycloheximide-resistant isolate was strongly keratinolytic and identified as a Chrysosporium anamorph of Nannizzopsis vriesii (1,2). High-dose antimicrobial treatment with voriconazole (200 mg twice daily, subsequently reduced to 200 mg daily) was added to the antiretroviral (ritonavir, amprenavir, trizivir), anti-convulsive, and adjuvant corticosteroid treatment. The isolate was highly susceptible to voriconazole in vitro (MIC, 1.6µg/mL [Etest, AB-Biodisk Solna, Sweden]). Recovery was complicated by a generalized seizure and severe, acute psychosis associated with rapid refilling of the 2 lesions with mycotic abscess fluid. After re-ascertainment, the patient’s psychosis improved gradually, and no further seizures occurred. When last seen 4 months later, the patient was healthy and without neurologic deficits. His CD4+ cell count was 233/µL, HIV-load was <50 copies/mL, and a MRI scan of the brain showed partial regression of the 2 brain lesions (Figure D).

Chrysosporium spp. are common soil saprobes, occasionally isolated from human skin. Invasive infection is very rare in humans, and most were observed in immunocompromised patients, manifesting as osteomyelitis (3,4) or diffuse vascular brain invasion (5). Here, we report the first case of brain abscesses by the Chrysosporium anamorph of N. vriesii. This fungus has been associated with fatal mycosis in reptiles (6,7) and cutaneous mycosis in chameleons originating from Africa (2).

In our patient, we were unable to determine the portal of entry and the sequence of fungal dissemination; no

Address for correspondence: Nick Wilson, Department of Public Health, Wellington School of Medicine and Health Sciences, Otago University, PO Box 7343, Wellington South, New Zealand; fax: 64-4-4763646; email: nwilson@actrix.gen.nz

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skin lesions were present at the time of admission. However, the multifocal nature, lung infiltration, and involvement of the middle cerebral artery distribution suggest hematogenous dissemination (8,9) after replication of airborne conidia within the respiratory tract.

Fungi cause >90% of brain abscesses in immunocompromised transplant patients with an associated mortality rate of 97% (10), despite aggressive surgery and antifungal therapy (9). Our patient was treated successfully with abscess drainage, antiretroviral therapy, and oral voriconazole, a novel antifungal triazole drug. Despite limited data available on voriconazole penetration into brain abscess cavities (9), this drug was clinically and radiologically effective in our patient.

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Christoph Steininger,* Jan van Lunzen,* Kathrin Tintelnot,† Ingo Sobottka,* Holger Rohde,* Matthias Anser Horstkotte,* and Hans-Jürgen Stellbrink*

*University Clinic Eppendorf, Hamburg, Germany; and †Robert Koch-Institut, Mykologie, Berlin, Germany

References


Address for correspondence: Christoph Steininger, University Clinic Eppendorf, Department of Medicine I, Infectious Diseases Unit, Martinistrasse 52, 20246 Hamburg, Germany; fax: 49-40-42803-6832; email: c.steininger@uke.uni-hamburg.de

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Tuberculosis in Undocumented Migrants, Geneva

To the Editor: In today’s globalized world, a growing number of people are migrating in search of a better life. Simultaneously, industrialized countries are strengthening border controls and administrative barriers to contain this influx of newcomers, resulting in a significant increase in illegal migration and human trafficking. The U.S. Department of State estimates the annual flow of irregular migrants worldwide to be 700,000–2 million (1). Many of the migrants are from countries where tuberculosis is endemic, and they contribute to the increasing proportion of foreign-born persons with tuberculosis in North America and Europe. These persons may be highly contagious in the local population, as they have limited access to healthcare and often go untreated (2,3).

Of 450,000 residents in Geneva, Switzerland, 10,000–20,000 are undocumented and come from developing countries or Eastern Europe. All patients treated for tuberculosis in Geneva are systematically registered by the Antituberculosis Center, a facility at Geneva University Hospital. An outpatient clinic provides free consultations for patients with tuberculosis who have no health insurance, and patients are not required to disclose their immigration status to physicians. Patients with sufficient funds pay for their medication.

All cases of tuberculosis in undocumented migrants (foreign residents with no resident permits) reported from 1994 to 1998 were reviewed by the same investigator. Their sociodemographic and clinical characteristics were compared with those of 7 South American legal residents with tuberculosis (representing the whole sample of South American tuberculosis patients) during the same period and with those of a group of 50 tuberculosis patients from the general population in a previous study.

From 1994 to 1998, a total of 397 persons in Geneva were notified that they were infected with tuberculosis. Twenty-two (6%) case-patients were found among undocumented migrants. The mean age was 31 years (19–48 years), and 20 (91%) were women; 15 (68%) came from South America, 5 (22%) came from Africa, and 2 (9%) came from Europe. Nineteen (95%) of 20 persons had symptoms for >1 month preceding their first medical encounter. Approximately 27.2% had pulmonary manifestations only, 36.4% had extra-pulmonary manifestations only, and the remaining 36.4% had both pulmonary and extrapulmonary manifestations. Mycobacterium tuberculosis was found in 11 of 14 with pulmonary involvement, and chest radiograph was normal in 5 (22%). When compared with patients from the general population, women were more numerous (91% vs. 30%), and extrapulmonary tuberculosis was more frequent among undocumented residents (72% vs. 34%). The time from first symptoms to first consultation was also longer when compared to the general population and the registered South American residents (5% vs. 30% and 40%, respectively, consulting in the first month; p = 0.008). In 4 (19%) patients, resistance to ≥1 tuberculosis drug was identified, with no multidrug resistance (defined as rifampicin) identified, a rate of resistance similar to that seen in their countries of origin but higher than the Swiss rate (6.3%) (4). All patients were treated with a 4-drug regimen (HRZE: H = isoniazid, R = rifampicin, Z = pyrazinamide, E = ethambutol) for 2 months, followed by a 2-drug therapy (HR) for 4 months. Eighteen (82%) patients adhered to the regimen, as determined by monthly medical interviews and urine isoniazid checks. Only the 4 remaining patients who missed more than one third of the appointments with 50% of negative urine checks, or who defaulted, were placed under directly observed therapy. Fifteen (68%) patients regularly attended their appointments until completion of treatment. Seven (32%) patients left Switzerland before the end of treatment, 2 of whom were deported.

Fourteen (64%) patients were hospitalized to initiate treatment. Four had health insurance; the other patients contracted a debt for hospitalization. The lack of insurance did not influence adherence to treatment negatively. However, as a consequence of tuberculosis, 8 (66%) lost their jobs.

Of 102 identified close contacts, 88 (87%) were evaluated by tuberculin skin testing. Chest x-ray was performed on 21 (24%) patients with a positive test (>10 mm induration), and isoniazid was prescribed prophylactically. No secondary case of active tuberculosis was identified.

Most undocumented immigrants with tuberculosis in Geneva are young South American or African women engaged in domestic activities. This finding reflects the irregular work opportunities in Geneva, an area with little agriculture and industry. As suspected, a delay of several weeks occurred before seeking care (5). The economic and social impact of tuberculosis was high for this population. Two thirds of these patients lost their jobs as a consequence of tuberculosis. Joblessness could be an additional factor to further deter patients from seeking care. Adherence to treatment was good, which suggested confidence that care providers would not report to immigration authorities and that supportive follow-up care was available. Of more concern, approximately one third of the patients left Switzerland before completing the full course of treatment. This transfer rate of undocumented migrants corresponds to that observed (43%) among foreign-born patients with unknown legal status in
Switzerland (6). Failure to complete a full course of treatment may lead to relapse and emergence of resistant strains. A growing proportion of cases of tuberculosis observed in Europe is in migrants, some undocumented, from the developing world (3). Strong political measures should be enforced to ensure access to healthcare services with respect to confidentiality (as recently stated in the Netherlands) (7).

Much emphasis has been put on screening at time of arrival. Screening can be conducted for immigrants and asylum seekers, but undocumented migrants are not screened (8). Facilitated access to medical services and free affordable therapy is a necessity; active tuberculosis develops in most foreign-born residents several years after their arrival (2). In an era of high mobility, specific innovative programs should be established to control and prevent tuberculosis for this high-risk, foreign-born population. Early detection with nonidentifying tuberculosis tracking systems (9), screening at unspecialized clinics (10), and free treatment with adequate administrative measures are needed. Industrialized countries must take responsibility to reduce the spread of resistant tuberculosis.

Sigiriya Aebischer Perone,* Patrick Bovier,* Christian Pichonnaz,* Thierry Rochat,* and Louis Loutan*

*Geneva University Hospital, Geneva, Switzerland

References


Address for correspondence: Louis Loutan, Travel and Migration Medicine Unit, Department of Community Medicine, Geneva University Hospital, 24, rue Micheli-du-Crest, 1211 Geneva 14, Switzerland; fax: 0041-22-372-96-26; email: louis.loutan@hcuge.ch

**Mycobacterium chelonae Skin Infection in Kidney-Pancreas Recipient**

To the Editor: Mycobacterium chelonae is rapid growing and is ubiquitous in the environment, including soil, water, domestic and wild animals, and milk and fruit products. It can be associated with infections of the soft tissue, lung, bone, joint, central nervous system, and eye. M. chelonae infections in an immunocompromised host are disseminated in >50% of those infected; chronic use of steroids, even in low doses, seems to be the most important predictive factor for disseminated disease (1,2). In immunocompetent hosts, nontuberculous mycobacteria can colonize body surfaces and be secreted for prolonged periods without causing disease. In hematopoietic stem cell and solid organ transplant recipients, infections with nontuberculous mycobacteria are common and may be a source of illness and death (3). We describe a case of localized cutaneous M. chelonae infection after a dog bite in a kidney-pancreas transplant recipient.

A 43-year-old female patient underwent kidney transplantation for diabetic nephropathy in 1985. After loss of organ function due to chronic rejection, she underwent combined kidney-pancreas transplantation 5 years later, in 1990. Because of chronic rejection, the patient lost the kidney graft 5 years later, in 1995, and went back on dialysis with a well-functioning pancreas graft. In 2004, the patient was bitten on the right forearm by a dog. She was on immunosuppressive therapy of prednisolone (5 mg/day), cyclosporine-A (trough levels of 100 ng/dL), and azathioprine (50 mg/day). The initial lesion healed without major complication. After several days, a single firm edematous plaque of 3 x 5 cm developed at the site of the animal bite, and the patient was admitted to the Department of Dermatology. Empiric antimicrobial combination therapy, including clindamycin (300 mg every 8 hours) and ciprofloxacin (500 mg every 12 hours), was initiated. As no clinical improvement was achieved, a biopsy was performed, which showed a granulomatous inflammation with a high number of mycobacteria (Figure). Atypical mycobacteria were cultured from a second biopsy (Löwenstein-Jensen/Stonebrink, Heidelberg, Germany); M. chelonae was identified by
polymerase chain reaction. Therefore, antimicrobial therapy was changed to clarithromycin (500 mg twice daily) for 6 months. Although cyclosporine-A dosage was reduced with initiation of antimicrobial therapy, trough level increased to 350 ng/mL; therefore, further dose reduction was performed. Within a few weeks, the lesion disappeared completely, and the patient retained good pancreatic graft function. To rule out dissemination to other organs, a computed tomography of head, thorax, and abdomen was performed at time of diagnosis.

In the immunocompromised host, an uncontrolled proliferation of primarily colonizing or contaminating pathogens or commensals can progress to severe disease. Diagnosis is often difficult because patients with these infections may have atypical symptoms due to immunosuppressive therapy. If diagnosis is made early, dissemination can likely be avoided. Therefore, suspicious cutaneous lesions should be biopsied for histopathologic examination, and special stains and tissue cultures should be performed for detecting fungi, viruses, and bacteria, including mycobacteria (3,4). Nontuberculous mycobacteria are resistant to conventional tuberculostatic therapy and have variable susceptibility to other antimicrobial agents (1,2,5). Clarithromycin seems to be the most active drug, and azithromycin might also have good activity (3–6). Clarithromycin has been administered successfully as monotherapy, and our case confirms these data. However, several cases of resistance have been described, and use of at least 1 other drug, such as an aminoglycoside or a quinolone, in addition to clarithromycin has been recommended (1,4,7–9). Clarithromycin is a potent inhibitor of cytochrome P450 (3,4). Therefore, cyclosporine-A and tacrolimus levels have to be monitored exactly, and dose adjustments may be required. Duration of therapy depends on the isolate, site of infection, and clinical response to therapy, but in general, it should be continued for at least 6 months (3,8).

Thus far only a few cases of infections with M. chelonae in kidney, heart, liver, and lung transplant recipients have been described (3). Most of these infections were disseminated and often resulted in chronic infection. To our knowledge, this report is the first of localized cutaneous disease from M. chelonae, which completely healed within 3 months, in a kidney-pancreas transplant recipient. Although M. chelonae might be part of the colonizing oral flora of dogs, it is more likely that the bite contributed to translocation of the transient dermal flora. Any factor that disrupts the skin barrier, such as insulin self-injection in diabetes patients, surgical wound, insect sting, or animal bite, might be associated with this type of infection (10,11). We conclude that early diagnosis prevents dissemination, leads to rapid clinical response, and allows antimicrobial monotherapy with a macrolide. Such an approach preserved the function of the pancreatic allograft.

Ingrid Stelzmueller,* Karin M. Dunst,* Silke Wiesmayr,* Robert Zangerle,* Paul Hengster,* and Hugo Bonatti*  
*Innsbruck Medical University, Innsbruck, Austria

References
To the Editor: Hawryluck et al. (1) have published an interesting study that found that some persons subject to quarantine for severe acute respiratory distress syndrome (SARS) displayed symptoms of posttraumatic stress disorder and depression. They conclude that the psychological symptoms result from quarantine. I believe the study has serious flaws and that their conclusion is premature.

First, their study sampled 129 volunteers among the >15,000 persons subjected to quarantine. As acknowledged by the authors, persons with the most severe symptoms may be more likely to volunteer for the study, resulting in an overestimation of the frequency and severity of the symptoms. Second, more than two thirds of the participants were healthcare workers. Healthcare workers in Toronto who cared for SARS patients but were not subject to quarantine were experiencing extreme stress because they were working with a poorly understood infectious disease, wearing protective equipment for extended periods, and watching colleagues become ill and die while wondering if they themselves were the next victims. Most healthcare workers subject to quarantine in Toronto (including 34% of persons on work quarantine) likely cared for SARS patients and would have experienced stresses similar to those not quarantined. Third, 85% of the study participants wore masks at home, indicating that they were likely to have been symptomatic and subject to isolation rather than quarantine. Certainly symptomatic persons would be undergoing stress because of their concerns about SARS developing, the possibility of dying, and the potential for exposing others. Increasing levels of stress with increasing length of isolation found in the study may be due to more severe or prolonged symptoms rather than to isolation or quarantine per se.

Measuring the psychological effects of isolation and quarantine will require studies comparing psychological symptoms of healthcare workers subjected to quarantine with those who continued working, as well as studies comparing randomly selected persons subject to isolation with the general population living in the city during the outbreak.

In the final analysis, although isolation and quarantine are stressful, that is an insufficient reason to hesitate when these measures are indicated. One might wonder how stressed the participants would have been if SARS had developed and they infected their family members or friends.

Regardless of whether isolation and quarantine induce posttraumatic stress disorder, public health officials must be cognizant of and prepared to supply appropriate emotional and social support to persons subject to isolation or quarantine.

Harry F. Hull*
*Minnesota Department of Health, Minneapolis, Minnesota, USA

Reference


Address for correspondence: Harry F. Hull, State Epidemiologist and Division Director, Infectious Disease Epidemiology, Prevention and Control Division, Minnesota Department of Health, 717 Delaware, SE, Minneapolis, MN 55414, USA; fax: 612-676-5666; email: harry.hull@health.state.mn.us
work that we have conducted on non-quarantined, uninfected healthcare workers treating patients with SARS in a hospital in Toronto (2). The implication of Dr. Hull’s statement, however, is that being a healthcare worker in Toronto at the time of SARS, rather than being placed into quarantine, was responsible for the psychological distress that we measured. To dispute this, we found that healthcare worker status was not correlated with PTSD or depression symptoms, which indicates that respondents who were nonhealthcare workers experienced similar levels of distress as healthcare workers who responded. Furthermore, we found that longer durations of quarantine were associated with increased symptoms of PTSD, which indicates that respondents who were nonhealthcare workers experienced similar levels of distress as healthcare workers who responded. Furthermore, we found that longer durations of quarantine were associated with increased symptoms of PTSD, which indicates that the physical state of being in quarantine was at least in part responsible for the psychological distress.

Finally, Dr. Hull states that 85% of the study participants wore masks at home, which indicates that they were likely to have been symptomatic and subject to isolation rather than quarantine. This statement is incorrect. The respondents to this survey were asymptomatic, exposed persons who were placed into quarantine. Instructions to all quarantined persons, per public health guidelines (3), were to wear masks while in the presence of other household members, not because they were symptomatic, but rather because they may have been incubating SARS and had the potential to transmit infection to household contacts in the 24 hours before symptom onset.

Although the terms isolation and quarantine have often been used interchangeably, they actually represent distinct concepts (4). The strategies differ in that isolation applies to persons who are known to have an illness, and quarantine applies to those who have been exposed to a transmissible pathogen but who may or may not become ill. Quarantine directives for SARS included the adherence to home infection control measures, including wearing masks in the presence of other household members, not sharing utensils, and sleeping in separate quarters (3).

We agree with Dr. Hull’s final statement that the psychological distress experienced by persons in quarantine is not a sufficient reason to refrain from invoking these measures when they are needed to control an outbreak. We did not arrive at this conclusion in our article. The goal of the study was to develop a benchmark for the possible distress associated with quarantine. While we felt that documenting the possible distress that may result from quarantine was important, it was not intended to negate the need to impose quarantine should it be required, but rather to determine the support measures that may be needed by quarantined persons. Public health officials must be cognizant of these needs and prepared to supply appropriate emotional and social support to persons in quarantine for such measures to succeed in halting the spread of disease.

Rima Styra,* Laura Hawryluck,* and Wayne Gold*
*University Health Network, Toronto, Ontario, Canada

References

Address for correspondence: Rima Styra, Toronto General Hospital, 200 Elizabeth St, 8 Eaton North - Rm 235, Toronto, Ontario, Canada MSG 2C4; fax: 416-340-4198; email: rima.styra@uhn.on.ca

Correction, Vol. 9, No. 12

In "Severe Acute Respiratory Syndrome Epidemic in Asia," by G. Zhou and G. Yan, an error occurred in the Table. Under the table heading "Parameter estimation," the third subheading should be "1/α." The corrected table appears online at http://www.cdc.gov/ncidod/EID/vol9no12/03-0382.htm/table

We regret any confusion this error may have caused.
Attention shifts from issues to methods in the last 6 chapters of the book. In textbook fashion, Nabih R. Asal and Laura A. Beebe distinguish observational studies from experimental designs in chapter 5 and remind the reader of the importance of person, place, and time in epidemiologic investigations. The strengths and weaknesses of the Behavioral Risk Factor Surveillance System are illustrated by Deborah Holtzman in chapter 6. Qualitative research methods are described in chapter 7 and applied to a case study of 45 African-American, female crack-cocaine users in chapter 8. Community intervention trials are introduced and a half dozen are reviewed in chapter 9. Then the book rather abruptly ends with a short chapter on cardiovascular risk-reduction community intervention trials.

Instructors looking for a graduate-level textbook may find this recent addition to the preventive medicine literature incomplete. It fails to link community-based research with theories of social and cultural change; the principles and practices of community mobilization; and the identification, development, implementation, and evaluation of culturally competent interventions. The editors have produced an adequate introduction to community-based research issues and methods, but a concluding section that serves to pull all the components together would put additional copies of this publication in college bookstores.

William W. Darrow*

*Florida International University, North Miami, Florida, USA

Address for correspondence: William W. Darrow, Robert R. Stempel School of Public Health, Florida International University, 3000 NE 151st St, TR-7, North Miami, Florida 33181-3600, USA; fax: 305-919-5673; email: darroww@fiu.edu

The Pneumococcus

Elaine I. Tuomanen, Timothy J. Mitchell, Donald A. Morrison, and Brian G. Spratt, editors

Washington: American Society for Microbiology Press; 2004
Pages: 466, Price: U.S.$115.95

Streptococcus pneumoniae, known as the pneumococcus, remains an important pathogen in spite of tremendous advances in medical care. Globally, as many as 1 million children die of pneumococcal infections each year, nearly all in developing countries. Pneumococcal disease is also common in children in industrialized countries, although in those settings nearly all such deaths occur in older adults or adults with chronic medical conditions. Given its place near the top of the list of killer bacteria, pneumococcus is a focus of numerous researchers around the world. A new book, The Pneumococcus, edited by Elaine Tuomanen et al., is the latest effort to summarize the state of research on the organism.

The book begins by providing a well-thought-out answer to a basic question—what is a pneumococcus?—and moves on to chapters on topics ranging from attachment and invasion of the respiratory tract to vaccine-induced immunity. The editors are leaders primarily in the areas of molecular biology and pathogenesis, and the focus of much of the book is on these topics, although issues such as treatment, carriage, disease in persons with immunodeficiencies, antimicrobial resistance, and epidemiology are also well covered. The relatively recent deciphering of several pneumococcal genomes has led to a new outburst of research activity, aspects of which are summarized in several of the chapters.
All of the authors are recognized experts in their respective areas. The foreword by Robert Austrian, a pioneer in pneumococcal microbiology, disease description, and vaccine work, provides an interesting summary of the history of major discoveries in the field. While covering many areas of pneumococcal research, the book is not exhaustive; for example, issues specific to pneumococcal disease in developing countries are mentioned only in passing.

The book may be most suitable as a tool for new researchers in the pneumococcal field, but it may also be useful for medical students, graduate students, and infectious disease specialists. The level of detail varies among the chapters, but it is adequate to provide an introduction to each of the topics covered, and all chapters are thoroughly referenced. Overall, the editors and writers have done a remarkable job of consolidating the latest information. The Pneumococcus is an authoritative reference in a rapidly changing field.

Cynthia G. Whitney*
*Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DNA Amplification:
Current Technologies and Applications

Vadim V. Demidov and Natalia E. Broude, authors
Horizon Bioscience, Norfolk, UK
IBSN: 0-9545232-9-6
Pages: 335, Price: U.S.$180

DNA amplification is a powerful technique that has had an immense impact on scientific research in the past 2 decades. While polymerase chain reaction (PCR) is still the most popular method, alternative methods of DNA amplification are constantly being developed. In addition, the extraordinary versatility of PCR has led to its use in novel ways that have opened new avenues of research. These novel methods for DNA amplification and the versatility of PCR are highlighted in DNA Amplification: Current Technologies and Applications.

The 17 chapters in this book are divided into 4 sections that focus on enzymes (3 chapters), thermal cycling methods (6 chapters), isothermal methods (6 chapters), and the detection of non-DNA analytes by DNA amplification (2 chapters). Each chapter has a thorough description of methods and highly detailed protocols for applying the technique to at least 1 specific application. Several excellent chapters describe the uses of Phi29 DNA polymerase and of applications using isothermal rolling circle amplification. A chapter on multiple-displacement amplification details the isothermal amplification of total genomic DNA and should prove extremely useful for amplifying DNA in limited amounts, such as DNA from clinical samples. The final 2 chapters describe use of either real-time PCR or rolling circle amplification to detect and quantify non-DNA analytes, such as serum cytokines, with much greater sensitivity than conventional enzyme-linked immunosorbent assay methods.

This book is not for the novice scientist, as it does not describe basic DNA amplification fundamentals; rather, it is directed at those with a solid background in molecular biology who desire knowledge of cutting-edge applications. Although many of the detailed protocols will not be applicable to certain laboratory situations, the versatility of most of the methods described will allow them to be easily adapted to other studies. Therefore, this book will be a good addition to the library of researchers in molecular biology or to molecular diagnostics laboratories planning to expand their horizon beyond standard PCR amplification techniques.

Robert F. Massung*
*Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Address for correspondence: Cynthia G. Whitney, Respiratory Diseases Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop C23, Atlanta, GA 30333, USA; fax: 404-639-3970; email: cwhitney@cdc.gov

Address for correspondence: Robert F. Massung, Centers for Disease Control and Prevention, 1600 Clifton Rd, Atlanta, GA 30333, USA; fax: 404-639-4436; email: rfm2@cdc.gov

www.cdc.gov/eid

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ABOUT THE COVER

Hazards of Travel—Who Will Free the Contemporary Traveler?

Polyxeni Potter*

A native of Mecklenburg County, North Carolina, Romare Bearden was the offspring of a middle-class family established in Charlotte, where the railroad and cotton industries flourished after the Civil War. His paternal great-grandparents, with whom he spent considerable time, were described in the 1915 publication Colored Charlotte as “former servants of Dr. Joseph Wilson the father of President Woodrow Wilson…” (1). His maternal grandparents, who were also influential in his development, ran a boarding house in Pittsburgh, Pennsylvania, serving migrant steel mill workers from the South.

Around 1914, Bearden’s family moved north to New York and settled in Harlem. In their apartment at 154 West 131st Street, he grew up with the artistic, intellectual, and political influences of the cultural movement of the 1920s and 30s known as Harlem Renaissance. His circle included writers Langston Hughes and Ralph Ellison, musicians Duke Ellington and Fats Waller, activist W.E.B. DuBois, and artists Aaron Douglas and Jacob Lawrence (2). Although he spent most of his school years in New York, Bearden visited Pittsburgh often, enjoying life in his grandparents’ boardinghouse, where mill workers returning from work would sit on the steps and “tell stories about down-home in the South” (1).

Bearden had many talents and broad academic interests. He graduated from New York University with a degree in education, but he also loved mathematics and music and was an accomplished writer and cartoonist. His editorial drawings on the social, political, and economic issues of his day (depression era soup lines, segregation, social inequality), are reminiscent of the politically charged work of Diego Rivera and other Mexican muralists and of Francisco de Goya’s caprices, which chronicled the vices of 19th-century Spain. Bearden studied art throughout his life. While employed in the New York City Department of Social Services, he satisfied his growing wish to become an artist by painting during evenings and weekends. By the end of the 1930s, he was fully engaged in art.

“…[T]he function of the artist is to find ways of communicating, in sensible, sensuous terms, those experiences which do not find adequate expression in the daily round of living and for which, therefore, no ready made means of communication exists…” wrote Bearden in his first solo exhibition pamphlet in 1940 (1). In a career marked by continuous growth, he experimented with new media, always seeking the texture, form, and color that most closely embodied his artistic goals, and became one of the most creative and original artists of the 20th century.

Bearden’s early work was mostly gouaches (opaque watercolors on brown paper). He became increasingly interested in the human figure but gradually moved away from representational painting toward abstraction (3) and “those universals that must be digested by the mind and cannot be merely seen by the eye” (4). By the early 1960s, he was constructing photomontages, which he

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA
ABOUT THE COVER

continued to refine through various techniques into col-
lages, his signature style. During the 1970s and 80s, he
synthesized elements of his earlier work into an individu-
alized art form using brown paper, brilliant color, and
graphite drawings.

The collage, which dates back to medieval Persia and
Japan, was known in Europe well before the 18th century
and was rediscovered and used in modern times by Pablo
Picasso and others. Bearden turned the medium into a nar-
ратive device, synthesizing color, form, photographic
images, and patches of social commentary into intricate,
richly textured, intensely emotional scenes. “…I use many
disparate elements to form either a figure, or part of a
background. I build my faces…from parts of African
masks, animal eyes, marbles, mossy vegetation….” (5).

A prolific artist, Bearden painted the places where he
lived and worked: the rural South, northern cities of his
childhood, and the Caribbean islands where he spent the
latter part of his life. His artistic goal was “to reveal
through pictorial complexities the riches of a life I know.”
“I do not need to go looking for ‘happenings,’ the absurd,
or the surreal,” he said, “because I have seen things that
neither Dalí, Beckett, Ionesco nor any of the others could
have thought possible; and to see these things I did not
need to do more than look out of my studio window” (6).

In 1976, after many years, Bearden traveled to the sites
of his early childhood, only to find that everything had
changed. Shortly afterwards, perhaps reflecting on his own
life’s journey, he embarked on a series of 20 collages based
on Homer’s Odyssey. Inspired by Odysseus’ epic travails
as he wandered the Mediterranean in search of Ithaca,
these compositions showcase the essential geometry in
Bearden’s work. Highly finished flat panels of vivid color
contain minimal surface manipulation or paint. Fluid char-
coal silhouettes beneath the waves recall the dark figures
adorning classical Greek pottery.

“…[T]he sparkle and pulsations of water give men and
women a certain energy…” wrote Bearden in praise of his
Caribbean experience (1), which also might have prompt-
ed this excursion into mythology. For most of us, fascina-
tion with the sea and longing for the unknown prompt trav-
el. As the graceful nymph on this month’s cover frees
Odysseus from one more hurdle of his 10-year journey, we
sympathize with the weary traveler. Yet, however grue-
some, his impediments were imaginary—angry gods,
cyclopes, sirens, Scylla and Charybdis. As we reach con-
temporary ports of call, the threats we meet—SARS, avian
flu, West Nile virus, Ebola—are real.

References

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Address for correspondence: Polyxeni Potter, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D61,
Atlanta, GA 30333, USA; fax: 404-371-5449; email: PMP1@cdc.gov
Upcoming Infectious Disease Activities

March 16–18, 2005
Focus on Fungal Infections 15
Sheraton Bal Harbour
Miami, Florida, USA
Contact: 770-751-7332 or
c.chase@imedex.com
http://www.imedex.com/calendars/
infectiousdisease.htm

April 9–12, 2005
Society for Healthcare Epidemiology of America (SHEA) Annual Meeting
Los Angeles, California, USA
Contact: 703-684-1006
Web site: http://www.shea-online.org

May 1, 2005
International Society of Travel Medicine (ISTM) offers certificate
of knowledge in travel medicine exam
(Given prior to the opening of 9th Conference of the ISTM)
Contact: exam@istm.org
http://www.ISTM.org/

May 1–5, 2005
9th Conference of the International Society of Travel Medicine
Lisbon, Portugal
Contact: +49-89-2180-3830
http://www.ISTM.org/

November 13–18, 2005
Fourth MIM Pan-African Malaria Conference
Yaoundé, Cameroon
http://www.mim.su.se/conference2005

2006
June 25–29, 2006
ISHAM 2006 (International Society for Human and Animal Mycology)
Palais des Congrès
Paris, France
Contact: 770-751-7332 or
c.chase@imedex.com
http://www.imedex.com/calendars/
infectiousdisease.htm

Look in the March issue for the following topics:

Fly Transmission of Campylobacter

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

Probable Psittacosis Outbreak Linked to Wild Birds

Rapid Identification of Emerging Pathogens: Coronavirus

Outpatient Community-acquired Pneumonia and Antimicrobial Drugs

Effect of Regulation and Education on Salmonellosis

SARS Risk Perceptions in Healthcare Workers, Japan

Logitudinally Profiling Neutralizing Antibody Response to SARS Coronavirus with Pseudotypes

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

Notifiable Disease Surveillance and Practicing Physicians

Rumor Surveillance and Avian Influenza H5N1

Complete list of articles in the March issue at
http://www.cdc.gov/ncidod/eid/upcoming.htm
EMERGING INFECTIOUS DISEASES
6-Year-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles
Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed contents in public health terms, contact the Emerging Infectious Diseases web page (http://www.cdc.gov/eid/Submit/WinEID.htm).

Instructions to Authors
Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide the author’s mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author’s primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text.

Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by “et al.” Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word’s table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles
Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only two. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging infectious diseases and related topics. 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.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., “Here is what we found, and here is what the findings mean”).

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and 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 1,000–1,500 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemiologic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader’s literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article’s publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have one Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only (effective January 2005). Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.