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Chikungunya Virus Disease

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Pierre-Auguste Renoir (1841–1919)
Luncheon of the Boating Party (1880-1881)
Oil on canvas (130.175 cm × 175.5775 cm)
Acquired 1923. The Phillips Collection, Washington, DC, USA

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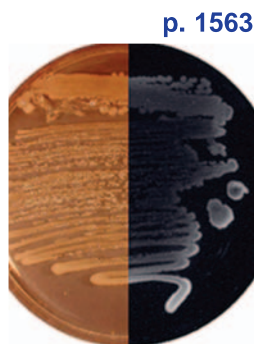

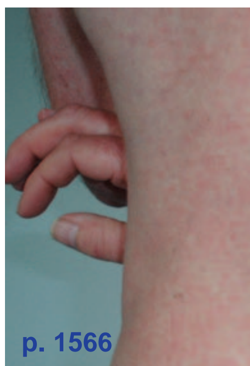

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Malaria Epidemics and Interventions, Kenya, Burundi, Southern Sudan, and Ethiopia, 1999–2004

Francesco Checchi,*† Jonathan Cox,† Suna Balkan,‡ Abiy Tamrat,§ Gerardo Priotto,* Kathryn P. Alberti,* Dejan Zurovac,‡¶# and Jean-Paul Guthmann*

Quantitative data on the onset and evolution of malaria epidemics are scarce. We review case studies from recent African *Plasmodium falciparum* epidemics (Kisii and Gucha Districts, Kenya, 1999; Kayanza Province, Burundi, 2000–2001; Aweil East, southern Sudan, 2003; Gutten and Damot Gale, Ethiopia, 2003–2004). We highlight possible epidemic risk factors and review delays in epidemic detection and response (up to 20 weeks), essentially due to poor case reporting and analysis or low use of public facilities. Epidemics lasted 15–36 weeks, and patients' age profiles suggested departures from classical notions of epidemic malaria everywhere but Burundi. Although emergency interventions were mounted to expand inpatient and outpatient treatment access, we believe their effects were lessened because of delays, insufficient evaluation of disease burden, lack of evidence on how to increase treatment coverage in emergencies, and use of ineffective drugs.

Plasmodium falciparum malaria epidemics were detected in 41 African sites from 1997 through 2002 (*1*). A total of 125 million persons are considered at risk for malaria epidemics, with an estimated yearly death rate of 155,000 to 310,000 (*2*).

Research on malaria epidemics mostly concerns long-range forecasting, early warning, and early detection (improved understanding of the role of temperature, rainfall, and El Niño–Southern Oscillation events [*3–6*], development of epidemic detection thresholds [*7*]). Malaria epidemics evolve rapidly and most often occur in remote, underresourced settings without proper surveillance. Data on their evolution may thus go unrecorded, which prevents the development of evidence-based recommendations on effective epidemic control.

Recently, Médecins Sans Frontières (MSF) intervened in several *P. falciparum* malaria epidemics in remote or conflict-affected sub-Saharan African settings. We present case studies from these interventions (Kisii and Gucha Districts, Kenya, 1999; Kayanza Province, Burundi, 2000–2001; Gutten and Damot Gale, Ethiopia, 2003–2004; Aweil East County, southern Sudan, 2003). We also describe the epidemics and possible factors that explain their occurrence, review challenges encountered in their detection and control, and make recommendations for epidemic prevention and control policies. This article reports health facility–based morbidity and mortality data. Findings on deaths in the community will be presented elsewhere (manuscript in review).

Methods

We reviewed MSF program reports; unpublished assessments (*8–12*); and available morbidity, mortality, diagnostic, and treatment data from each of the 5 interventions. We also consulted archives of the United Nations humanitarian data clearinghouse (www.reliefweb.int) for general situation reports for each epidemic period and extracted meteorologic indexes to explore the possible contribution of climate to epidemic onset (online Appendix, available from http://www.cdc.gov/ncidod/EID/vol12no10/06-0540_app.htm).

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Results

Description of Intervention Sites

Four interventions (Table 1) took place in highland environments, where peaks and valleys create a complex, climate-affected altitude gradient of malaria transmission and age-acquired immunity. Kisii and Gucha Districts are located in the southern highlands of Nyanza Province, Kenya, and experience low year-round transmission, with short, dramatic, and increasingly frequent outbreaks (*16*). Before May 1999, the last recorded epidemic had taken place from January through April 1998. The epidemic we describe also affected 10 nearby districts (*17*).

In Burundi's northern Kayanza Province, a 3-year time series up to September 2000 showed constant monthly caseloads of ≈10,000 outpatients/month. In 2000, MSF operated 7 of the province's 22 outpatient facilities. The September 2000–May 2001 epidemic, the largest ever recorded in Burundi, affected 9 of 16 provinces, and 3.5 million cases were reported (*18*).

The Ethiopian highlands experience 2 moderate transmission seasons every year (after rains in March through April and August through September). Epidemics occur in 5- to 8-year cycles; >1 million cases were recorded in 1998 (*1*). The 2003–2004 epidemic affected 15 million persons in 3 federal regions (*19*).

Finally, malaria is considered endemic in low-altitude Aweil East County (Bahr el Ghazal state, southern Sudan), although no data are available. Most cases occur from July through January after spring rains.

Possible Epidemic Determinants

Findings on possible epidemic determinants are summarized in Table 1. Factors noted at all sites were drought in preepidemic years followed by above-average rainfall in the preepidemic months and elevated drug resistance.

Table 1. Characteristics of intervention sites and potential determinants of epidemics*

Characteristic/ determinant	Kisii/Gucha, Kenya	Kayanza, Burundi	Aweil East, southern Sudan	Gutten, Ethiopia	Damot Gale, Ethiopia
Epidemic period (no. weeks)	May–August 1999 (15)	September 2000–May 2001 (36)	June–November 2003 (22)	July 2003–February 2004 (33)	July 2003–January 2004 (30)
Population	956,000	578,000	307,000	44,000	287,000
Altitude (m)	1,200–2,200	1,400–1,750	430	1,700	1,600–2,100
Malaria vectors	*Anopheles funestus* (constant), *A. gambiae* sensu lato (seasonal)	*A. arabiensis* (95%), *A. funestus* (5%)	Not available (*A. gambiae* sensu lato presumed)	*A. arabiensis*	*A. arabiensis*
Malaria species (nonepidemic months)	*Plasmodium falciparum* (>90%)	*P. falciparum* (>90%)	*P. falciparum* (>95%)	*P. falciparum* (≈25%), *P. vivax* (≈75%)	*P. falciparum* (≈60%), *P. vivax* (≈40%)
Temperature anomalies	Above average in 3 preepidemic months	None apparent	Maximum LST strongly below average during epidemic	None apparent	None apparent
Rainfall anomalies	Heavy rainfall in preepidemic rainy season after drought in previous rainy season	Heavy rainfall 5 and 3 months before epidemic, drought 2 years before epidemic but not in preepidemic year	Below average rainfall in 3 preepidemic years, above average in 2 preepidemic months	Below average rainfall in 2 preepidemic and epidemic years but heavy rainfall in preepidemic month	Below average rainfall in 2 preepidemic and epidemic years but heavy rainfall in 3 preepidemic months
Land pattern changes	None reported	Creation of rice paddies and fish ponds	Widespread flooding	Creation of water ponds	None reported
Political instability	None	Armed conflict	Tenuous ceasefire	Inactive insurgency	Inactive insurgency
Population movement	None	Forced relocation	Seminomadic, returnees from north Sudan	Government resettlement schemes	Government resettlement schemes
Global acute malnutrition†	Not available	10%–15%	25%	Not available (probably >5%)	28%
Drug resistance (in vivo failure rates)	CQ 24%–87% (neighboring districts), SP 10% (*13*)	CQ 100%, SP 54.2%, CQ+SP 42.0% (*9*)	CQ 63%, SP 3% (*14*)‡	SP 78.0% (*15*)	SP 68.1% (neighboring zone) (*15*)

*LST, land surface temperature; CQ, chloroquine; SP, sulfadoxine-pyrimethamine.
†Among children <5 y of age; malnutrition rates >15% denote a serious situation; values are provided for 2 months before the epidemic.
‡Percentages refer to the frequency of single *Pfcrt* mutations and triple *Dhfr* mutations in the *P. falciparum* genome of outpatients sampled in Aweil East. These mutations are predictive of in vivo CQ and SP failure rates, respectively.

Individual sites also experienced temperature abnormalities, land pattern changes, and high malnutrition rates. Further detail on these findings is provided in the online Appendix.

Epidemic Alert and Detection

No early warnings were issued. In Kisii, the alert came from the media in epidemic week 5 (when the district hospital was overwhelmed with malaria cases). MSF issued alerts in Kayanza (doubling of fever cases in epidemic week 2, early exhaustion of antimalarial stocks), Aweil East (quadrupled inpatient and outpatient malaria after epidemic week 1), and Damot Gale (increased proportion of *P. falciparum*–positive test results among children admitted to feeding centers, Figure 1). No alert information was found for Gutten.

Formal epidemic declaration was hampered by missing data. Time series for historical comparisons were available in Kisii (12 years) and Kayanza (3 years), where, however, authorities initially suspected a typhus outbreak, until the 80% seroprevalence detected among febrile patients (epidemic week 7) pointed to *P. falciparum*. In Aweil East, comparison with the 2 past years was confounded by 1) a change in diagnostic strategy (from presumptive in 2001 to rapid test–based in 2002 and 2003) and 2) decreased access to treatment in 2003 because of flooding.

In Ethiopia, a malaria-specific surveillance system aimed for early outbreak detection at both the village (positivity >25% detected among slides collected by field workers in the community was considered an outbreak and theoretically led to village-level mass treatment and vector control) and *woreda* or zone (where weekly reports from health facilities were compiled) levels. A malaria epidemic was declared in August 2003 (6–10 weeks after probable onset) by East Wollega Zone, including Gutten. Data from this surveillance system were difficult to interpret. Only some of the reports reached the zone bureau, with a delay of 4 to 6 weeks. In Damot Gale, despite incomplete reporting, a massive increase in caseload was evident at the province level (6,500 from July through October 2003 vs. 1,233 from July through October 2002); however, by early July (epidemic onset), only reports up to April were available. Furthermore, clinics aggregated mass fever treatment and outpatient data, causing artificial incidence spikes when the former took place. Conversely, drug shortages in August gave a false impression of declining incidence. MSF had only been present in Damot Gale since April 2003 and only operated feeding centers.

Operational Response

Interventions occurred 3–20 weeks late (Table 2). In Kisii and Gucha, numerous agencies, including Merlin, African Medical and Research Foundation (AMREF), Cooperative for Assistance and Relief Everywhere, Inc. (CARE), World Vision, the Kenyan Army, and medical staff of Kenyatta National Hospital supported health structures. Elsewhere, MSF was the sole external agency. Everywhere, facilities were initially overwhelmed. In Kisii Hospital, bed occupancy reached 300% in the first 3 weeks. Hospitalization capacity was 0.45 beds per 10,000 people in Gutten and 0.53 beds per 10,000 in Damot Gale, where, in June and July 2003 (epidemic onset), the rate of outpatient consultations per person per year was 0.03–0.09 (0.5–1.0 is expected in such settings if access is good) (*20*). Although waived once epidemics were officially recognized, user fees were initially charged in Kenya, Burundi, and Ethiopia. In Aweil East, non-MSF clinics had run out of chloroquine 3 weeks into the outbreak.

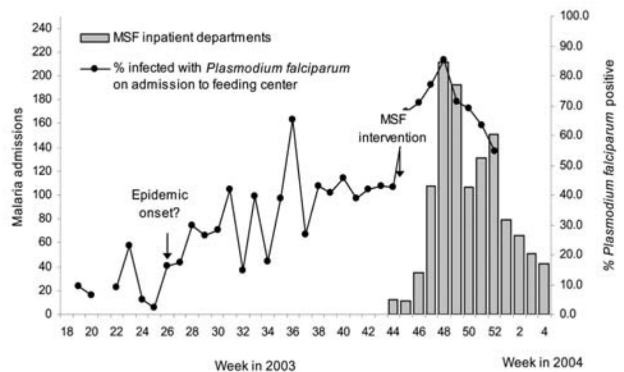

Figure 1. Trends in inpatient malaria caseload and positivity among malnourished children admitted to feeding centers in Damot Gale, Ethiopia, 2003–2004. MSF, Médecins Sans Frontières.

All interventions included inpatient components with blood transfusion. Conversion of existing MSF nutritional structures enabled expansion of care in Aweil East and Damot Gale. To reach isolated communities, mobile clinics, consisting of teams of nurses or nursing assistants working with simple treatment algorithms, were established at each site. However, this intervention occurred late (10 weeks late in Kisii and Gucha, 7 in Kayanza, 8 in Aweil East, 13 in Gutten, and 27 in Damot Gale) and, apart from in Kayanza, after the epidemic peak (Table 2). Choice of location depended on results of a cross-sectional prevalence survey (Kisii and Gucha), distance from the nearest health center or proximity to swampy areas (Kayanza), known gathering point and greatest distance to the outpatient department (Aweil East), known gathering point near existing health posts (Gutten), and village morbidity/mortality surveillance results (Damot Gale). Diagnosis was presumptive everywhere except Ethiopia, where the *P. falciparum*–specific rapid diagnostic test (RDT) Paracheck (Orchid Biomedical Systems, Verna, Goa, India) was used systematically (Table 2). In Aweil

Table 2. Details of operational response to malaria epidemics by intervention site*

Factor	Kisii/Gucha, Kenya	Kayanza, Burundi	Aweil East, southern Sudan	Gutten, Ethiopia	Damot Gale, Ethiopia
Delay of intervention (wks)	7	7	3	≥12, probably 19	20
Inpatient care					
Expansion in bed capacity	From 310 to 510 beds	From 65 to 125 beds	From ≈80 to ≈120 beds	From 2 to ≈100 beds	From 12 to >100 beds
Treatment	IM/IV quinine, IM artemether	IM/IV quinine	IM artemether	IV quinine	IV/IR quinine
Diagnosis	Presumptive	Blood slide	RDT	RDT	RDT
Fixed outpatient care					
Increase in capacity	2 additional OPDs	Increased capacity in 5 OPDs, 2 additional OPDs	Conversion of nutritional centers, 2 additional OPDs	1 additional OPD	Supervision and drug supply to 5 OPDs
Treatment	SP	CQ+SP	AS+SP	Quinine (IR if vomited)	SP, quinine
Diagnosis	Presumptive	Presumptive	RDT	RDT	RDT
Mobile clinics					
Number	3	6	14	5	Not available
Catchment population	302,000	Not available	144,000	44,000	73,000
Sites visited	45	10	43	5	14
Days per site per week (wks of operation)	0.2–0.3 (7)	1.2 (22)	1–2 (15)	2 (13)	0.2–0.5 (4)
Treatment	SP, AS+SP (73.4% of cases)	CQ+SP	AS+SP, artemether for severe cases	Quinine	Quinine
Diagnosis	Presumptive	Presumptive	Presumptive	RDT	RDT

*IM, intramuscular; IV, intravenous; IR, intrarectal; RDT, rapid diagnostic test; OPD, outpatient department; SP, sulfadoxine-pyrimethamine; CQ, chloroquine; AS, artesunate.

East, mobile teams traveled on bicycle and canoe, spending 3–4 days in each location; because transporting patients with severe cases was impossible, more experienced teams carried injectable artemether and anticonvulsant drugs and treated 110 patients on a semi-inpatient basis (no outcome was recorded for these patients). At other sites, mobile clinics remained on site for 1 day and provided an ambulance service. Mobile teams were present in each targeted village for no more than 1–2 days a week on average and as little as once a month in Kenya and Damot Gale (Table 2). Mobile clinics treated 46,541 (9.3%) of 501,214 reported cases in Kayanza, 34,749 (68.3%) of 50,863 in Aweil East, 7,258 (19.4%) of 37,457 in Gutten, and 467 (2.8%) of 16,621 in Damot Gale (Table 3). In Damot Gale, active severe case finding was organized (no data available).

Artemisinin-based combination therapy (ACT) was deployed in Aweil East and in mobile clinics in Kenya (Table 2). Its use was not officially authorized in Burundi and in Ethiopia, where empiric evidence of poor sulfadoxine-pyrimethamine efficacy, later confirmed by in vivo studies (*15*), led clinicians to use quinine as first-line treatment. To ensure adherence to the 7-day regimen, high-risk patients were treated intrarectally under observation (Table 3).

Surveillance and Epidemic Evolution

In Burundi, Sudan, and Ethiopia, surveillance data were analyzed weekly. In Kayanza, RDT testing was carried out every 2–3 weeks among outpatients to monitor epidemic trends. In Aweil East and Gutten, an automated surveillance spreadsheet generated key indicators and graphs (caseload, proportionate morbidity and mortality, case-fatality, RDT confirmation of diagnosis).

The Kisii and Gucha epidemic followed a historical pattern of short dramatic peaks (Figure 2). Kisii Hospital records showed that, during the first 12 epidemic weeks, 2,669 (22.2%) of children <5 years of age in Kisii municipality (≈12,000) were hospitalized for malaria (Table 3).

The Kayanza epidemic lasted 36 weeks and roughly followed a normal distribution (Figure 3). A total of 501,214 cases were reported, for a minimum attack rate of 86.5%.

In Aweil East, a peak was reached by epidemic week 2, and a steady decline followed, which reflected percentage of confirmed malaria cases among women who came to the clinic for antenatal visits (Figure 4). Children <5 years of age (assumed to be 20% of the population) experienced attack rates of ≥41.2% (all malaria) and 1.1% (complicated).

In Ethiopia, the epidemic's evolution can partly be reconstructed by plotting available microscopy results from the Gutten government clinic, which yields a normal distribution (Figure 5), and percentage *P. falciparum* positivity among malnourished children admitted to feeding centers in Damot Gale (Figure 1). Results showed a steady rise from June, a plateau in August and September, and a new peak in late November after heavy rains.

Table 3. Epidemiologic profile of malaria at fixed inpatient, fixed outpatient, and mobile health facilities operated by Médecins Sans Frontières in 5 intervention sites

Characteristic	Kisii/Gucha, Kenya	Kayanza, Burundi	Aweil East, southern Sudan	Gutten, Ethiopia	Damot Gale, Ethiopia
Uncomplicated cases					
Fixed outpatient centers					
All ages	13,127*	272,459	15,239	15,928†	–
Age <5 y (%)	2,426 (18.5)	Not available	7,257 (47.6)	4,758‡ (29.9)	–
Mobile clinics					
All ages	29,769	46,541	34,749	7,258	467
Age <5 y (%)	5,376 (18.1)	Not available	17,338 (49.9)	1,405 (19.4)	145 (31.0)
Complicated cases					
All ages	9,773§	3,953¶	875#	330**	1,291
Age <5 y (%)	5078 (52.0)	761 (19.3)	683 (78.1)	175 (53.0)	595 (46.1)
No. deaths (CFR [%])	397 (4.1)	108 (2.7)	50 (5.7)	34 (10.3)	62 (4.8)
No. deaths <5 y (CFR [%])	164 (3.2)	31 (4.1)	39 (5.7)	15 (8.6)	38 (6.4)
Minimal attack rate (%)††	22.2 (complicated, <5 only; 12/15 weeks)	86.5 (36/36 weeks)	41.2 (<5 only; 22/22 weeks)	53.4 (15/33 weeks)	Not available
P. falciparum prevalence at epidemic peak (%)	38–49 (community survey)	80 (random sample in OPD‡‡ queue)	52–64 (random sample in OPD queue)	Not available	60 (random sample by community workers)

*Includes data from 3 government clinics (Masimba, Kenyenya, and Etago) for which age breakdown was available.
†Includes 2,061 patients treated with intrarectal quinine in inpatient department.
‡Includes 1,773 patients <5 years of age treated with intrarectal quinine in inpatient department.
§Includes data from Kisii, Keumbu, and Ogembo hospitals, supported by Médecins Sans Frontières and other agencies but operated by the government.
¶Excludes patients treated in the Kayanza government hospital (data not available).
#Excludes 110 severe cases treated by mobile clinics (no age breakdown or outcome available).
**Includes only hospitalized patients who met a strict definition of severe malaria, which probably explains the considerably higher case-fatality ratio (CFR) noted in Gutten.
††Ratio of weeks refers to the number of epidemic weeks from which the attack rate was calculated divided by the total number of epidemic weeks.
‡‡OPD, outpatient department.

Profile of Patients

Among uncomplicated cases, the proportion of patients <5 years of age exceeded the expected levels of 15% to 20% in southern Sudan and Ethiopia but not Kenya, where only presumptive diagnosis was used (Table 3). Patients <5 years made up half of all complicated cases in Kisii, Gucha, Gutten, and Damot Gale and almost 80% in Aweil East. Case-fatality rates were comparable across ages and sites except Gutten (footnote, Table 3). Convenience samples of outpatients or household surveys suggested high *P. falciparum* prevalences at or near caseload peaks (Table 3). Where MSF was involved in both outpatient and inpatient care (Kayanza, Aweil East, and Gutten), comparable proportions of patients were hospitalized (1.2%, 1.9%, and 1.4% respectively).

Discussion

In sub-Saharan Africa, malaria epidemics arise suddenly in mostly remote, disadvantaged settings without effective alert systems. Our case studies show that large-scale interventions can be organized in such epidemics, and that these interventions can considerably increase diagnostic and treatment output. Both preparedness and control, however, were seriously deficient. Epidemic detection was late everywhere, and additional delays occurred before external intervention to support overwhelmed local health structures.

Experiences in Kisii, Gucha, Kayanza, Gutten, and Damot Gale probably reflect conditions in neighboring regions affected by the corresponding epidemics, although scarcity of published records makes comparisons difficult. This analysis relies on programmatic data, the limitations of which are apparent.

Epidemic Risk and Vulnerability Factors

Climate

Our analysis did not include controls (i.e., sites where no epidemics occurred). Nevertheless, remotely sensed climate data suggest rainfall abnormalities during key preepidemic periods: relative drought in the 2 or 3 preepidemic years (with the exception of Kayanza) and above-average rainfall 1–2 months before epidemic onset. No consistent temperature pattern emerged.

The full role of such abnormalities as epidemic determinants is unclear. Furthermore, although remotely sensed environmental variables provide relatively robust and accurate estimates (*21*) and are becoming more publicly accessible (*22*), they remain only proxies for ground-based measurements. Nonetheless, we believe that our findings implicate climate abnormalities to a varying extent in all 5 epidemics and support strengthened monitoring of climate variables for early warning.

Other Factors

Land cover changes in Aweil East (flooding) and in Kayanza (rice paddy creation) probably favored vector

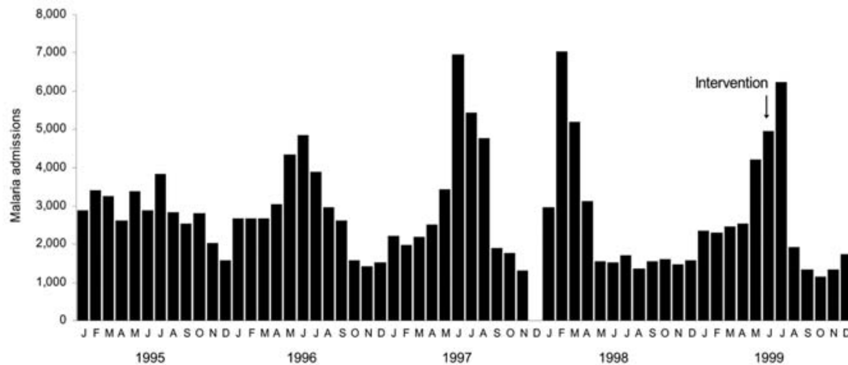

Figure 2. Trends in outpatient malaria caseload in Kisii Hospital outpatient department, Kenya, 1995–1999. Data for December 1997 are missing because of a nursing staff strike.

breeding. Malnutrition, displacement, and drug resistance may not in themselves cause epidemics, but in our settings these factors probably exacerbated the epidemics' magnitude, duration, and case-fatality ratios. The effects of past drought and malnutrition are difficult to extricate: they are related causally, and either could result in impaired immunity (respectively, through reduced exposure to infection and nutrient deficiencies).

To our knowledge, no entomologic data were collected during any of these epidemics, which limits the strength of our findings; changes in vector species or breeding habitats could have had a major role, but these factors can only be imputed from observed land pattern or climate alterations. Future studies on malaria epidemics should include detailed entomologic profiling, even during the epidemics.

In short, we believe that, given available evidence, to predicate epidemic prevention activities solely on the basis of individual risk factors (meteorologic or other) would be imprudent. Instead, appropriate decision support systems should be built that integrate all relevant data (e.g., environmental variables, food security and nutritional status, drug efficacy, health coverage, vector characteristics, population at risk) into a risk profile for each epidemic-prone population, to be updated regularly; in such a scenario, warning flags (*23*) resulting from detected environmental anomalies or other risk factors would result in enhanced surveillance activity and increased emergency preparedness (e.g., stockpiling drugs, ensuring insecticides and spray teams are in place), rather than leading directly to control activities.

Difficulties in Detection

Even without early warning, detecting epidemics within 2 weeks of onset should be possible (*24*), provided that weekly reporting and analysis are complete and timely and that caseload data reflect community incidence trends. In most case studies described here, epidemics were detected after substantial delay and by agencies other than local authorities with unconventional methods, such as RDT monitoring among malnourished children. An exception was Aweil East, where weekly reporting and analysis took place. Several formal definitions of a malaria epidemic have been proposed. Most, like the C-Sum or Cullen methods (*22*), rely on comparison with past caseloads. These methods cannot be effective unless surveillance is greatly improved. Experience from the Highland Malaria Project (HIMAL) (*7*) shows that meeting the requirements of epidemic early detection requires supplementing existing routine surveillance systems with networks of representative sentinel health facilities, new data collection forms, procedures for rapid exchange of data between different levels of the health system, and tools for the collation, analysis, and interpretation of incoming data. However, the apparent success of HIMAL's pilot early detection systems in selected districts of Uganda and Kenya suggests that these efforts are viable, given requisite resources and motivation (J. Cox and T. Abeku, pers. comm.).

Free treatment and steady drug supplies probably favored early detection in Aweil East. Conversely, in Ethiopia, facility use was too low to reflect the magnitude of the emergency, and irregular drug distributions confounded epidemiologic monitoring. User fee systems may have long-term benefits, but cost barriers hamper treatment access (*25*). In impoverished populations at risk for malaria epidemics, a free care policy may encourage early treatment seeking and thus facilitate early epidemic detection and monitoring, in addition to minimizing

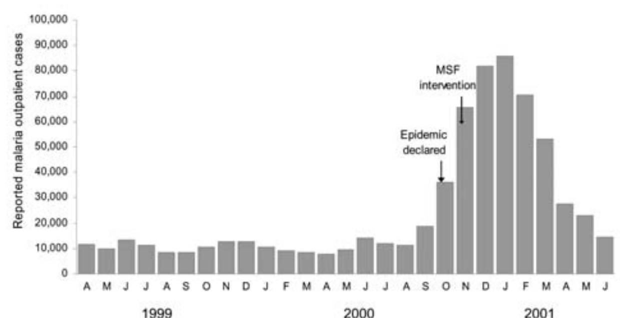

Figure 3. Trends in outpatient malaria caseload in Kayanza Province, Burundi, 1999–2001. MSF, Médecins Sans Frontières.

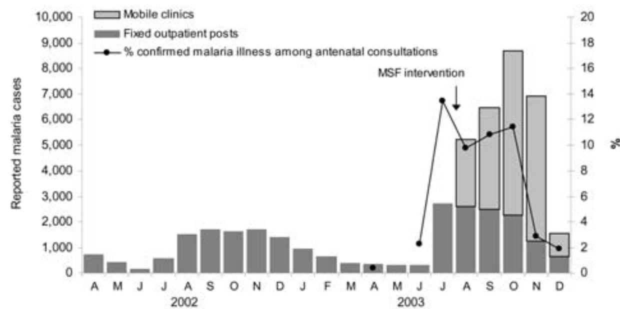

Figure 4. Trends in outpatient caseload and proportionate malaria among pregnant women attending antenatal consultations in Aweil East, southern Sudan, 2002–2003. MSF, Médecins Sans Frontières.

deaths. Conversely, user fee systems may result in "silent" public health disasters.

Intervention Strategies

Expansion of Access

By the time interventions were implemented, their potential effects were reduced. Mobile clinics were deployed to expand health access and detect severe cases. Implementation of clinics understandably varied according to local conditions, but apart from in Aweil East, probably had limited impact. Mobile clinic programs should be designed on the basis of clearly identified catchment areas and set frequencies with which communities should be visited. Although various criteria were used in our case studies, we believe that actual access to healthcare should be a key indicator for selecting target populations. Rapid methods to assess antimalarial treatment coverage thus need to be developed. How frequently communities are visited determines both the improvement in treatment coverage and the probability of preventing progression to severe disease through prompt treatment, which is likely to increase exponentially with frequency of mobile team visits; we hypothesize that frequent visits to selected sites may be more efficient than infrequent visits to a wider area. Impact monitoring should be included in future mobile clinic interventions to adjust their strategy as the situation evolves, and they should be evaluated after the fact. More generally, alternative modes of rapidly decentralizing care, such as fixed temporary health posts or training of resident community health workers (possibly equipped with artesunate suppositories to treat severely ill patients), merit further exploration. Where no clear indications exist that local health structures can cope with a large malaria epidemic, mobile clinics or other temporary treatment programs should be implemented immediately.

Reduction of Case-fatality Ratio

Case-fatality ratio among patients with complicated cases was lower than current best estimates of 10% (*2*) and 13% (*26*); however, whether all cases were severe depends on the case definition used. Treatment of uncomplicated cases relied on failing drugs everywhere but Sudan, and sulfadoxine-pyrimethamine monotherapy was probably counterproductive because the drug stimulates gametocytogenesis (*27*) and thus transmission. Ineffective drug use in Burundi probably limited the effect on mortality; in Ethiopia, quinine first-line administration proved challenging because of vomiting and required impractical patient monitoring.

Effect on Public Health

Kayanza excluded, the increased proportion of children <5 years of age among inpatients, as previously observed in Kenya (*28*), suggests that children were more susceptible to symptomatic disease, which challenges classical notions of unstable, epidemic malaria. In Aweil East, the predominance of children is consistent with stable, mesoendemic to hyperendemic transmission, and this situation is probably better characterized as a severe seasonal outbreak.

Clinic-based attack rates approach 100% for all age groups when extrapolated to the entire epidemic period (Kayanza and Gutten) and are even more alarming among children <5 years of age in Kisii. Even after overdiagnosis from presumptive treatment is accounted for, these rates are likely to be gross underestimates. The vast gap in treatment coverage was evident in Aweil East, where large-scale deployment of mobile clinics greatly increased output, and in Ethiopia, where despite capturing only the declining phase of the epidemic, uninterrupted provision of free care with effective drugs resulted in far higher outpatient and inpatient department attendance. The true community incidence in these epidemics is probably much greater than represented by regular reporting systems and higher than current estimates of 0.5 episodes of malaria per

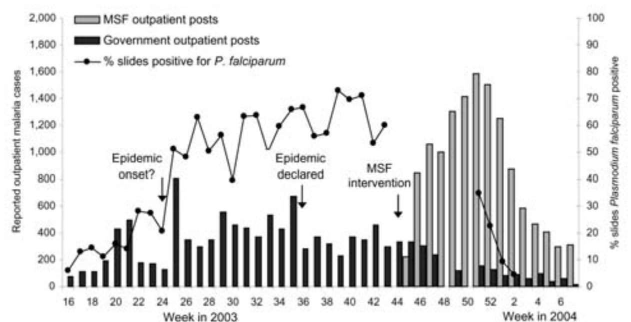

Figure 5. Trends in outpatient malaria caseload and slide positivity in Gutten, Ethiopia, 2003–2004. MSF, Médecins Sans Frontières.

person per epidemic (*29*). Only population-based studies can yield realistic estimates of this incidence.

Conclusion

Malaria epidemics create daunting medical emergencies. In addition to ongoing research on alert systems, much greater donor investment is necessary to prevent and control them. All 4 countries in this study are moving to ACT combinations for outpatient treatment, a major improvement that is still insufficient unless 1) simple but valid surveillance data are transmitted and analyzed on a weekly basis, maximizing the chance of early epidemic detection, and 2) treatment coverage of uncomplicated and complicated cases truly reflects community needs. Further research is needed on methods to rapidly estimate needs (incidence) and coverage and on strategies to efficiently expand treatment access. Arguably, focusing resources only on how to predict and respond to epidemics might lead policymakers to overlook basic problems with access to effective treatment and tools for prevention that are common to both epidemic and stable malaria settings and that probably merit similar solutions. Donors and policymakers should thus aim for a balanced approach: improved capacity for epidemic prediction and response is needed, but long-term improvements in access to proper care and vector control by all members of the community, even before epidemics strike, must not be neglected, as they could be the most relevant determinants of decreased epidemic severity.

Because malaria epidemics are difficult to predict and multifactorial, setting up controlled studies to formally demonstrate the benefit of any single intervention will be difficult. Properly documenting the cost, feasibility, and output of these interventions and measuring the true extent of malaria epidemics are nevertheless crucial to inform the choice of future prevention and control strategies and must be included in the research agenda.

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Birds and Influenza H5N1 Virus Movement to and within North America

John H. Rappole* and Zdenek Hubálek†

Highly pathogenic avian influenza (HPAI) H5N1 expanded considerably during 2005 and early 2006 in both avian host species and geographic distribution. Domestic waterfowl and migratory birds are reservoirs, but lethality of this subtype appeared to initially limit migrant effectiveness as introductory hosts. This situation may have changed, as HPAI H5N1 has recently expanded across Eurasia and into Europe and Africa. Birds could introduce HPAI H5N1 to the Western Hemisphere through migration, vagrancy, and importation by people. Vagrants and migratory birds are not likely interhemispheric introductory hosts; import of infected domestic or pet birds is more probable. If reassortment or mutation were to produce a virus adapted for rapid transmission among humans, birds would be unlikely introductory hosts because of differences in viral transmission mechanisms among major host groups (i.e., gastrointestinal for birds, respiratory for humans). Another possible result of reassortment would be a less lethal form of avian influenza, more readily spread by birds.

Avian influenza virus A refers collectively to a group of viruses within the family *Orthomyxoviridae* that has a worldwide distribution and causes a variety of diseases in birds. Classification of influenza viruses is based on 2 glycoproteins (antigens) characteristic of the group members: hemagglutinin, of which 16 forms are known; and neuraminidase, of which 9 forms have been described. In 1997, a virulent, highly pathogenic avian influenza (HPAI) A virus, identified as the H5N1 subtype, was identified in samples taken in Hong Kong (*1,2*). This virus has spread to several localities in Asia and, since late 2005, Europe (*3*) and Africa (*4*) (Table 1). HPAI H5N1 virus is found most commonly in domestic fowl, although as of late 2005, it has been found in migratory and resident birds of several orders (mainly Anseriformes) and in pigs, civets, house cats, tigers, leopards, and humans (*3*). This virus poses a potential danger to human populations; 224 human cases of H5N1 avian influenza have been reported as of May 29, 2006; 127 of these cases were fatal (*17*). Its discovery in migratory birds is especially troubling because of the potential for rapid dispersal of the virus across continents and hemispheres.

We review facts concerning outbreaks of H5N1; the species of birds, especially migrants, known to have been infected by this subtype; and available information on the ability of migrants to serve as reservoir or introductory hosts that move the virus from outbreak areas to new localities. On the basis of this information, we consider the avian pathways by which HPAI H5N1 might enter the Western Hemisphere and, once present, the likelihood that it will be able to disperse to new regions. We define migratory or migrant birds as those species that move annually between geographically separate breeding and wintering quarters. Migrating birds are those actually in the process of moving from 1 locality to another.

Ecology of Influenza A Viruses

Avian influenza A viruses are common and widespread in birds. Most viruses in this family attack the intestinal tract of the host preferentially and are spread mainly by shedding in host feces (*18,19*). Waterfowl, e.g., ducks, geese, and swans (Anseriformes), and shorebirds (Charadriiformes) are particularly susceptible because they are exposed to water that may be contaminated with infected fecal matter, especially at specific sites and seasons, when these birds congregate densely at relatively confined and shallow water bodies (Figure 1). A secondary mode of viral spread is consumption of infected avian host

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Table 1. Geographic spread of highly pathogenic avian influenza H5N1 subtype since 1996

Date	Event
1996	1st isolation; domestic geese, southern China (*5*)
1997–1998	Chickens, Hong Kong; 18 humans (6 deaths) (*6*)
1999	Geese, Hong Kong (*7*)
2001	Geese from China in Vietnam (*8*)
Nov 2002	Hong Kong poultry, other bird species in or near zoologic parks (*7*)
Feb 2003	Human travelers from Fujian Province (China) (*9*)
Dec 2003–Nov 2005	Poultry (mainly chickens) and humans: South Korea, Vietnam, Thailand, Hong Kong, Cambodia, Laos, Indonesia, China, and Malaysia (*6*)
Jan 2004	Wild birds: Hong Kong (*10*)
Feb 2004	Birds in a zoo collection: Cambodia (*10*)
Mar 2004	Wild bird: South Korea (*10*)
Oct 2004	Bird smuggled from Thailand into Belgium (*11*)
Apr–Jun 2005	Migratory birds: Qinghai Lake and Xinjiang Province, China (*12*)
Jul–Oct 2005	Poultry and wild waterfowl: Novosibirsk, Altai, Kurgansk, Omsk, and Tyumen regions, Asian Russia (*13,14*)
Aug 2005	Geese and other poultry: northern Kazakhstan, Tibet (*13*)
Aug 2005	Migratory waterfowl: northern Mongolia (*15*)
Aug–Oct 2005	Poultry and pigeons: Ural Territory, Russia (*13*)
Aug 2005	Wild waterfowl: Kalmykia, European Russia (*13*)
Oct 2005	Domestic turkeys: Western Asian turkey (*13*)
Oct–Nov 2005	Poultry and wild migratory birds: Romania, Ukraine (*13*)
Oct 2005	Wild birds: Thailand (*15*)
Oct–Nov 2005	Poultry, wild birds, some humans: 7 Chinese provinces (*15*)
Oct 2005	Migratory waterfowl: Croatia (*13*)
Oct 2005	Poultry: Tula and Tambov regions, European Russia (*14*)
Oct 2005	Quarantined birds from Taiwan in United Kingdom (*16*)
Jan 2006	Humans: Iraq (*15*)
Jan 2006	Poultry: Nigeria, India (Maharashtra) (*15*)
Feb 2006	Migratory waterfowl: Bulgaria, Greece, Italy, Slovenia, Bosnia, Azerbaijan, Iran, Georgia, Germany, Switzerland, Austria, Hungary, France, Croatia, Slovakia, Bosnia (*15*)
Feb 2006	Poultry: Egypt, Cameroon, Niger, Ethiopia (*15*)
Mar 2006	Migratory birds: Sweden, Denmark, Serbia, Poland, Czech Republic (*15*)
Mar 2006	Poultry: Afghanistan, Pakistan, Albania, Israel, Jordan, Lebanon (*15*)
Apr 2006	Poultry: Burkina Faso, Côte d'Ivoire, Myanmar, Nigeria, Palestinian Autonomous Territories (*15*)
May 2006	Poultry: Sudan; migratory birds: United Kingdom (*15*)

parts by predators, including captive carnivores, avian raptors, and carrion-feeding vertebrates. Infection by most avian influenza A strains appears to be asymptomatic for the host (*18*). Proportions of birds shedding active virus can be high (e.g., >30% in some Canadian duck populations) among juvenile waterfowl gathered in large flocks on lakes and ponds during the summer postbreeding molting period but decrease rapidly during southward migration, falling to 1% to 2% during winter (*18*). Nevertheless, shedding of active virus can remain as high as 0.25% by individual birds among northbound spring migrants, sufficient to reinfect northern breeding populations (*18*).

Most birds appear to be more or less susceptible to ≥1 strain of avian influenza A, but rates of infection and levels of susceptibility to the different viral subtypes vary among taxa. For instance, H3 and H6 subtypes are common in ducks, geese, and swans (Anseriformes), while H4, H9, H11, and H13 subtypes are more prevalent in sandpipers, terns, and gulls (Charadriiformes) (*20*). The best opportunities for viral transmission among large numbers of anseriform hosts would likely be on lakes and ponds in summer, where large concentrations gather for weeks to undergo the postbreeding, premigratory molt (*18*). For charadriiformes, the greatest viral transmission opportunities would likely be at stopover sites during fall migration, where tens of thousands of individual birds congregate to feed and roost (*20*).

Avian Influenza in Humans

Humans and other mammals normally are not susceptible to infection by avian influenza A viruses. Nevertheless, several subtypes of avian influenza or bird-origin influenza viruses have infected humans; 3 of these subtypes have caused pandemics within the past century. At present, HPAI H5N1 is entirely an avian influenza subtype. Humans can become infected, but so far as is known, they must inhale or ingest massive viral doses from excreta or tissues of infected birds to do so. Although clinically ill humans have high death rates, ≈50%, passage of H5N1 virus from human to human is rare (*3*).

The more humans infected with HPAI H5N1, the greater the probability that reassortment with a human

Figure 1. Saurus cranes (*Grus antigone*) over Naung Mung, Myanmar, in March 2006.

influenza virus strain will occur and produce a lethal form that is spread readily between humans (*18,19*). However, viral interhost transmission strategies differ fundamentally for those viruses that primarily infect humans versus those that infect birds. Bird viruses have an affinity for the host's intestinal tract, and interhost transmission occurs mainly by fecal contamination of shared water bodies. Human viruses more often attack the respiratory system and depend on shedding in respiratory effluvia for interhost transfer. If, or when, a reassortment or mutation of HPAI H5N1 produces a virus capable of efficient horizontal transfer among humans, the new virus would likely not be particularly effective in transfer among birds; migrants likely would play little role in spread of such a virus. Vaccines produced to prevent human infection by H5N1 might not be effective against a new virus produced by reassortment.

Birds as HPAI H5N1 Reservoirs and Introductory Hosts in the Old World

The main reservoirs and introductory hosts for avian influenza A viruses in general are migratory waterfowl and domestic fowl (*18,19*). HPAI H5N1, however, causes high rates of disabling illness and death in most avian species (*21*). High rates of illness would prevent migrants from being introductory hosts, since sick wild birds normally cannot move far and do not survive long. Thus, perhaps not surprisingly, no evidence exists that migrants were introductory hosts for H5N1 for several years after its initial appearance in Guangdong Province, People's Republic of China, in 1996. In fact, no deaths or even infections of migrants were reported until December 2002, when several migrants and exotic birds were found dead at a Hong Kong park and zoologic garden (*10*). Of 3,095 outbreaks of HPAI H5N1 reported from December 2003 through February 2005, all involved captive birds or domestic fowl (*6*). Until early August 2005, only 2 outbreaks of HPAI H5N1 had been confirmed in migratory birds presumed to be completely separate from domestic fowl: Qinghai Lake and Xinjiang Province, China, (April, May 2005) (*12*) and Lakes Erhel and Khunt in northern Mongolia (August 2005) (*15*). However, that situation has changed, and several new outbreaks have been recorded in migrants that were presumably separate from domestic fowl within the last few months (online Appendix; available from http://www.cdc.gov/ncidod/EID/vol12no10/05-1577_app.htm), perhaps signaling genetic modification of the virus (*19*).

Data based on observations of dead wild birds at sites where infections have broken out and negative results from subsequent extensive screening for seropositive or infected migrants around outbreak sites have indicated that HPAI H5N1 was lethal for most wild birds, at least until recently. Nevertheless, some studies have demonstrated that chickens, domestic ducks, and geese infected under laboratory conditions, as well as some wild birds exposed under quasilaboratory conditions (e.g., birds fed, watered, and protected at zoologic parks or gardens), survive infection and shed the virus in active form (*10,22,23*). The work by Komar et al. (*24*) on wild birds exposed to West Nile virus (WNV) under laboratory conditions may be instructive in this regard. These researchers found that in species like the fish crow (*Corvus ossifragus*), in which individual birds were known to have high death rates on exposure to the New York 99 subtype of WNV in the wild (on the basis of large numbers of birds found dead and failure to find free-flying birds captured that were seropositive), survival rates from exposure in the laboratory were 45%. When one considers that birds kept in a laboratory have ready access to food and water during their illness, as well as protection from inclement weather and predators, this finding perhaps is not surprising. However, wild birds associating with free-ranging domestic fowl at farm ponds, or captive exotic birds at city parks or zoological gardens, may receive some of the same benefits as laboratory birds, experiencing conditions conducive to survival of infection by HPAI H5N1.

Recent detections of HPAI H5N1 in free-ranging migrants may be a result of heightened awareness and thus the virus could have been circulating in migrants, although undetected. This explanation is unlikely considering the extensive screening of blood and feces of migrants in the past several years in Europe, parts of Asia, and North America. These screenings have searched for birds seropositive for H5N1 and other avian influenza type A viruses. These searches have involved sampling thousands of birds of hundreds of species (*25,26*). The virus may also have changed to some degree (*2,19*), allowing higher survival rates among some species of migrants. Both explana-

tions may have some relevance to the current situation. In any event, some migratory birds may now be able to move HPAI H5N1 in active form over considerable distances (online Appendix). Increasing numbers of recent reports document apparent movement of the virus, whereas before April 2005, no evidence existed of HPAI H5N1 in free-ranging migratory birds distant from domestic fowl, despite years of sampling of tens of thousands of migratory waterfowl of several species from wetland sites across the European continent (*25*).

Possible Role of Birds in Arrival of HPAI H5N1 Avian Influenza in New World

To date, HPAI H5N1 has not been recorded in the New World, although outbreaks of related avian influenza viruses lethal to domestic fowl have occurred in Ontario, Canada, in 1966 (H5N9); Pennsylvania, United States in 1983 (H5N2); Puebla, Mexico, in 1994 (H5N2); Chile in 2002 (H7N3); Canada in 2004 (H7N3); and Texas, United States, in 2004 (H5N2) (*27*). All of these outbreaks occurred in domestic poultry and were controlled without further diffusion. We see 3 possible modes by which HPAI H5N1 might gain entry to the New World if birds were the introductory host: 1) normal interhemispheric migration, 2) vagrancy, and 3) legal and illegal importation of birds as explained in the following section.

Normal Interhemispheric Migration

Few individual birds within few species undertake regular, interhemispheric migration. However, some do, and the waterfowl (Anseriformes, Charadriiformes, Ciconiiformes) could be introductory hosts for HPAI H5N1 to the New World (Table 2). Three pathways are used annually by a small number of waterfowl species to travel between the hemispheres: 1) Alaska–East Asia, in which birds that breed in Alaska winter in East Asia; 2) East Asia–Pacific North America, in which birds that breed in northeast Asia winter along the Pacific Coast of North America; and 3) Europe–Atlantic North America, in which birds that breed in Iceland or northwestern Europe winter along the Atlantic Coast of North America (Figure 2, Table 2).

Two lines of evidence argue against normal, interhemispheric migration as a likely mode of entry for HPAI H5N1 into the Western Hemisphere. First, as discussed previously, data indicate that most infected individual birds of most species of migrants become extremely ill and either cannot migrate far in their weakened state or die at the place of infection. Second, investigation of the genetics of avian influenza viruses has shown that little natural interchange occurs between the Eastern and Western Hemispheres: each hemisphere appears to have an avian influenza virus community that is largely distinct (*18*). This fact is particularly noteworthy when one considers that most avian influenza A viruses appear to be asymptomatic, and migrants readily transport them in infectious form, in stark contrast to the situation for HPAI H5N1. Presumably, the distinct nature of the avian influenza A community in each hemisphere results from the fact that the main reservoir for these viruses is migrants, and few migrants move regularly between the hemispheres (*32*).

Vagrancy

Perhaps a third or more of Eurasian waterfowl species have traveled into the Western Hemisphere as vagrants; some occur more regularly than others, including those listed in Table 2. However, all Eurasian vagrants are, by definition, extremely rare in the New World (a few birds per decade). One mode of interhemispheric vagrancy is tropical storm systems that originate off the West African coast during the Atlantic hurricane season, which lasts from June to November each year. These systems can, and occasionally do, sweep up and transport Old World birds, especially waterfowl, across the Atlantic to the New World (route 4, Figure 2). Vagrancy is much rarer (by several orders of magnitude) than normal interhemispheric migration and seems an even less likely mode of entry for HPAI H5N1.

Legal and Illegal Importations

Human traffic in birds and bird products is the sole documented means of HPAI H5N1 movement between geographically separate regions to date (*19*). While migratory birds have been suspected of involvement, particularly in cases in which no obvious human interchange of infected birds or products has occurred, these conclusions are inferred (*19*). Thus, if HPAI H5N1 is to be kept out of the Western Hemisphere, control of legal and illegal imports should be the primary focus of prevention efforts.

The legal importation of exotic birds has declined dramatically in the United States since enactment of the 1992 Wild Bird Conservation Act. Nevertheless, 2,770 birds entered the country through the New York port of entry in 1999, including 323 pet birds and 2,447 commercial birds. In addition, 12,931 birds passed through in transit (S. Kaman, US Department of Agriculture [USDA], pers. comm.) Legal importations are controlled by USDA Animal and Plant Health Inspection Service and the US Fish and Wildlife Service. Most imported birds undergo a 30-day quarantine at USDA facilities located near each of the 3 allowed ports of entry: New York, Miami, and Los Angeles. Quarantine procedures include isolation in indoor, air-filtered cages and standard testing for common poultry diseases, including avian influenza. The number of illegally imported birds is not known. These birds are not subject to quarantine and testing and could be a mode of entry for HPAI H5N1. Hawk eagles from Thailand infected with the virus were recently detected while being

smuggled into Belgium (*11*). Although these birds were detected and quarantined, they serve as an example of how such imports could spread the virus. Species commonly associated with the transhemispheric bird trade are listed in Table 2.

If birds turn out to be responsible for entry of HPAI H5N1 into the Western Hemisphere, illegal import of an infected bird or bird product seems the most likely mode of entry. We base this conclusion on the fact that illegally imported birds, unlike infected, free-flying migrants, are provided food and water ad libitum and protected from predators, greatly increasing their chances of survival in an infectious state. Furthermore, these birds often end up in close association with other, similarly protected birds,

Table 2. Known interhemispheric movement by migratory or vagrant waterfowl (Ciconiiformes, Anseriformes, Charadriiformes), domestic bird trade (Galliformes), or exotic bird trade (Galliformes, Psittaciformes) from Eurasia to North America*

Species	Likely mode of entry
Bean goose (*Anser fabalis*)	Migration†
Greylag goose (*A. anser*) (domestic)	Exotic and domestic bird trade
Whooper swan (*Cygnus cygnus*)	Migration†
Falcated duck (*Anas falcata*)	Migration,† exotic bird trade, zoos, vagrant
Eurasian wigeon (*A. penelope*)	Migration,†‡ exotic bird trade, zoos
Mallard (*A. platyrhynchos*) (domestic and wild)	Exotic and domestic bird trade
Garganey (*A. querquedula*)	Migration,†‡ exotic bird trade, zoos
Green-winged teal (*A. crecca*)	Migration†‡
Common pochard (*Aythya ferina*)	Migration†
Tufted duck (*Aythya fuligula*)	Migration†‡
Smew (*Mergellus albellus*)	Migration†
Jungle fowl (*Gallus gallus*) (domestic)	Domestic bird trade
Pheasants (Phasianidae)	Exotic bird trade, zoos
Quail (*Coturnix coturnix*)	Domestic bird trade
Wild turkey (*Meleagris gallopavo*) (domestic)	Domestic bird trade
Red-faced cormorant (*Phalacrocorax urile*)	Migration§
Gray heron (*Ardea cinerea*)	Vagrant
Little egret (*Egretta garzetta*)	Vagrant
Cattle egret (*Bubulcus ibis*)	Vagrant
Eurasian kestrel (*Falco tinnunculus*)	Vagrant
Northern lapwing (*Vanellus vanellus*)	Vagrant
Mongolian plover (*Charadrius mongolus*)	Migration†
Common ringed plover (*C. hiaticula*)	Migration§
Eurasian dotterel (*C. morinellus*)	Migration§
Spotted redshank (*Tringa erythropus*)	Migration†
Wood sandpiper (*T. glareola*)	Migration†
Gray-tailed tattler (*Heteroscelus brevipes*)	Migration†
Bar-tailed godwit (*Limosa lapponica*)	Migration§
Red-necked stint (*Calidris ruficollis*)	Migration§
Little stint (*C. minuta*)	Vagrant
Sharp-tailed sandpiper (*C. acuminata*)	Migration†§
Ruff (*Philomachus pugnax*)	Migration†‡
Little gull (*Larus minutus*)	Migration‡
Black-headed gull (*L. ridibundus*)	Migration†‡
Black-tailed gull (*L. crassirostris*)	Vagrant
Yellow-legged gull (*L. cachinnans*)	Vagrant
Slaty-backed gull (*L. schistisagus*)	Migration†
Common tern (*Sterna hirundo*)	Vagrant
Rock pigeon (*Columba livia*) (domestic)	Exotic bird trade
Oriental turtle-dove (*Streptopelia orientalis*)	Exotic bird trade
European turtle-dove (*S. turtur*)	Exotic bird trade
Eurasian collared-dove (*S. decaocto*)	Exotic bird trade
Parrots (*Psittacidae*)	Exotic bird trade

*Species shown in **bold** are known to have been infected with highly pathogenic avian influenza H5N1. Sources for information on migrant or vagrant status are Kessel and Gibson (*28*), Palmer (*29*), and the American Ornithologists' Union (*30*). Nomenclature follows the American Ornithologists Union checklist (*30*) to the degree possible. Supplementary source: Rasmussen and Anderton (*31*).
†Route 2. See Figure 2.
‡Route 3. See Figure 2.
§Route 1. See Figure 2.

Figure 2. Map of known routes for natural interhemispheric bird movement: route 1, migrants breeding in Alaska and wintering in East Asia; route 2, migrants breeding in East Asia and wintering along the Pacific Coast of North America; route 3, migrants breeding in Iceland or northwestern Europe and wintering along the Atlantic Coast of North America; route 4, vagrants from West Africa carried by tropical storm systems across the Atlantic to eastern North America.

sharing the same food or water, a situation that provides ample opportunity for viral transmission.

Possible Role of Birds in Movement of HPAI H5N1 in Western Hemisphere

Movement of HPAI H5N1 by sale of infected domestic fowl or poultry products in the United States and Canada is unlikely, given existing regulations. Thus, a major mode of HPAI spread available in much of Eurasia would be ruled out. Also, most domestic fowl are kept separate from wild migratory waterfowl in both countries, which would rule out a second major mode of introduction and cross-infection. Mixing of wild migratory birds with captive, exotic birds is relatively common, however, at North American zoos. Birds in such exhibits should be screened regularly for H5N1 or whatever HPAI virus is in circulation during a given year.

The HPAI H5N1 subtype of avian influenza A causes high mortality rates in most wild birds, at least in its present form. The situation is similar to that found for the form of WNV introduced into the Western Hemisphere in 1999 (*24,32–34*). Even under conditions in which food, water, and protection from predators are provided, death rates are high. These kinds of death rates could result if the current form of HPAI H5N1 were introduced into New World bird populations. In such a scenario, migrants might not be capable of moving the virus far from its point of introduction, at least initially. Also, the die-offs occurring at the site of entry likely would be obvious to wildlife disease monitors, which would allow for rapid quarantine. However, if the H5N1 virus were introduced into the Western Hemisphere, migratory birds, particularly anseriforms (ducks, swans, geese), might serve as dispersal agents, especially if the virus were to change to a less lethal form through reassortment or mutation.

A key difference between mosquitoborne WNV and birdborne HPAI H5N1 is the virtual absence of effective reservoir hosts other than birds for the latter. WNV can be maintained without birds because infected mosquitoes can pass active virus to subsequent generations through vertical transmission (*35*). So far as is known, no alternative to birds exists as major reservoir hosts for HPAI H5N1.

An additional consideration concerning the future of HPAI H5N1, should it gain wide circulation in migratory birds, is the possibility of infection of a bird already infected with another form of avian influenza virus. Such infection could result in reassortment and production of a new virus, possibly less lethal than HPAI H5N1 but more readily spread.

Conclusions

HPAI H5N1 spread rapidly across Eurasia during 2005 for reasons that are not entirely understood. Despite this rapid movement, effective introduction (i.e., under conditions allowing its spread) of the virus to the New World through migratory or vagrant birds seems unlikely. Few individual members of few waterfowl species migrate between hemispheres, and should a bird make the journey while shedding sufficient active virus to infect birds in the Western Hemisphere, newly infected birds would probably die before being able to transport the virus from the entry site. If spread of HPAI H5N1 to the New World occurs in its current form (e.g., through domestic or pet bird trade or smuggling), it should be readily detectable because of the large number of dead native birds likely to result. However, the virus is changing (*19*), and a modified H5N1 virus introduced into the Western Hemisphere could be moved more readily by migratory waterfowl. If this event were to occur, the virus should be amenable to control through isolation and quarantine. If viral reassortment or mutation occurs to produce a new virus that is readily transmissible to humans, the role of birds in general and migrants in particular may be moot because of the fundamentally different methods of infection favored by viruses infecting humans and birds. Viruses infecting birds preferentially attack the intestinal tract and are shed with the feces; by contrast, human viruses mainly attack the respiratory tract and are shed with respiratory effluvia. If HPAI H5N1 were to gain wide circulation among migrants, it might infect a bird already infected with another form of

avian influenza A and undergo reassortment to produce a low-pathogenic form that is more readily spread.

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Novel Chikungunya Virus Variant in Travelers Returning from Indian Ocean Islands

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Chikungunya virus (CHIKV) emerged in Indian Ocean islands in 2005 and is causing an ongoing outbreak that involves >260,000 patients, including travelers returning home from these islands. We investigated cases in 4 patients returning from Mayotte and Reunion Islands with CHIKV infection and a nurse infected in metropolitan France after direct contact with the blood of a traveler. Four patients had tenosynovitis and pain at wrist pressure, and 1 had life-threatening manifestations. Four CHIKV strains were isolated, including 1 from the patient with the autochthonous case. The complete genomic sequence identified a new CHIKV variant emerging from the East/central African evolutionary lineage. *Aedes albopictus*, the implicated vector of CHIKV in Indian Ocean islands, has dispersed worldwide in recent decades. High viral loads in patients returning from Indian Ocean islands to countries where *Ae. albopictus* is prevalent may be a source of epidemics.

Human pandemics and emerging infectious diseases such as influenza, HIV, dengue hemorrhagic fever, West Nile encephalitis, and possibly severe acute respiratory syndrome have been attributed to the ability of RNA viruses to evolve rapidly and expand their vector or host range (*1*). To date, most Western countries have escaped much of the health problems that RNA arboviruses inflict on humans in the tropics. However, recent events suggest that this situation may be changing (*2*). The emergence of West Nile virus in 1999 in the United States and its subsequent rapid spread demonstrated that arboviruses are still a threat, even in temperate, industrialized countries. West Nile fever has become the dominant vectorborne viral disease in the United States, with >20,000 reported human cases, 770 deaths, and an estimated 215,000 illnesses during the past 7 years (*3*).

A recent outbreak of chikungunya fever in the islands of the Indian Ocean has drawn attention to chikungunya virus (CHIKV, genus *Alphavirus*, family *Togaviridae*) (*4*), first identified in the 1950s in Africa. There, it is maintained in a sylvatic cycle involving wild primates and forest-dwelling *Aedes* mosquitoes that resembles the epidemiologic cycle of yellow fever virus (*5–7*). CHIKV has since been associated with the urban *Aedes aegypti* mosquito (possibly supplemented by *Ae. albopictus*) in Asian countries in an epidemiologic cycle resembling that of dengue and characterized by the absence of an animal reservoir, direct human-to-human transmission by urban mosquitoes, and the potential for major epidemics (*8–11*). At the beginning of 2005, an outbreak of chikungunya fever was observed in the southeastern islands of the Indian Ocean. The epidemic was most noticeable in urban and semiurban areas of the Comoros Islands, where >5,000 cases have been reported. Thereafter the virus has circulated in other islands, including Reunion and Mayotte (2 French territories), Mauritius, the Seychelles, and Madagascar (*12–15*). The population of these islands is >22 million (*16*). At the beginning of 2006, Reunion was experiencing an explosive outbreak. On June 1, an estimated 264,000 CHIKV infections (in a population of 770,000) were reported; 237 death certificates mentioned CHIKV as the possible cause of death (*17*). The implicated vector in this outbreak is *Ae. albopictus*, the Asian tiger mosquito

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(*12*,*17*). This mosquito was originally indigenous to Southeast Asia, the Western Pacific, and the Indian Ocean but has recently spread to Africa, the Middle East, Europe and the Americas, mainly because of transportation of dormant eggs in tires (*10*).

The Indian Ocean islands are popular tourist destinations. According to the World Organisation of Tourism, in 2002, some 719,000 tourists arrived in Mauritius, 432,000 in Reunion, 139,000 in Madagascar, and 122,000 in the Seychelles (*18*). In 2004, an estimated 1,474,218 persons traveled from Madagascar (153,766), Mauritius (657,312), Mayotte (63,372) Reunion (498,388), and Seychelles (101,380) to the European mainland (*19*). In addition, hundreds of cases of CHIKV infections have been reported in India and Malaysia (*19*). Recently, CHIKV-infected travelers returned home to countries where competent vectors are indigenous (*19*), which raises serious concern for potential disease spread. We describe 4 patients who returned to southern France, where the Asian tiger mosquito has been established since 2005, with CHIKV infection. One of them was the source of an autochthonous nosocomial infection in a nurse in metropolitan France.

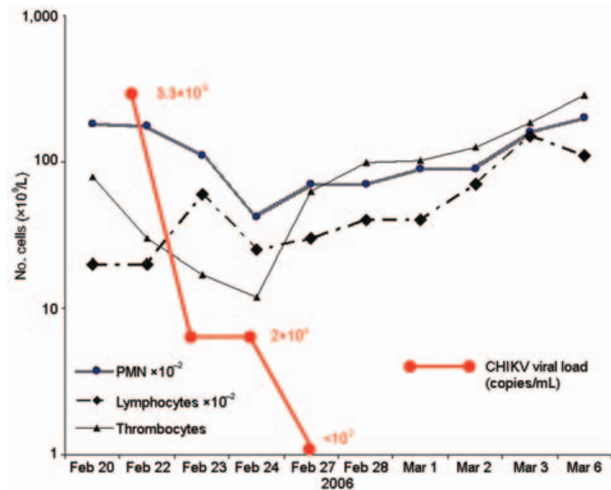

Figure 1. Evolution of viral load and blood cell counts in a 73-year-old man who had returned from Reunion during the acute phase of chikungunya virus (CHIKV) infection. PMN, polymorphonuclear leukocytes.

Patients

Patient 1, a 73-year-old man, returned from Reunion on February 17, 2006. His medical history included type 2 diabetes mellitus and oral treatment with imatinib for an intestinal stromal tumor with liver metastasis that was surgically removed in 2004. He was admitted for a 3-day history of fever (temperature 38.5°C) and arthralgia. Clinical signs included asthenia, anorexia, nausea, myalgia, headache, bilateral conjunctivitis, and arthralgia that was particularly intense in shoulders and elbows, left ankle, and left wrists, which suggested tenosynovitis. Purpuric lesions of the legs and intense pain in response to pressure on the left wrist were noticed. Laboratory findings showed severe pancytopenia, including reduced platelet count (17×10^9 cells/L), reduced leukocyte count (0.7×10^9 cells/L), and nonregenerative anemia (hemoglobin 9.5 g/dL, 10×10^9 reticulocytes/L) (Figure 1). Blood chemistry values included raised enzymes: 1,858 IU/L lactate dehydrogenase (LDH), 283 IU/L aspartate aminotransferase (AST), 210 IU/L alanine aminotransferase (ALT), 672 IU/L creatine phosphokinase, and 84 IU/L γ-glutamyl transaminase. Bone marrow smear was cell-rich; granulocytic cells were predominant and hemophagocytosis was limited. *Escherichia coli* septicemia occurred during the neutropenic period and was successfully treated with ceftriaxone. The patient recovered slowly after a 5-day regimen of high-dose intravenous immunoglobulin, but intensive joint pain persisted. On March 2, bone scintigraphy showed diffuse inflammation of all joints, specifically at the left shoulder, wrist, and hand (Figure 2A).

Patient 2 was a 5-month-old Canadian child who was hospitalized in Marseilles on February 24, 3 days after returning from Reunion and a few days before traveling to Canada. Symptoms included fever and a macular rash; laboratory findings showed elevated serum C-reactive protein (CRP) (37 mg/L) and AST (59 IU/L). Blood cell counts showed no abnormalities. He was discharged, and his fever slowly abated.

Patient 3 was a 31-year-old woman who had been living in Marseilles since 1987 and who had returned from Mayotte, a French Comoros island, on February 28, 2006. Two days later, she was admitted with fever (temperature 39.2°C), nausea, myalgia, lumbar pain, headache, bilateral conjunctivitis (Figure 2B), and severe bilateral arthralgia (shoulders, knees, and particularly ankles, elbows, wrists, and fingers). Exquisite pain was noted when pressure was applied to the right wrist. Laboratory findings included negative blood smears for malaria, anemia (hemoglobin 10.8 g/dL), and lymphopenia (0.6×10^9 cells/L). Blood chemistry values were within normal limits, except for raised enzymes (177 IU/L AST, 116 IU/L ALT, 780 IU/L LDH), hypocholesterolemia (3.5 mmol/L), and CRP (64 mg/L). Fever disappeared at day 4, and the patient was discharged, although still in pain.

In January 2006, a 75-year-old female patient (patient 4) returned from Reunion with a sudden-onset fever, asthenia, arthralgia with wrist pain, and diarrhea. Two days later, blood specimens were collected at the patient's home by a 60-year-old female nurse (patient 5). At this time, patient 4 had high fever (temperature 40°C). Three days later, fever, skin rash, and arthralgia with pain at wrist pressure developed in the nurse. Interview and examina-

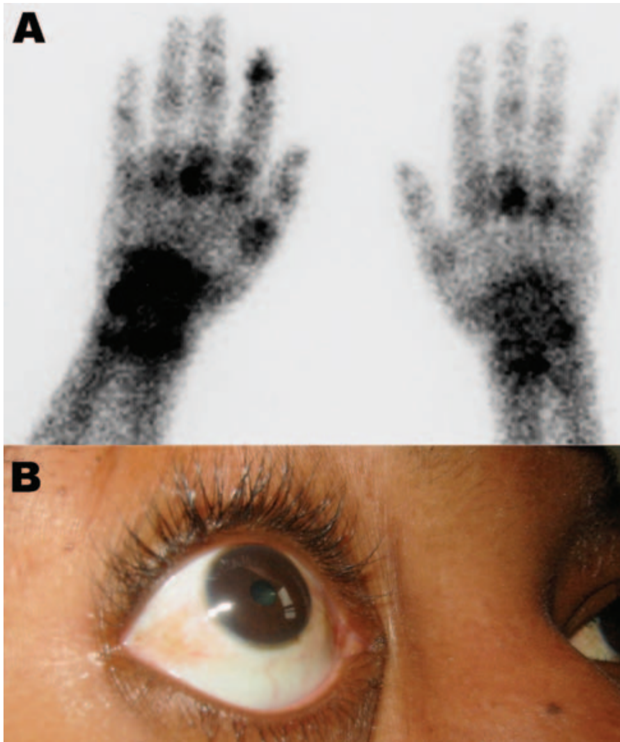

Figure 2. Clinical findings in patients. A) Bone scintigraphy of the wrists and hands showing an intense focus of technetium-99m–labeled methylene diphosphonate tracer uptake, particularly on the left side in the left metacarpophalangeal, wrist, and the first distal interphalangeal joints in a 73-year-old man who returned from Reunion with a severe viremic chikungunya virus (CHIKV) infection. B) Conjunctivitis in a 31-year-old woman who returned from Mayotte, French Comoros, with a severe viremic CHIKV infection.

tion did not show recent travel abroad, mosquito bite, accidental skin puncture during blood sampling, skin lesion, or eczema. The nurse washed her hands with a hydroalcoholic solution before and after drawing blood, but she did not wear gloves. She noted direct contact with patient 4's blood during hemostasis.

Methods

Acute-phase sera obtained from patients 1, 2, 3, and 5 were tested for CHIKV RNA through a quantitative real-time reverse transcription (RT)-PCR test (*20*) and used for partial E1 gene sequence determination and virus isolation in Vero E6 cells. Viral loads were estimated by comparative analysis by using threshold values obtained with serial dilutions of a 450-nucleotide (nt) in vitro transcribed RNA, quantified spectrophotometrically as previously reported (*21*), encompassing the target region (detailed protocol available on request). CHIKV-specific immunoglobulin G (IgG) and IgM were tested in acute- and convalescent-phase sera by an indirect fluorescent antibody test with a hyperimmune mouse ascitic fluid prepared against CHIKV Ross strain in conjunction with a fluorescein isothiocyanate–conjugated goat anti-human IgG (Fluoline G, bioMérieux, Marcy l'Etoile, France) as previously described (*22*). Primers used to completely sequence strain LR2006-OPY1 (first passage) were designed from CHIKV and o'nyong-nyong virus sequences retrieved from GenBank. Sequence reconstruction was performed with Sequencer software program (Gene Codes Corp., Ann Arbor, MI, USA). E1 gene sequences (1,225 nt) were generated by using primers CHIKE1S1, 7 ACATCACGTGCGAGTACAAAAC and CHIKE1R1, TCTCTTAAGGGRCACATATACC. Phylogenetic and evolution studies were performed with E1 sequences as previously described (*23*) with ClustalX version 1.81 (*24*) for sequence alignments and MEGA version 2.1 (*25*) for phylogenetic analyses.

Results

CHIKV infection was diagnosed by positive RT-PCR in acute-phase sera (viral loads of 3.3×10^9, 1.0×10^7, 4.2×10^8, and 2.0×10^8 copies/mL in patients 1, 2, 3, and 5, respectively). Patient 1's viral load decreased to 2×10^4 copies/mL at day 2 and 3 and was negative at day 4 (Figure 1). Patient 3's viral load decreased to 7×10^5 copies/mL at day 4, 3×10^4 copies/mL at day 7, and was negative at day 8. Infection was also diagnosed by seroconversion with no antibody in acute-phase serum and IgM and IgG in convalescent-phase serum or isolated IgM in acute-phase serum (patient 2, no convalescent-phase serum) and by virus isolation from acute-phase serum (isolates LR2006-OPY1, LR2006-OPY2, MCF2006-OPY4, and GARD2006-OPY6 for patients 1, 2, 3, and 5, respectively).

A 1,044-nt sequence of the E1 gene was used for comparative genetic analysis of sequences from Indian Ocean CHIKV and reference strains from diverse geographic and temporal origins (*23*). Indian Ocean strains formed a sublineage that is closely related to but distinct from viruses belonging to the East/Central African evolutionary lineage (Figure 3). The E1 nucleotide sequences of the 4 CHIKV isolates from 2006 (GenBank accession nos. DQ451149–DQ451151) were 100% identical, while ranges of 2.1%–3.3%, 14.8%–15.8%, and 5.5%–6.4% of nucleotide divergence were observed when compared with isolates from Central/East Africa, West Africa, and Asia, respectively.

Phylogenetic analysis and high bootstrap values indicated that the 4 Indian Ocean strains had a common ancestor, constituted a new sublineage that is distinct from the 4 lineages previously recognized, and most likely emerged recently to cause the regional outbreak (*26*). Two mutations, which are apparently specific for strains circulating in Reunion and Mayotte, consist of $A^{1028}\rightarrow V$ and $D^{1086}\rightarrow E$

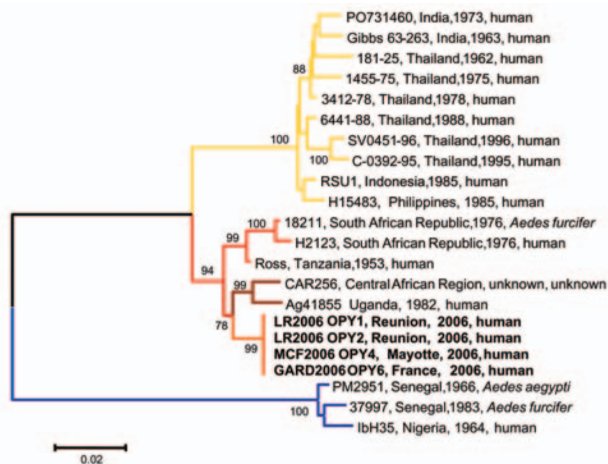

Figure 3. Phylogenetic analysis of chikungunya virus (CHIKV) isolates based on a 1,044-nucleotide (nt) fragment between nt 10243 and 11286 (numbered after strain Ross [accession no. AF490259]) in the E1 gene. Distances and groupings between the 3 Indian Ocean isolates and 18 isolates previously characterized (*23*) were determined by the Jukes-Cantor algorithm and neighbor-joining method with the MEGA software program (*25*). Bootstrap values ≥75% are indicated and correspond to 500 replications. The main evolutionary lineages, East/Central African (brown), eastern/southern Africa (red), West African (blue), and Asian (yellow), are indicated. The Indian Ocean sublineage is indicated in orange. **Boldface** indicates sequence determined in this study.

substitutions with reference to all other CHIKV strains characterized to date, regardless of evolutionary lineage (amino acid positions refer to the sequence of the original Ross isolate, accession no. AF490259). A and D residues are also found in available sequences of o'nyong-nyong virus, another alphavirus distantly related to CHIKV. The complete coding sequence of the strain recovered from patient 1 (LR2006 OPY1) was determined and deposited in GenBank (accession no. DQ443544). Comparative analysis with the 3 other full-length sequences available for CHIKV showed that the overall nucleotide distances were 2.7% (1.4% amino acid) with East/Central strains and 14.8% (4.4% amino acid) with West African strains (no full-length sequence was available for CHIKV strains from Asia).

Diagnosis of a CHIKV infection in patient 5 was based on clinical (typical chikungunya fever signs and symptoms), epidemiologic (taking care of a patient who was returning from Reunion at the time of the outbreak), and serologic (IgM- to IgG-specific conversion) evidence. After contact with patient 4, CHIKV infection of patient 5 was shown by RT-PCR, virus isolation, and seroconversion as reported above.

Discussion

Visitors to tropical countries and workers migrating to Western countries may serve as sentinels for the global dissemination and emergence of viral, bacterial, and parasitic agents that affect local populations in the tropics (*27*). As an example, the outbreak of chikungunya fever in 2005 in the Comoros Islands led to the first documented CHIKV infections imported into France by travelers who had visited the islands (*28*). The patients reported here had typical clinical signs and symptoms of CHIKV infection, including febrile polyarthritis with papular, macular, or purpuric rashes; hemorrhagic manifestations are, however, uncommon. Another characteristic was severe arthralgia accompanied by extremely painful arthritis and tenosynovitis and specific pain when pressure was applied to the wrists. Severe thrombocytopenia and neutropenia complicated by septicemia developed in patient 1. In Reunion, complicated clinical syndromes have been reported mainly in newborns, the elderly, and disabled patients (*17*). Previous studies have shown that a proportion of CHIKV-infected patients remain in severe pain for months (*29*,*30*). In patient 1, and in subsequent patients in our units, we also observed late-to-chronic CHIKV rheumatic manifestations, particularly subacute tenosynovitis of the wrists, hands, and ankles (*31*).

Virologic studies identified a new CHIKV variant in patients infected from Mayotte and Reunion, with specific amino acid mutations in the E1 gene; these findings may be useful in the future for tracing dissemination of the virus. The genetic heterogeneity between this variant and other strains in the East/Central Africa lineage has a magnitude equivalent to or higher than that observed between strains of West Nile virus circulating in southern Europe and the Middle East for the past 4 decades and which emerged and dispersed in the United States in 1999. Phylogenetic analysis showed that this variant originated from the East/Central African lineage of CHIKV and emerged recently, in accordance with the observed epidemic pattern. High viremia was observed in this study (up to 3.3×10^9 copies/mL), which enabled virus isolation from all viremic patients. We used the same diagnostic system as reported by Pastorino et al. (*20*), but viral load was quantified with RNA that encompassed the target region by using a method similar to that described previously (*21*). RNA was then quantified by spectrophotometry, diluted serially from 10^0 to 10^{-14}; the 4 highest dilutions that provided a positive result were included in each diagnostic experiment. The quantification was performed with an in vitro–transcribed RNA based on a strain that was genetically distinct from those circulating in Indian Ocean islands. However, in the region targeted by the real-time PCR assay, 206 of 209 sites are conserved between the sequence of Ross strain and strains circulating in 2006 in the Indian Ocean region. Moreover, no mismatch exists in the sequence targeted by the primers or the probe. This finding suggests that the quantification of viral loads, although approximate, is a good reflection of biologic reality.

Although viral loads up to 10^9 have been reported in dengue virus infection, such levels of viremia are uncommon in arthropodborne viral diseases such as dengue fever and West Nile virus infection (*32–34*). In the absence of published data, we cannot determine whether high viral loads are common during CHIKV infection or specific to Indian Ocean strains. They may account for the autochthonous case reported here. Patient 5's CHIKV infection was associated with direct contact with the blood of patient 4; in addition, *Aedes* spp. do not generally bite in southern France during the winter. Virus transmission through direct contact with highly viremic blood appears to be the most plausible hypothesis to explain this autochtonous CHIKV infection. Whether the skin was intact when contact with infected blood occurred could not be clearly determined.

A major determinant of outbreak dynamics is the ecologic cycle of the virus and its vector. This cycle in Reunion probably follows a denguelike model characterized by the absence of an animal reservoir and the ability to spread rapidly among humans through peridomestic mosquito bites. We suspect that direct transmission to healthcare workers may have also occurred in disease-endemic countries, but it was not identified.

Because dengue virus does not require a nonhuman vertebrate reservoir, it was able to disseminate in regions where competent vectors were present. CHIKV may become similarly globalized. Since the 1980s, *Ae. albopictus* has spread worldwide; it reached the United States in 1985, Brazil in 1986, Central America in 1988, and Africa in 1992 (Figure 4) (*10*). In Europe, it was identified in Albania in 1979 and in Italy in 1991, where it has become established (*10*). In France, it was reported for the first time in 1999 (*35*). It has also been introduced into several other European countries, including Belgium, Bosnia and Herzegovina, Croatia, Greece, the Netherlands, Serbia and Montenegro, Slovenia, Spain, and Switzerland (*19*). In 2004, *Ae. albopictus* was identified in several areas of the French Riviera, only 200 km from Marseilles, where 3 of our viremic patients were hospitalized (*36*). Although *Ae. albopictus* is active year-round in tropical (e.g., Central America) and subtropical (e.g., Gulf Coast) latitudes, it overwinters in its egg stage in the colder latitudes of the Northern Hemisphere. Therefore, risk for CHIKV spread is probably absent during colder months in Europe. However, in Italy, *Ae. albopictus* is active from February through December, with a peak in August and September (*37*). Whether ecologic conditions during the period of *Ae. albopictus* activity in southern Europe and North America support the development of a productive and persistent viral cycle in local vector populations is unknown. Furthermore, we do not know whether *Ae. albopictus* can transmit vertically and thus transfer CHIKV to the next generation (and the next season), as has been shown for dengue virus (*10*). If viremic patients arrive in Italy, France, or elsewhere in southern Europe during the summer, however, they could cause a European outbreak. The risk may also exist in the Americas, where the Asian tiger mosquito is prevalent.

Conclusion

In 1 year, >250,000 persons have been infected by a new CHIKV variant on the Indian Ocean islands. Although major differences in the fitness or virulence of CHIKV may be associated with minor genetic differences, the most likely explanation for this devastating outbreak is the penetration into a region where the population is immunologically naive for CHIKV and where *Ae. albopictus* proliferates. As this outbreak has spread, we believe this type of outbreak could occur in other regions of the world where competent vectors are prevalent. Because of high viremia, the virus could also be directly transmitted to healthcare workers. We must be prepared for the possibility of similar arboviral epidemics in such places.

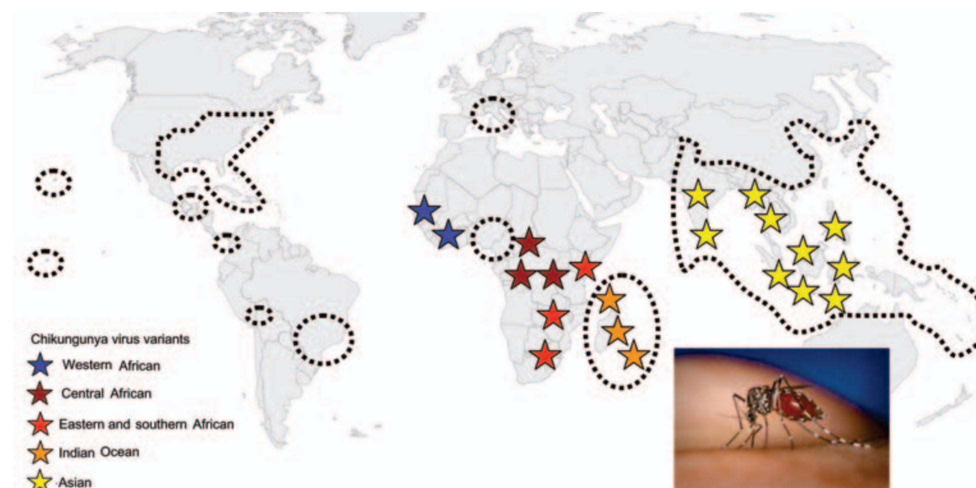

Figure 4. Estimated global distribution of *Aedes albopictus* (areas enclosed in dotted lines) and distribution of chikungunya virus (stars) from western Africa to southeastern Asia, including the Indian Ocean variant responsible for the 2006 outbreak. The color of the stars reflects the main evolutionary lineages shown in Figure 3. *Ae. albopictus* photograph courtesy of James Gathany, Centers for Disease Control and Prevention.

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Campylobacter jejuni Multilocus Sequence Types in Humans, Northwest England, 2003–2004

Will Sopwith,* Andrew Birtles,† Margaret Matthews,† Andrew Fox,† Steven Gee,‡ Michael Painter,* Martyn Regan,* Qutub Syed,* and Eric Bolton†

Detailed understanding of the epidemiology of *Campylobacter* is increasingly facilitated through use of universal and reproducible techniques for accurate strain differentiation and subtyping. Multilocus sequence typing (MLST) enables discriminatory subtyping and grouping of isolate types into genetically related clonal complexes; it also has the advantage of ease of application and repeatability. Recent studies suggest that a measure of host association may be distinguishable with this system. We describe the first continuous population-based survey to investigate the potential of MLST to resolve questions of campylobacteriosis epidemiology. We demonstrate the ability of MLST to identify variations in the epidemiology of campylobacteriosis between distinct populations and describe the distribution of key subtypes of interest.

Campylobacter has been the most commonly reported bacterial enteric pathogen causing gastrointestinal illness in England and Wales for at least the last 15 years (*1*). The main species infecting humans are *Campylobacter jejuni* and *C. coli*; these species also colonize many different animals, especially birds (*2*).

In Europe, campylobacteriosis shows a marked seasonality with a peak during the summer months (*3*,*4*), although this pattern is more marked in some countries than others (*5*). Especially sharp and annually consistent rises in incidence are reported in the United Kingdom, Greece, the Netherlands, and Denmark (*5*). A study in northwest (NW) England also indicated a consistent peak of human infection in March (*6*). Some studies have shown a coincident seasonality of infection in broiler chickens and humans in Scandinavia and contamination of retail raw chicken and infection in humans in the United Kingdom and suggest a common environmental trigger (*2*,*7*,*8*). In a recent study of the influence of rainfall, sunshine, and temperature on seasonality in different regions of England and Wales, increasing incidence of campylobacteriosis was most strongly correlated with increases in air temperature (*9*).

Studies in northern Europe have attempted to identify environmental reservoirs of infection in water sources and livestock that could explain the seasonality of human infection. These studies have demonstrated campylobacter carriage rates peaking in late spring and summer in broiler chicken flocks (*10*,*11*) and dairy cattle (*12*) but more constant infection in lambs and beef cattle (*12*,*13*). Campylobacter has been successfully isolated and cultured from surface water in Finland (*14*,*15*), Italy (*16*), and NW England (*17*), and sporadic campylobacteriosis (illness not associated with an outbreak) has been linked with exposure to untreated water in Scandinavia (*18*,*19*). *C. jejuni* has also been isolated from a wide range of animal and environmental samples in a rural area in NW England, which suggests a potential environmental risk for exposure (*20*).

Molecular subtyping methods including pulsed-field gel electrophoresis (PFGE) (*21*), fluorescent amplified fragment length polymorphisms (*22*), and multilocus sequence typing (MLST) (*23*) have been applied to *C. jejuni* to overcome the problems associated with traditional phenotypic methods. Although PFGE has been accurately and reproducibly used for several years to investigate disease clusters (*24*), an advantage of MLST is its ability to sequence isolate types and thus group them into genetically related clonal complexes; this is especially useful for integrating newly identified sequence types. MLST has

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also provided conceptual advances in understanding the population biology and epidemiology of *C. jejuni* (*25*). Previous studies that used phenotypic strain characterization methods failed to establish source/host associations for particular phenotypes (*26*), but recent studies with MLST, including a study in NW England, suggest that a measure of host association may be distinguishable when this system is used (*27*).

No continuous population-based survey has been performed to investigate the potential of MLST to answer the unresolved questions of campylobacteriosis epidemiology, particularly the drivers of the seasonal peak in the United Kingdom. This article is the first report of a 3-year study that used MLST to investigate campylobacteriosis in NW England in 2 defined human populations, 1 small town–based and rural and l metropolitan and suburban.

Methods

Study Population

The study population was defined as all persons with confirmed *Campylobacter* from April 2003 to March 2004 reported by residents in 4 local authorities (government administrative boundaries) in NW England (Figure 1). Wyre (population 106,826) and Fylde (74,032) local authorities adjoin geographically in the county of Lancashire and largely consist of rural and small town–based populations. For the purposes of this study, this area is referred to as rural. Salford (216,178) and Trafford (209,760) local authorities also adjoin geographically within the conurbation of Greater Manchester and are predominantly a mix of metropolitan and suburban districts. This area is referred to as suburban in this study. The 2 areas are ≈50 km apart and share some of the same drinking water sources but are environmentally located in different water catchment areas.

Data Collection

Confirmed cases of campylobacteriosis (according to the UK National Standard Method for diagnosis [*28*]) are routinely reported to the NW Health Protection Agency (HPA) surveillance system by local National Health Service laboratories. Case-patients were identified as residents in the study area through available geographic information or by patient names, when geographic information was not available. Reports included basic demographic information such as age, sex, and date of disease onset. Where date of onset was not available, date of report was used as a proxy. Travel overseas was not well recorded in these data.

Positive isolates of *Campylobacter* from patients resident in the study area were sent by the main diagnostic laboratories to the NW HPA Laboratory in Manchester for sequence typing. Case-patients were determined to be residents in the study area by using the methods described above.

Figure 1. Location of the 4 local authorities constituting the study area in Northwest England. The populations covered (outlined areas) were Fylde and Wyre (in Lancashire) and Salford and Trafford (within the metropolitan area of Greater Manchester). Gray areas indicate approximate location of built-up areas.

Campylobacter Speciation

C. jejuni isolates were spread onto Colombia blood agar (Oxoid CM331, Unipath, UK) containing 5% defibrinated horse blood and incubated at 37°C in anaerobic jars (Don Whitley Scientific, Shipley, UK) under microaerobic conditions (5% CO_2, 5% O_2, 3% H_2, 87% N_2). DNA was extracted (10% dilutions) and tested for *C. jejuni* or *C. coli* by using a previously described Taqman assay (*29*) with primers and probes for the genes *ceuE* (for *C. coli*) and *mapA* (for *C. jejuni*).

Sequence Typing of *C. jejuni* Isolates

MLST was performed as described (*23*). The amplification reactions were performed in a 50-μL volume containing ≈1 μL *C. jejuni* chromosomal DNA (10 ng/μL), 5 μL of each primer (10 pmol/μL), 10 μL of 1 mmol/L deoxynucleoside triphosphates (Roche, Welwyn Garden City, UK), 5 μL 10× PCR buffer (Qiagen, Crawley, UK), and 0.25 U Taq DNA polymerase (Qiagen). The thermal cycling conditions were as follows: initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 2 min; primer annealing at 50°C for 1 min; and extension at 72°C for

1 min with a final elongation step at 72°C for 10 min. Thermal cycling was conducted with an MJ PTC 200 thermal cycler. Amplicons were detected on a 1.5% ethidium bromide agarose gel and purified by using a UniFilter Multiscreen PCR cleanup plate (Whatman, Brentford, UK) according to the manufacturer's instructions. Sequencing reactions were conducted in 10 µL of 1/4 reaction volumes containing 2 µL purified DNA, 0.5 µL primer (10 pmol/µL), 1 µL sequencing buffer (Genetix, New Milton, UK), 2 µL DTCS Quick Start Master Mix (Beckman Coulter, Fullerton, CA, USA), and 4.5 µL molecular grade water. Thermal cycling conditions for sequencing reactions and ethanol cleanup of sequenced products were set up according to the manufacturer's instructions (Beckman Coulter). The products were analyzed on a Beckman Coulter CEQ 8000 automated DNA sequencer (Beckman Coulter). All sequence assemblage and editing were performed with Sequencher 4.0 software (GeneCodes Corporation, Ann Arbor, MI, USA).

MLST Allele and ST Assignment

MLST alleles, sequence types (STs), and clonal complexes were assigned by using the *Campylobacter* PubMLST database (*30*). Sequences were submitted for allele designation as appropriate.

Statistical Analysis

Incidence rates quoted were calculated per 100,000 population by using the relevant 2003 annual population estimate for each local authority area, purchased from the UK Office for National Statistics. Incidence ratios, associated 95% confidence intervals (CIs), and p values were calculated by using StatsDirect statistical software (http://www.statsdirect.com/), which used Fisher exact test to analyze the difference between 2 crude rates. A mid-p approach to Fisher exact test was also used to test the significance of observed differences in proportions (by using StatsDirect). The level of statistical significance chosen for all analyses was $p<0.05$.

Results

Seasonal Incidence of Reported Disease

During the first 12 months of the study period (April 2003–March 2004), 493 cases of laboratory-confirmed *Campylobacter* sp. were reported through the NW surveillance system (Table 1) from residents of Fylde, Wyre, Salford, and Trafford local authorities (Figure 1). This corresponded to approximate annual incidences of 100/100,000 for the area encompassing Fylde and Wyre (rural area) and 73.3/100,000 for the area encompassing Salford and Trafford (suburban area); this difference was significant ($p<0.001$). The incidence for the whole region of NW England in this period was 69.8/100,000 (data not shown). A similar seasonal pattern of cases was seen in each of the 2 areas in the study, with a large increase in reported cases between weeks 17 to 20 and weeks 21 to 24 (May/June), an elevated incidence through the summer months (weeks 21–36) that declines between weeks 37 and 48 (September to November) to the baseline level of incidence seen in weeks 17–20 (Figure 2). Incidence appeared in increase during the Christmas holiday period (weeks 49–52); this increase was sustained in the rural area until week 8 (February). With the exception of one 4-week period (weeks 25–28), the monthly incidence of campylobacteriosis reported was higher in the rural area, but this finding was only statistically significant during weeks 29–32 in 2003 and weeks 1–8 in 2004.

Table 1. Distribution of *Campylobacter jejuni* multilocus sequence typing clonal complexes by study area, April 2003 to March 2004*

Clonal complex	Fylde	Wyre	Salford	Trafford	Total	% of all typed
ST-21	15	21	22	44	102	28.7
ST-45	11	10	7	7	35	9.8
UA	6	6	6	16	34	9.6
ST-257	5	4	9	10	28	7.9
ST-443	1	5	6	9	21	5.9
ST-48	3	3	2	11	19	5.3
ST-206	6	2	3	7	18	5.1
ST-353	3	1	1	8	13	3.7
ST-22	2	0	2	3	7	2.0
ST-49	0	2	2	3	7	2.0
ST-42	1	0	2	3	6	1.7
ST-354	1	0	0	4	5	1.4
ST-61	2	1	1	1	5	1.4
ST-283	0	1	0	3	4	1.1
ST-52	1	0	2	1	4	1.1
ST-573	1	2	0	1	4	1.1
ST-658	1	0	2	1	4	1.1
ST-403	0	0	0	3	3	0.8
ST-508	1	0	0	2	3	0.8
ST-460	0	0	2	0	2	0.6
ST-177	1	0	0	0	1	0.3
ST-362	0	0	1	0	1	0.3
C. coli	5	11	4	10	30	8.4
No typing	22	24	39	52	137†	
Total	88	93	113	199	493	

*Including UA (new sequence types as yet unassigned to a clonal complex). Data show the number of human isolates per local authority and the percentage of all typed isolates attributed to each complex. Numbers of *C. coli* isolates are also shown and included in the denominator of "all typed cases." Isolates with "no typing" represent reports of campylobacteriosis to the surveillance system that do not have a corresponding typed isolate.
†Not included in the "all typed cases" denominator.

Sequence Typing of Isolates

Of 493 cases reported, 388 (79%) laboratory specimens were obtained for typing. A proportion of the other cases may not have been identified at the point of diagnosis as study isolates because incomplete demographic informa-

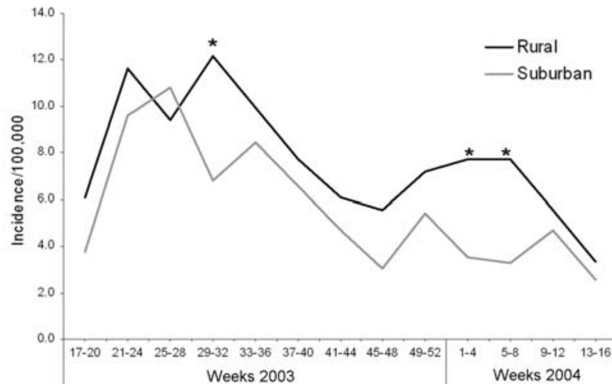

Figure 2. Seasonality of human cases of campylobacteriosis reported in the first 12 months of the study period in patients residing in Fylde and Wyre (rural) and Salford and Trafford (suburban). To allow comparison between the areas, the number of cases reported to the North West Health Protection Agency surveillance system during 4-week intervals were converted to incidence by using estimates of the annual population for each local authority. The periods at which the incidence differed with marginal statistical significance are indicated with an asterisk: weeks 29–32, incidence ratio (IR) 1.79, 95% confidence intervals (CI) 0.98–3.22 (p = 0.05); weeks 1-4, IR 2.20, 95% CI 0.98–4.88 (p = 0.04); weeks 5-8, IR 2.36, 95% CI 1.04–5.33 (p = 0.02).

tion was supplied with the sample. Of the specimens submitted for typing, 30 were *C. coli*, and 32 did not yield a culture, which left 326 isolates (66% of reported cases) of *C. jejuni* typed. From these isolates, 93 distinct MLST sequence types of *C. jejuni* were identified and assigned to 21 clonal complexes, with 20 remaining unassigned until further identification of types enables the designation of new complexes. The most common clonal complex isolated was ST-21 (102 cases, 28.7% of all typed cases, including *C. jejuni* and *C. coli*), and this complex was almost 3 times more common than the next complex ST-45 (35 cases, 9.8% of all typed cases) (Table 1). Almost 10% of typed cases were unassigned to clonal complexes at time of writing.

Geographic Distribution of Sequence Types

The geographic distribution of clonal complexes across the study area varied. Of the 10 most commonly reported clonal complexes (including ones not yet assigned), several were reported with higher incidence in the rural area, although greater numbers are required to give sufficient power to test the significance of these differences in many groups (Table 2). The greatest variation was seen among cases with clonal complexes ST-45 (incidence ratio [IR] 3.53, 95% CI 1.71–7.51) and ST-206 (IR = 1.88, 95%CI 0.65–5.30), although ST-45 was the only complex with a significantly (p<0.001) higher incidence in the rural area. Although not sequence typed, the incidence of *C. coli* (IR 2.69, 95%CI 1.23–5.95) was also significantly higher in the rural area (p<0.01).

Temporal Distribution of Sequence Types

Temporal distribution of different clonal complexes in each of the study areas also varied through the first year of the study. Cases of clonal complex ST-45 were most often reported in the rural area during weeks 25–28, although cases were reported continuously throughout the summer months (weeks 21–40, May to September, Figure 3). The proportion of cases reported in weeks 25 to 28 that were typed ST-45 was significantly higher that reported in the annual rural dataset (proportion difference 0.188, exact mid-p = 0.038) (data not shown). In the suburban area, clonal complex ST-45 was most often reported during weeks 21–28 (May to July), and the temporal distribution appeared reduced. The only period in which the difference

Table 2. Comparative distributions of the most common MLST clonal complexes by study area, April 2003 to March 2004*

Clonal complex	Isolates		Incidence/100,000		Incidence ratio	95% confidence intervals	p value
	Rural	Suburban	Rural	Suburban			
ST–21	36	66	19.91	15.50	1.28	0.83–1.96	–
ST–45	**21**	**14**	**11.61**	**3.29**	**3.53**	**1.71–7.51**	**<0.001**
UA	12	22	6.64	5.17	1.28	0.58–2.71	–
ST–257	9	19	4.98	4.46	1.12	0.44–2.59	–
ST–443	6	15	3.32	3.52	1.01	0.32–2.80	–
ST–48	6	13	3.32	3.05	1.09	0.34–3.07	–
ST–206	8	10	4.42	2.35	1.88	0.65–5.30	–
ST–353	4	9	2.21	2.11	1.05	0.24–3.75	–
ST–22	2	5	1.11	1.17	0.94	0.09–5.75	–
ST–49	2	5	1.11	1.17	0.94	0.09–5.75	–
C. coli	**16**	**14**	**8.85**	**3.29**	**2.69**	**1.23–5.95**	**<0.01**
All cases†	**181**	**312**	**100.08**	**73.25**	**1.37**	**1.13–1.65**	**<0.001**

*Including UA (new sequence types as yet unassigned to a clonal complex), *Campylobacter coli* isolates, and all cases of campylobacteriosis reported. Annual estimated incidence was calculated for each clonal complex and incidence ratios were calculated for rural incidence/suburban incidence, including confidence intervals. Those data with a statistically significant difference between the study areas are shown in **boldface** type (level of significance p<0.05). MLST, multilocus sequence typing.

†Refers to the entire dataset for reference, i.e., those shown in this table plus all others.

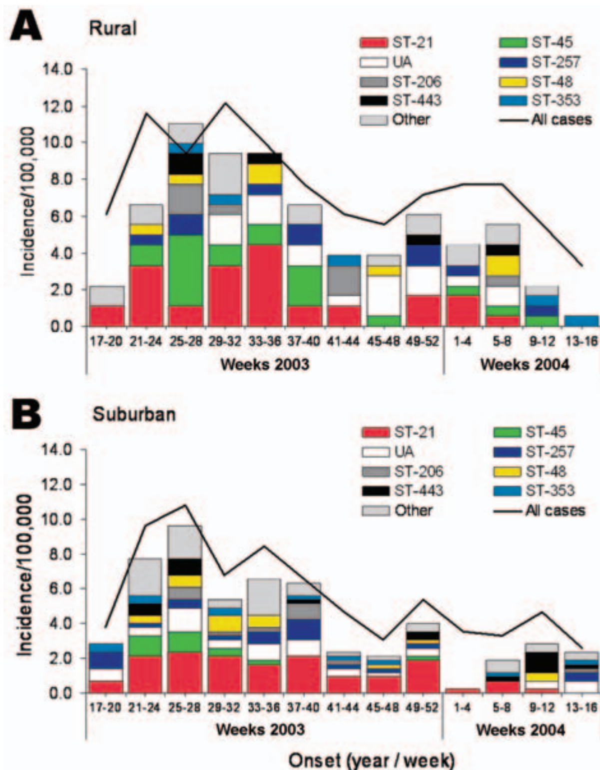

Figure 3. Seasonal distribution of multilocus sequence typing clonal complex in human cases of campylobacteriosis reported in the first 12 months of the study period, by residence in A) Fylde and Wyre (rural) and B) Salford and Trafford (suburban). The number of typed isolates reported during 4-week intervals was converted to incidence by using annual population estimates. The 8 most commonly reported complexes are distinguished, with cases from all other complexes presented as "other." The incidence of "all cases reported" (typed and untyped) is presented for reference (solid line).

in ST-45 incidence between the areas approached significance was weeks 25–28 (IR 3.3, 95% CI 0.9–13.17, p = 0.052) (data not shown). Clonal complex ST-45 was not reported before week 21 in either area.

Cases of clonal complex ST-21 were reported more or less throughout the 12-month period in both areas, but the incidence fluctuated far less in the suburban area. Almost 50% of the cases reported in the suburban area in weeks 49 to 52 were clonal complex ST-21 compared with 30% in the annual suburban dataset, but this difference was not significant (proportion difference 0.176, exact mid-p = 0.113) (data not shown).

Clonal complex ST-206 was most often reported in the rural area in weeks 25 to 28, and more remarkably in weeks 41 to 44, when the proportion of cases typed as ST-206 was significantly higher than in the annual dataset (proportion difference 0.367, exact mid-p = 0.006) (data not shown). Cases of clonal complex ST-206 were consistently reported from weeks 25 through 44 (June to October) in the suburban area, but the proportion of ST-206 cases in weeks 37 to 40 was significantly higher than in the annual dataset (proportion difference 0.101, exact mid-p = 0.035).

Age Distribution of Sequence Types

Analysis of age-specific incidence by clonal complex was hampered by low numbers of cases; only data for the 6 most commonly reported complexes are shown (Table 3). Overall, the higher incidence of campylobacteriosis described in the rural area was also evident in each of the age bands analyzed, although the difference in 0- to 14-year-old patients was only marginally significant (IR 1.72, 95% CI 0.91–3.18). Among cases in the most commonly reported clonal complex, ST-21, incidence among younger case-patients (0- to 14-year-olds) was higher in the suburban area, but for all other age groups, the incidence was higher in the rural area. None of these differences were statistically significant. The only significant difference was a higher incidence of ST-45 among those ≥55 years of age in the rural area compared with those ≥55 years in the suburban area.

Discussion

We described some characteristics of human infection with *Campylobacter* in 2 environmentally distinct areas of NW England and some preliminary results of a study that used MLST to better define the epidemiology of campylobacteriosis within a distinct population. MLST identified possible variations in the epidemiology of campylobacteriosis between populations. However, our sample sizes are small, and the role of chance in the associations described cannot be excluded without further analysis of a larger dataset.

The seasonality of incidence in the 2 study areas is broadly similar to that previously described (*3*,*6*) with a sharp rise in cases around weeks 21 to 24 (May and June) that is sustained through the summer months. Throughout the first year of the study, incidence was higher in the rural area of Fylde and Wyre than in the suburban area of Salford and Trafford; this difference was most significant in the first 8 weeks of 2004. This difference in incidence was seen for all analyzed age groups, although it was not statistically significant among 0- to 14-year-olds. These observations may indicate increased exposure of the more rural population to sources of *Campylobacter* and true differences in distribution season between the 2 areas, perhaps indicating distinct transmission routes. However, a variety of other factors likely influenced these observations, including differences in healthcare-seeking behavior between the populations and differences in frequency of travel abroad. Although some adjustments have been made for the underlying population structure of each study area

Table 3. Comparative age distributions of the most common MLST clonal complexes by study area (first year of the study)*

Clonal complex	Age group	Isolates		Incidence/100,000		Incidence ratio	95% confidence intervals	p value
		Rural	Suburban	Rural	Suburban			
ST–21	0–14	2	14	6.65	17.68	0.38	0.04–1.64	–
	15–34	9	24	24.27	21.02	1.15	0.47–2.57	–
	35–54	12	18	24.38	15.14	1.61	0.71–3.53	–
	≥55	13	13	20.17	11.43	1.76	0.75–4.13	–
ST–45	0–14	3	1	9.97	1.26	7.89	0.63–414.40	–
	15–34	1	4	2.70	3.50	0.77	0.02–7.78	–
	35–54	7	6	14.22	5.05	2.82	0.81–10.15	–
	≥55	**10**	**3**	**15.51**	**2.64**	**5.88**	**1.51–33.24**	**<0.01**
ST–257	0–14	2	1	6.65	1.26	5.26	0.27–310.48	–
	15–34	1	8	2.70	7.01	0.38	0.01–2.87	–
	35–54	3	8	6.09	6.73	0.91	0.15–3.77	–
	≥55	4	4	6.21	3.52	1.76	0.33–9.47	–
ST–443	0–14	1	0	3.32	0.00	–	–	–
	15–34	3	5	8.09	4.38	1.85	0.29–9.50	–
	35–54	0	6	0.00	5.05	–	–	–
	≥55	1	6	1.55	5.28	0.29	0.01–2.42	–
ST–48	0–14	1	2	3.32	2.53	1.32	0.02–25.27	–
	15–34	5	7	13.48	6.13	2.20	0.55–8.05	–
	35–54	0	4	0.00	3.36	–	–	–
	≥55	1	2	1.55	1.76	0.88	0.01–16.94	–
ST–206	0–14	0	0	0.00	0.00	–	–	–
	15–34	1	6	2.70	5.26	0.51	0.01–4.23	–
	35–54	5	5	10.16	4.21	2.42	0.56–10.49	–
	≥55	2	1	3.10	0.88	3.53	0.18–208.11	–
All cases	0–14	19	29	63.13	36.62	1.72	0.91–3.18	–
	15–34	**48**	**87**	**129.45**	**76.20**	**1.70**	**1.17–2.44**	**<0.01**
	35–54	**54**	**87**	**109.71**	**73.18**	**1.50**	**1.05–2.13**	**0.02**
	≥55	**57**	**66**	**88.43**	**58.05**	**1.52**	**1.05–2.20**	**0.02**

*Including all cases of campylobacteriosis reported for which age was available. Annual estimated incidences in 4 age groups were calculated for each clonal complex using estimates of the age-specific annual population for each local authority. Incidence ratios were calculated for rural incidence/suburban incidence and 95% confidence intervals calculated. Those data with a statistically significant difference between the study areas are shown in **boldface** type (level of significance p<0.05). MLST, multilocus sequence typing.

(the use of age-specific 4-weekly incidence), estimates of population do not take into account the seasonal movement of persons, such as students (Salford has a large student population) and age-related travel (*31*,*32*). For instance, the winter rise in incidence in the rural setting may be due, in part, to winter vacations taken by those with the flexibility and finances to travel abroad out of season (generally the older generation, who are also more represented in the rural area of this study). The exclusion of travel-related cases will be key in further exploring some of these trends, and matching more detailed epidemiologic information from case questionnaires will facilitate this as the study progresses. Subsequent years of data analysis will also clarify any true differences in incidence between the populations.

Sequence typing isolates collected throughout the first 12 months of the study showed a wide range of identified types, with many represented only by single cases and relatively few identified in significant numbers. Several new types were described in this study and await assignment to clonal complexes. Almost 30% of typed isolates from the study area align with the largest clonal complex so far defined, complex ST-21 (*23*,*30*), and previously reported isolates from this clonal complex originate from a wide variety of sources other than human cases, including cattle, chicken, milk, sand, and water (*23*). Most cases arising from a large waterborne outbreak of campylobacteriosis in Walkerton, Ontario, that originated from infected cattle (*33*) were later identified as clonal complex ST-21 (*34*), which suggests that this clonal complex can be associated with environmental and foodborne transmission. Study of a farm ecosystem in NW England demonstrated that most clonal complex ST-21 isolates came from livestock, especially cattle (*27*). Isolates of clonal complex ST-21 have also been described in cloacal and excreta samples from broiler chickens (*35*) and from a variety of farmyard isolates, including sheep and wild birds (*36*).

Isolates from the next most represented clonal complex in the study area, complex ST-45, have previously been reported largely from humans and chicken (*23*,*37*) and were almost exclusively reported in broiler chicken and turkey chicks in a study sampling various livestock and wild birds in NW England (*36*). However, complex ST-45 was more frequently identified in wildlife than in livestock

in the farm ecosystem study above (*27*) (although the ecosystem studied did not include poultry).

Initial data from this current study suggest that ST-45 complex is more frequently identified in the rural component of the study population than in the metropolitan component, and particularly in those >55 years of age. This finding raises the possibility that this complex may be associated with an environmental transmission pathway, a specific set of behavioral traits, or both. This hypothesis is supported by the identification of this clonal complex in wildlife isolates of *Campylobacter* (*27*), which suggests a widespread distribution maintained in the environment. Cases of *C. coli* in this study have a similar distribution, and in previous studies this species has been the most dominant one isolated from surface waters (*20*,*38*). The seasonal clustering of ST-45 complex human isolates (compared with ST-21 complex) in summer months in the study populations may also indicate an environmental source. However, seasonal carriage rates in broiler flocks in other north European settings (*10*,*11*) correlate with the seasonality of ST-45 complex in this population, in which infection of beef cattle (*13*) correlates more closely to the seasonality of ST-21 complex. Given the apparent host preferences (not exclusively) of these 2 complexes, the seasonality of human complexes in this study may only reflect *Campylobacter* distribution in food animals rather than a common environmental source, as suggested by previous studies (*2*,*7*,*8*).

No single sequence type was associated with the late spring seasonal rise in human campylobacteriosis, a finding consistent with that of a previous study in England that used serotyping (*26*). However, we have shown complex ST-45 to be significantly more prevalent during summer months in rural than in suburban areas. In addition, some evidence exists that a Christmas rise in infection in the suburban population may in part be mediated by cases of ST-21. Eating out at a restaurant is a recognized risk factor for campylobacteriosis (*32*,*39*), and the Christmas season involves a variety of public as well as private parties in the United Kingdom. Identifying specific sequence types associated with such seasonal activity may help clarify the role of subpopulations of *Campylobacter* in human epidemiology. Some evidence also suggests that MLST is able to distinguish temporal clusters of campylobacteriosis such as for ST-206, which is significantly overrepresented in weeks 41 to 44 in the rural area.

As this 3-year study progresses, we will match MLST complexes with common epidemiologic exposures through the use of case questionnaire data. The ease of use of the technique and its repeatability in a variety of laboratories are distinct advantages, and the increased use of MLST will enable valuable interlaboratory comparisons of types from similar population-based studies. We have demonstrated the ability to improve linking of apparently sporadic cases encountered in routine surveillance by assigning isolates to sequence type complexes. We believe that MLST will be a valuable tool in testing the significance of suspected epidemiologic exposures in human campylobacteriosis and thus support improved surveillance and development of effective interventions.

Acknowledgments

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The Food Standards Agency, United Kingdom, funded this project. The Wellcome Trust funded development of the aforementioned website.

Dr Sopwith is involved in developing web-based surveillance, running training days and conferences, producing regularly published analytical reports, developing disease mapping, and participating in infectious disease research and development projects with the Health Protection Agency in Liverpool, United Kingdom. Current projects include the molecular epidemiology of campylobacteriosis in NW England, the epidemiology of cryptosporidiosis, and the effect of rotavirus infection among children.

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Active Surveillance for Candidemia, Australia

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Population-based surveillance for candidemia in Australia from 2001 to 2004 identified 1,095 cases. Annual overall and hospital-specific incidences were 1.81/100,000 and 0.21/1,000 separations (completed admissions), respectively. Predisposing factors included malignancy (32.1%), indwelling vascular catheters (72.6%), use of antimicrobial agents (77%), and surgery (37.1%). Of 919 episodes, 81.5% were inpatient healthcare associated (IHCA), 11.6% were outpatient healthcare associated (OHCA), and 6.9% were community acquired (CA). Concomitant illnesses and risk factors were similar in IHCA and OHCA candidemia. IHCA candidemia was associated with sepsis at diagnosis ($p<0.001$), death ≤30 days after infection ($p<0.001$), and prolonged hospital admission ($p<0.001$). Non–*Candida albicans* species (52.7%) caused 60.5% of cases acquired outside hospitals and 49.9% of IHCA candidemia ($p = 0.02$). The 30-day death rate was 27.7% in those ≥65 years of age. Adult critical care stay, sepsis syndrome, and corticosteroid therapy were associated with the greatest risk for death. Systematic epidemiologic studies that use standardized definitions for IHCA, OHCA, and CA candidemia are indicated.

Bloodstream infections with *Candida* (candidemia) account for 8% to 15% of hospital-acquired sepsis in the United States (*1,2*). Death rates associated with candidemia are high (40%–70%) (*3*), with an estimated attributable death rate of 25% to 49% (*4,5*). Candidemia also results in prolonged hospital stay and substantial healthcare costs (*4,6*).

Studies have shown that incidence and etiology of hospital-acquired candidemia varied with geography, type of hospital, population studied, and clinical practice (*3,7–9*). Secular trends in the incidence of candidemia and etiologic species of *Candida* differ between Europe and the United States (*10*). Although candidemia is perceived as a hospital-acquired infection, changes in medical practice to more frequent use of home healthcare for many illnesses and long-term indwelling vascular devices have increased the number of susceptible patients in the community, potentially increasing incidence and changing epidemiology.

Two population-based surveys in the United States showed that 20% and 28% of cases were acquired outside hospitals (*7,11*), compared with only 1.2% in Finland (*12*). In a more recent study in Spain, 11% of episodes occurred in outpatients (*13*). These studies are not directly comparable because definitions of outpatient-acquired episodes were different. In addition, within this group, little distinction was made between cases of healthcare-associated candidemia and community-acquired (CA) candidemia.

A retrospective survey of candidemia in Australian hospitals from 1995 to 1998 showed an increase in the annual incidence of infection and a decrease in the proportion caused by *Candida albicans* (*14*). The Australian Candidemia Study Group was formed in 2001 to conduct the first countrywide, population-based, active laboratory surveillance for candidemia.

Methods

Study Design and Data Collection

Cases of candidemia were prospectively identified by blood culture surveillance at 50 of 52 public and private microbiology laboratories in Australia (population ≈20.1 million) from August 2001 to July 2004. One nonparticipating laboratory provided services to a university pediatric hospital where candidemia rarely occurred (A. Daley, pers. comm.) and the other provided services to an adult university hospital (36 cases identified; H. Sheorey,

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pers. comm.). Approval for the study was obtained from the human ethics review committees of institutions providing clinical data.

Adults, children, and neonates with ≤1 blood culture yielding *Candida* species were eligible for enrollment. State or territory coordinators were responsible for case identification. Information was collected on a standard form regarding age, sex, patient location at time of candidemia diagnosis, healthcare setting, risk factors within the preceding 30 days (including surgery, vascular access devices [VADs], hyperalimentation, and use of antimicrobial and systemic antifungal agents), major concomitant conditions (International Statistical Classification of Diseases and Related Health Problems, 10th revision, Australian modification) (*15*), portal of entry, clinical signs of sepsis (*16*), complications of candidemia, results of diagnostic studies, antifungal therapy, and clinical outcome ≤30 days after diagnosis. All data were collected prospectively, and forms were completed on days 5 and 30 after the date of the initial positive blood culture or at death if it occurred earlier. Data were collected and analyzed at a central site. Periodic audits of laboratory records ensured that all cases of candidemia were reported. For the study period, number of hospital beds and annual different day patient separations (defined as completed admissions) were obtained from 40 hospitals.

Definitions

A case was defined as incident isolation of *Candida* species from blood during the study period. For patients with ≥1 episode of candidemia, the second episode was defined as a new case if it occurred ≥30 days after the previous episode. A total of ≥2 episodes fulfilling the case definition and occurring in different patients who were epidemiologically linked (e.g., with regard to risk factors, location, and time) constituted a case cluster.

Episodes were classified as inpatient healthcare-associated (IHCA) if they occurred ≥48 hours after hospital admission and had not clinically manifested on admission (*17*). Cases occurring ≤48 hours after hospital admission were considered outpatient-acquired. Among outpatient-acquired candidemia, episodes associated with an indwelling medical device, surgical procedure, or chemotherapy-related neutropenia ($<1\times10^9$ cells/L, adjusted for children) were classified as outpatient healthcare associated (OHCA), and CA infections were classified as those occurring in patients with no healthcare-related risk factors (*17*). Adult intensive care unit (ICU) acquisition was when candidemia developed ≥48 hours after ICU admission for nonneutropenic patients. Source of candidemia was considered VAD related if culture of the device tip grew the same species isolated from blood. Endocarditis was classified by modified Duke criteria (*18*).

Microbiologic Methods

All laboratories cultured blood specimens in BACTEC (Becton Dickinson, Sparks, MD, USA) or BacT/Alert 3D (bioMérieux, Marcy l'Etoile, France) automated systems. *Candida* organisms were speciated by using standard phenotypic methods (*19*). Isolates were forwarded to a reference laboratory (Women's and Children's Hospital, Adelaide) for susceptibility testing and species confirmation by using conventional methods (*20*). *Candida dubliniensis* was distinguished from *C. albicans* by PCR fingerprinting (*21*). Where identification was discordant, species determination at the reference laboratory was used.

Statistical Analysis

Population and age-specific incidences were calculated by using denominator data from the 2004 Australian census (*22*) and hospital-specific incidences by using day of separation denominator data from individual hospitals for the study period. Overall incidences were expressed as pooled mean rates calculated by aggregating numerators and dividing by the sum of denominators from all sites. Data were analyzed with SPSS version 10.0.7 (SPSS Inc., Chicago, IL, USA). Continuous variables were compared with Student *t* test, and categorical variables were compared with χ^2 or Fisher exact tests. Incidence data from university hospitals were pooled and compared with pooled data from other hospital types with χ^2 test. A p value <0.05 was considered significant. Univariate analyses were performed to identify risk factors associated with overall illness. Candidate variables with a univariate p<0.15, or previously published risk factors, were analyzed by multiple logistic regression. The same independent predictors of death were identified with forward, backward, and stepwise variable selection methods.

Results

Incidence and Patient Demographics

Permission was denied to include 36 candidemia episodes identified at the nonparticipating adult hospital. Thus, the number of incident episodes was 1,095 (in 1,095 patients), with 337, 352, and 406 during the first, second, and third years, respectively, of the study. Data on hospital characteristics were available for 1,095 patients, demographic and clinical data for 1,005 (91.7%) episodes, and outcome data for 857 (78.3%). Species were identified for 1,068 (97.5%) isolates.

The average population-based incidence of candidemia was 1.81/100,000 (1.87/100,000 if inclusive of cases at the nonparticipant hospital) per year (Table 1) and was highest in infants (24.8/100,000) and persons ≥65 years of age (13.7/100,000; Figure 1). Most cases (36.4%) occurred in the most populous state, New South Wales, but incidence

Table 1. Number and incidence of candidemia cases reported in all jurisdictions, Australia, 2001–2004*

Parameter	State or territory								
	NSW	VIC	QLD	SA	WA	TAS	NT	ACT	Total
No. cases (%)	399 (36.4)	300 (27.4)	273 (24.9)	45 (4.1)	40 (3.7)	19 (1.7)	5 (0.5)	14 (1.3)	1,095 (100.0)
Mean incidence per 100,000 population	1.98	2.01†	2.34	0.99	0.67	1.31	0.83	1.44	1.81†
Mean incidence per 1,000 separations‡	0.19	0.36	0.18	0.25	0.16	0.09	0.05	0.09	0.21
No. institutions	35	15	30	3	3	2	1	1	90
Acquisition (% of episodes)§									
IHCA	79.1	83.4	79.3	90.2	87.5	89.5	50	85.7	81.5
OHCA	10.9	14.2	10.7	9.8	2.5	10.5	0	14.3	11.6
CA	10.0	2.4	10.0	0	10.0	0	50.0	0	6.9

*NSW, New South Wales; VIC, Victoria; QLD, Queensland; SA, South Australia; WA, Western Australia; TAS, Tasmania, NT, Northern Territory; ACT, Australian Capital Territory; IHCA, inpatient healthcare-associated; OHCA, outpatient healthcare-associated; CA, community-acquired.
†Mean incidences per 100,000 in VIC and in Australia are 2.25 and 1.87, respectively, with inclusion of 36 cases identified at the nonparticipating adult hospital.
‡Data available for 17 hospitals in NSW, 8 in VIC, 8 in QLD, 2 in SA, 1 in WA, 2 in TAS, 1 in NT, and 1 in ACT.
§Data for 919 candidemia episodes.

was highest in Queensland. Age composition of the population was similar by jurisdiction and similar to the national average (data not shown) except for the 2 least populated areas, Northern Territory and Australian Capital Territory, where the percentage of persons ≥65 years of age was 4.4% and 9.3%, respectively, compared with the national average of 13%.

The pooled mean annual hospital-specific incidence of candidemia (data from 40 institutions) was 0.21/1,000 separations (range by jurisdiction 0.05–0.36) (Table 1). No case clusters occurred. The estimated mean incidence in university (n = 28) and university-affiliated hospitals (n = 9) was similar (0.22/1,000 separations, range by jurisdiction 0.05–0.90) and greater than in private hospitals (n = 3, 0.1/1,000 separations, range by jurisdiction 0.04–0.16).

Neonates ≤1 month of age accounted for 33 (3.3%) of 1,005 cases, children 2 months to 14 years of age for 95 (9.5%) cases, adults 15–64 years of age for 527 (52.4%) cases, and patients ≥65 years of age for 350 (34.8%) cases. The median age was 56 years (range 0–98); 537 (53.4%) episodes affected male patients.

Most cases occurred in medical wards (including cancer and hemopoietic stem cell transplant [HSCT] units; 390 [35.6%] of 1,095 cases), critical care units (273 [24.9%] cases), and surgical wards (193 [17.3%] cases). Emergency (4.3%) and obstetrics and gynecology (0.5%) services reported small percentages of episodes. Pediatric cases occurred in pediatric critical care units (16 cases, 1.5%) and pediatric medical or surgical wards (24 cases, 2.2%). Neonatal infections occurred in premature or low birthweight infants in neonatal critical care units (35 cases, 3.2%).

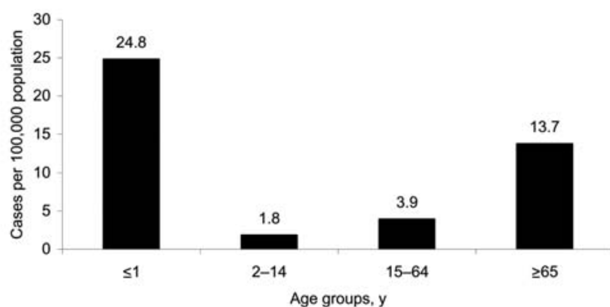

Figure 1. Annual age-specific incidence of candidemia, Australia, 2001–2004.

Concomitant Conditions, Risk Factors, and Healthcare Settings for Candidemia

Concomitant conditions and risk factor data were available for 1,005 episodes. Cancer was the most common underlying condition (323 cases, 32.1%), with 164 episodes in solid tumor (50.8% cancers) cancer patients. Of 159 cases diagnosed in patients with hematologic malignancy, 91 occurred in patients with leukemia and 58 in those with lymphoma. Only 52 episodes occurred in transplant recipients (17 allogeneic and HSCTs, 11 autologous HSCTs, and 24 solid organ transplants). Gastrointestinal disorders (19%), chronic cardiovascular disease (13.8%), and diabetes (13.6%) were common, but pancreatitis was infrequent (2.5%), and coincident HIV infection was rare (0.6%). Common iatrogenic risk factors included indwelling VADs (72.6%), antimicrobial agents (77%), major surgery (37.1%), and hyperalimentation (33%, Table 2).

Of 919 episodes for which information on the healthcare setting was available, 749 (81.5%) were IHCA, 107 (11.6%, or 62.9% of outpatient-acquired cases) were OHCA, and 63 (6.9%) were CA. The proportion of disease acquired outside a hospital by jurisdiction ranged from 9.8% (4 of 41 cases in South Australia) to 50% (but only 1 of 2 cases in Northern Territory). Of the major states, Western Australia reported the lowest percentage of OHCA cases (1 [2.5%] of 40 episodes, Table 1).

Table 2. Selected concomitant conditions, risk factors, and outcomes for 919 episodes of candidemia by healthcare setting, Australia, 2001–2004*

Characteristic	Healthcare-associated, outpatient acquired (n = 107)	Healthcare-associated, inpatient acquired (n = 749)	p value†	Community acquired (n = 63)	p value†
Concomitant condition					
Hematologic malignancy	27 (25.2)	131 (17.5)	0.06	1 (1.6)	<0.001
Solid organ malignancy	21 (19.6)	135 (18.1)	0.69	8 (12.7)	0.30
Solid organ transplantation	2 (1.9)	22 (2.9)	0.76	–	–
HSCT	3 (2.8)	25 (3.4)	1.0	–	–
Prematurity	–	35 (4.7)	–	1 (1.6)	0.37
Renal disease‡	10 (9.3)	56 (7.5)	0.44	2 (3.2)	0.24
GI and liver disease§	17 (15.9)	162 (21.6)	0.20	12 (19.0)	0.67
Pancreatitis	1 (0.9)	24 (3.2)	0.35	–	–
Cardiovascular disease¶	17 (15.9)	118 (15.8)	1.0	4 (6.3)	0.09
Diabetes mellitus	14 (13.1)	111 (14.8)	0.77	12 (19.0)	0.37
Risk factor					
Surgery in past 30 d	16 (15.7)	353 (49.0)	<0.001	4 (6.9)	0.08
Burns/trauma	-	40 (5.5)	–	1 (1.8)	–
VAD	72 (69.2)	653 (90.8)	<0.001	5 (9.1)	<0.001
Hyperalimentation	13 (12.6)	318 (44.1)	<0.001	1 (1.8)	0.04
Neutropenia	19 (18.1)	144 (19.6)	0.79	1 (1.6)	0.002
Antimicrobial agents	69 (65.7)	686 (95.5)	<0.001	19 (34.5)	<0.001
Corticosteroids	29 (28.4)	236 (33.0)	0.43	1 (1.6)	<0.001
Chemotherapy	27 (25.2)	106 (14.2)	0.01	–	–
Systemic antifungal use	15 (14.0)	106 (14.2)	1.0	–	–
Intravenous drug use	2 (1.9)	13 (1.7)	1.0	15 (23.3)	<0.001
Other BSI	15 (14)	256 (34.2)	<0.001	5 (8)	0.33
Sepsis syndrome	74 (69.2)	594 (79.3)	0.01	37 (58.7)	0.71
Candida endocarditis	7 (6.5)	22 (2.9)	0.08	5 (7.9)	0.55
Mean time in hospital, d	18.1	56.7	<0.001	16.1	0.33
Death within 30 d	13 (12.4)	218 (31.1)	<0.001	6 (9.4)	1.0

*Data are no. (%) of total cases in each category, except for mean time in hospital. Some patients had >1 concomitant condition or risk factor. HSCT, hemopoietic stem cell transplant; GI, gastrointestinal; VAD, vascular access device; BSI, bloodstream infection.
†By χ^2 test using outpatient healthcare-associated data as baseline.
‡Hemodialysis or peritoneal dialysis.
§Biopsy-proven cirrhosis with portal hypertension, past upper GI bleeding caused by portal hypertension, or prior episodes of hepatic failure.
¶Severe congestive heart failure. Symptoms at rest/inability to carry out physical activity without discomfort.

Comparison of major concomitant conditions and risk factors according to healthcare setting is summarized in Table 2; ≥4 risk factors were evident in 195 (21.2%) episodes. A total of 183 (20%) episodes were associated with use of an adult ICU. Twenty-nine (93%) neonates received hyperalimentation compared with 35.4% of the adults. Systemic antifungal agents were administered as prophylaxis in 67 (55.4%) of 121 instances; 48 (68.6%) of these prescriptions were for hematology/HSCT patients.

Most (90%–100%) episodes associated with prematurity, organ transplantation, and burn or trauma were IHCA (Table 2). Compared with all cases acquired outside a hospital, patients with IHCA infection were significantly more likely to have had recent surgery (49% vs. 12.5%, p<0.001), neutropenia (19.7% vs. 12.6%, p = 0.04), indwelling VADs (90.1% vs. 48.4%, p<0.001), corticosteroid therapy (33% vs. 19%, p<0.001), or parenteral nutrition (44.1% vs. 8.9%, p<0.001) or to have died within 30 days of infection (31.1% vs. 1.8%, p<0.001). These risk factors, with the exception of surgery, were more common in cases with OHCA candidemia than in those with CA candidemia (Table 2).

Recent surgery and VADs, hyperalimentation, and antimicrobial agents were more common in IHCA patients than in OHCA patients (Table 2). Conversely, coincident malignancy (44% vs. 34% episodes, p = 0.05) and cancer chemotherapy (25.2% vs. 14.3%, p = 0.01) were associated with OHCA infection. OHCA patients also included hemodialysis recipients and patients with diabetes. In CA candidemia, common concomitant conditions included intravenous drug use (IVDU, 23.8%), gastrointestinal disorders (19%), diabetes (19%), behavioral disorders such as chronic alcohol abuse (31.8%), and infectious diseases (23.4%); 15 (50%) of 30 episodes in IVDU patients were CA (Table 2). Differences in clinical manifestations were identified in the 3 groups. Patients with IHCA candidemia were more likely to have sepsis, die within 30 days, remain in hospital longer, and have concomitant healthcare-associated bacteremia (Table 2). Of 34 patients with *Candida* endocarditis (13 definite and 21 possible), 7 were OHCA

with an increasing trend when compared with those with IHCA candidemia (Table 2).

Candida Species

Species of *Candida* was correctly identified in 96.7% instances in which identity was established. *C. albicans* was the most common species (505 [47.3%] of 1,068 episodes) followed by *C. parapsilosis* (19.9%) and *C. glabrata* (15.4%). *C. tropicalis*, *C. krusei*, and *C. dubliniensis* were identified in 5.1%, 4.3%, and 1.9%, respectively, of the patients. The remaining 42 (4.0%) episodes were caused by *C. guilliermondi* (n = 11), *C. lusitaniae* (n = 7), *C. kefyr* (n = 5), *C. famata* (n = 3), *C. rugosa* (n = 3), and *C. pelliculosa* (n = 3). There were 24 (2.2%) episodes of polycandidal infection and an increase in the proportion of *C. glabrata* candidemia from 10.1% episodes the first year to 19.5% episodes in the third year of the study (p = 0.02, data not shown).

C. albicans was the predominant species in Tasmania (60%), Australian Capital Territory (77%), and Western Australia (54.1%). However, this species caused only 37.8% of episodes in South Australia, whereas 31.1% were caused by *C. parapsilosis* (7.7%–21.9% of cases elsewhere). *C. krusei* and *C. dubliniensis* candidemia were rare in the least populous jurisdictions where complex procedures such as HSCT are not performed. Age distribution, including proportion of neonates and children in the population, was comparable for all jurisdictions (data not shown).

Species other than *C. albicans* were isolated more often from patients with candidemia acquired outside a hospital (60.5% vs. 49.9% for IHCA candidemia, p = 0.02). *C. parapsilosis* was recovered from 30.9% of these episodes (compared with 16.7% of IHCA episodes, p<0.001), although *C. krusei* and *C. dubliniensis* candidemia was rare. Relative proportions of infections caused by *C. albicans* and non–*C. albicans* species in cases of OHCA candidemia and CA candidemia were similar.

In neonates and children, *C. parapsilosis* accounted for a similar proportion of episodes as did *C. albicans*; other species were rare. *C. albicans* was the most common causative pathogen in adults (48.6% of cases) and *C. glabrata*, *C. parapsilosis*, and other *Candida* species were approximately equally distributed (Figure 2). Eighty (53.3%) of 150 episodes in patients ≥65 years of age were caused by *C. glabrata*.

C. albicans was the most common pathogen in ICUs (61.7% of episodes) and surgical (54.4% of episodes) patients but caused only 32% of episodes in hematology patients, who had the highest proportion (24 [66.7%] of 36 episodes) of infection with *C. krusei*. *C. parapsilosis* accounted for 42.9% of episodes in neonatal ICUs and for 42.4% of cases in patients without hematologic cancer. *C. glabrata* was present in all patient groups. Twelve (63.2%) of 19 episodes of *C. dubliniensis* candidemia were in surgical and nonhematology patients.

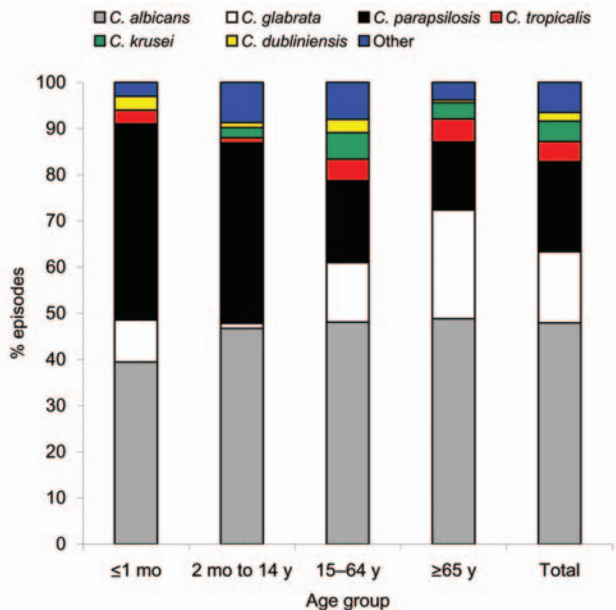

Figure 2. Distribution of causative pathogen according to patient age for 978 *Candida* species, Australia, 2001–2004.

Outcome

The all-cause 30-day death rate was 27.7% (236 of 853 episodes) and similar in all age groups. Clinician-reported attributable deaths were lower (93 episodes, 10.9%). The death rate was higher in IHCA episodes than in either OHCA or CA episodes (Table 2). Univariate analysis showed that numerous variables were associated with increased death (Table 3). Multiple logistic regression analysis of 16 variables showed that an age ≥65 years, ICU stay, sepsis at diagnosis, and corticosteroid therapy (p<0.001, Table 4) were associated with the greatest risk for death. *C. glabrata* infection was also an independent predictor of death. Treatment with an antifungal agent (752 [85.8%] of 876 episodes) was independently associated with lower odds of death but removal of a VAD (76.4% of 878 instances where VADs were in situ at diagnosis) was not (Table 4). However, in the subset of VAD-related candidemia episodes (n = 409), removal of the VAD (n = 339) was associated with a better outcome (odds ratio 0.48, 95% confidence interval 0.26–0.88, p = 0.02).

Discussion

This study provides the first contemporary, comprehensive, population-based description of candidemia across a continent. Overall disease incidence (1.81/100,000) was

Table 3. Univariate predictors of death by candidemia 30 days after diagnosis, Australia, 2001–2004*

Variable	Deaths, % (no./total)	Nondeaths, % (no./total)	p value
Age ≥65 y	53.8 (127/236)	28.4 (175/617)	<0.001
Malignancy	36 (85/236)	33.2 (205/617)	0.44
Hematologic malignancy	16.9 (40/236)	16.5 (102/617)	0.88
Lymphoma	8.1 (19/236)	5.0 (31/617)	0.09
Surgery in past 30 days	44.4 (104/234)	41.2 (254/616)	0.40
VAD	87.1 (203/233)	82.2 (505/614)	0.09
Hyperalimentation	44.2 (103/233)	35.3 (217/615)	0.02
Hemodialysis	16.7 (39/234)	8.3 (51/615)	<0. 001
Urinary catheter/drainage device	69.2 (162/234)	53 (325/613)	<0.001
Trauma/burns	1.7 (4/234)	5.8 (36/616)	0.01
Corticosteroid therapy	44.9 (105/234)	25.6 (158/616)	<0.001
Antimicrobial drug use	96.1 (224/233)	85.5 (526/615)	<0.001
Neutropenia	20.9 (49/234)	16.4 (101/614)	0.126
ICU stay	34.3 (81/236)	15.9 (98/617)	<0.001
Sepsis present (day 0)	87.6 (205/234)	78.8 (484/614)	0.003
Treatment with antifungal agent†	70.5 (165/234)	91.7 (564/615)	<0.001
VAD removal	61.6 (98/159)	82.5 (406/492)	<0.001
Candida glabrata infection	22.5 (53/236)	12.6 (78/617)	<0.001
Polycandidal infection	3.8 (9/236)	1.8 (11/617)	0.08
Inpatient healthcare-associated	91.9 (218/237)	85.7 (531/620)	0.02

*VAD, vascular access device; ICU, intensive care unit.
†Includes all patients receiving ≥1 therapeutic dose of systemic antifungal agent(s) as advised by an infectious diseases physician.

similar to that in Europe (1.4–4.9/100,000) (*12,23*) but different from that in the United States (6–10/100,000) (*7,11,24*). Most US and European surveys were not population-based and those that were included patients from specific areas (*11,13,24*). Age distribution of the population affected estimates, as shown by higher incidence among young and elderly people in Australia. Geographic variations in incidence were observed across Australia. However, this finding was unlikely to be caused by differences in age distribution because this distribution was similar in all states. Although the proportion of elderly persons was lower in Northern Territory and Australian Capital Territory, only 19 of 1,095 cases were in these jurisdictions.

As expected, mean incidence of candidemia was higher in university hospitals and university-affiliated hospitals, which have a higher proportion of patients at risk for candidemia (0.22/1,000 separations each) than private hospitals (0.1/1,000 separations). Since similar clinical management practices are used throughout Australia, jurisdictional differences are likely caused by different exposures to risk factors for infection. The highest incidences were observed in jurisdictions with organ transplantation, burn, and critical care centers.

Concomitant conditions and risk factors associated with candidemia were similar to those previously described (*11,25*), but pancreatitis (*26*) and HIV infection (8%–10% of cases in the United States) (*7,11*) were rare, and only 20% of episodes were associated with an ICU (33%–40% elsewhere) (*7,13,25*). We observed approximately equal numbers of cases in hematologic and solid tumor cancer patients. However, in previous studies, solid tumor patients were more common (*6,11,25*). Whether the prevalence of candidemia is increasing in the setting of chemotherapy for hematologic malignancy, despite use of antifungal prophylaxis in selected patients, is the subject of ongoing study.

Australian hospital statistics showed a 4% increase in community-based healthcare from 2000 through 2003 (*27*). Using population-based surveillance, we identified ≈20% of candidemia episodes that would not have been captured by nosocomial surveillance. This proportion is higher than in Europe (6%–10%) (*12,13*), but lower than in the United States (20%–28%) (*7,11*). However, these proportions are not directly comparable since previous studies have used different criteria to define outpatient-acquired episodes. Although most studies required blood cultures to be positive ≤48 hours of patient admission (*1,13*), others have used ≤72 hours (*28*) or ≤24 hours (*7,11*). If one

Table 4. Multivariate predictors of death by candidemia 30 days after diagnosis, Australia, 2001–2004*

Characteristic	OR	95% CI	p value
Age ≥65 y	3.0	2.0–4.3	<0.001
ICU stay	3.2	2.1–5.0	<0.001
Corticosteroid therapy	2.8	1.9–4.1	<0.001
Hemodialysis	2.4	1.4–4.1	0.002
Hyperalimentation	1.7	1.1–2.5	0.006
Antimicrobial drug use	2.1	0.9–4.4	0.07
Neutropenia	2.2	1.3–3.6	0.002
Sepsis present	3.1	1.7–5.4	<0.001
Treatment with antifungal agent	0.01	0.06–0.2	<0.001
Candida glabrata infection	1.8	1.1–2.9	0.01

*Sixteen candidate variables were included in the final model. OR, odds ratio; CI, confidence interval; ICU, intensive care unit.

considers the likely incubation period of candidemia, we suggest that 48 hours is appropriate. Furthermore, previous studies have not distinguished between CA and OHCA infections (*7,11,13,28*).

Using a national standard classification for bloodstream infections (*17*), we identified differences and similarities between IHCA candidemia and candidemia acquired outside a hospital and between OHCA and CA infections. Most (>60%) outpatient cases were healthcare associated. These cases resembled IHCA candidemia because cancer and other chronic diseases were common concomitant conditions and established risk factors were often present, although less frequently, than in IHCA infection; they differed from CA candidemia in instances in which IVDU was a risk factor. Thus, emergence of candidemia outside the hospital setting is likely due to the shift in persons with iatrogenic risk factors, such as chemotherapy and intravenous antimicrobial drug therapy, increasing management of more serious conditions outside the hospital, and implementation of early hospital discharge. Home-based therapies have been implicated in at least 1 outbreak of outpatient candidemia (*29*).

Compared with IHCA infection, the 30-day death rate and duration of hospital stay were lower in OHCA cases. Candidemia may have been more severe in the IHCA group because sepsis was initially identified in a higher proportion of these patients than in OHCA patients and was an independent predictor of death. Concomitant bacteremia was more common in IHCA patients. However, since no control group was studied, the difference may be explained by other characteristics of hospitalized patients compared with outpatients. Clinical outcomes of OHCA and CA candidemia were similar, although sepsis was present at diagnosis in a higher proportion of OHCA patients. Candidemia should be included in the differential diagnosis of patients with appropriate risk factors and sepsis who are admitted to emergency departments or in other healthcare settings.

Species distribution varied by jurisdiction, healthcare setting, age, and hospital service. In South Australia, *C. albicans* was less common and *C. parapsilosis* was more prevalent. This finding was not explained by differences in age distribution or proportions of IHCA and OHCA infections between jurisdictions. Overall, *C. parapsilosis* candidemia was more prevalent in outpatients, many of whom had VADs in situ, and in neonates, which is consistent with previous studies (*7,11*). Given the reduced susceptibility of *C. glabrata* to azole drugs (*30*), our observations that *C. glabrata* candidemia occurs more often in patients without hematologic malignancy and that these infections increased during the study provide useful data. In other studies, *C. glabrata* was more common in older persons (*31*) and patients with hematologic cancer (*24*).

The high overall death rate, albeit lower than reported elsewhere (35%–44%) (*7,12,13*), is another reminder of the role of candidemia in healthcare settings. The highest case-fatality rate was observed in the most vulnerable patients (elderly, ICU patients, and those who recently had medical interventions). Sepsis syndrome and failure to institute treatment with antifungal drugs (the latter occurred mainly in preterminal hematology patients) were independent predictors of death. Although removal of VADs did not independently protect against death, data from univariate analysis (Table 3) support current recommendations to remove VADs when candidemia is detected (*32*).

In conclusion, although candidemia is primarily a healthcare-associated entity in patients with established risk factors, many of these patients are observed in the outpatient setting. OHCA infections have characteristics intermediate between those of IHCA and CA infections. We propose that cases of candidemia be categorized as IHCA, OHCA, and CA, with the term CA reserved for those episodes occurring ≤48 hours of hospital admission and that do not meet criteria for healthcare-associated infections. Further study of secular trends and characteristics of candidemia acquired outside hospitals with standardized definitions is warranted. Surveillance is needed to track trends of this serious infection and provide guidelines for antifungal prophylaxis, treatment, and infection control strategies.

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Active Cytomegalovirus Infection in Patients with Septic Shock

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Cytomegalovirus (CMV) is a pathogen of emerging importance for patients with septic shock. In this prospective study, 25 immunocompetent CMV-seropositive patients with septic shock and an intensive care unit stay of $\geq$7 days were monitored by using quantitative pp65-antigenemia assay, shell vial culture, and virus isolation. Within 2 weeks, active CMV infection with low-level pp65-antigenemia (median 3 positive/5×10^5 leukocytes) developed in 8 (32%) patients. Infection was controlled within a few weeks (median 26 days) without use of antiviral therapy. Duration of intensive care and mechanical ventilation were significantly prolonged in patients with active CMV infection. CMV reactivation was associated with concomitant herpes simplex virus reactivation ($p = 0.004$). The association between active CMV infection and increased illness could open new therapeutic options for patients with septic shock. Future interventional studies are required.

Sepsis and septic shock are defined as a clinical syndrome with severe inflammatory response (*1*). Despite the availability of antimicrobial, antifungal, and supportive therapies, septic shock is fatal for about one third of patients.

Cytomegalovirus (CMV) is a human β-herpesvirus that has high seroprevalence in adults. CMV disease predominantly occurs as an opportunistic infection in patients with severe immunosuppression and rarely occurs in immunocompetent patients (*2*). Clinical diagnosis of CMV disease, without the use of virus diagnostics, is hampered by the fact that the clinical signs and symptoms are not very specific. Patients in intensive care units (ICUs) are rarely monitored for active CMV infection; therefore, the development of active CMV infection remains unrecognized in most critically ill patients.

During recent years, CMV has been discussed as an emerging pathogen in critically ill patients who are not receiving immunosuppressive therapy; however, the incidence of active CMV infection is controversial (*3,4*), and not all centers detected active CMV infections in these patients (*5–7*). Among critically ill patients, the highest incidence of active CMV infection was in patients with septic shock (*3*). The causality of sepsis, consecutive CMV reactivation, and CMV-associated pulmonary disease is supported by a mouse model of murine CMV reactivation after cecal ligation and puncture (*8,9*). Many factors could stimulate CMV reactivation in patients with septic shock; e.g., proinflammatory cytokines (*10,11*), transient immune paralysis (compensatory antiinflammatory response syndrome) (*12*), and drugs (*13*).

This pilot study investigated the incidence and the natural course of active CMV infection in patients with septic shock and different strategies for CMV monitoring. The study may stimulate future interventional trials aimed at preventing CMV-associated illness of patients with septic shock.

Patients and Methods

Patients

For 9 consecutive months, patients in the anesthesiologic ICU, University Hospital Ulm, Ulm, Germany, who had septic shock, were monitored for active CMV infection. We did not monitor patients who underwent splenectomy, transplantation patients, or patients receiving immunosuppressive therapy. Also, patients who had been in ICU <7 days were excluded because CMV reactivation and CMV-associated illness were expected to develop with a time delay. To define septic shock, we used the criteria established by the American College of Chest Physicians/Society of Critical Care Medicine (*14*). Clinicians were not made aware of virologic results. To avoid exogenous CMV infections, transfusions were limited to filtered leukocyte-reduced blood products. The study

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was approved by the local ethics committee and was in accordance with the Helsinki Declaration; informed consent was obtained.

Virologic Examinations

CMV monitoring was performed twice during the first week of the study and once a week thereafter until the patient was discharged from ICU. Quantitative pp65-antigenemia assay; shell vial culture; and viral isolation in leukocytes, urine, and bronchial aspirate were performed as previously described (*15*). Briefly, pp65 antigenemia was determined in blood collected in EDTA tubes and subjected to dextran sedimentation (1% dextran in phosphate-buffered saline). Duplicates of 5×10^5 leukocytes were placed onto glass slides, and the pp65 antigen-positive cells were evaluated by immunofluorescence assay (IFA) by using a mixture of 2 monoclonal mouse anti-pp65 antibodies (20:1; Virion, Rüschlikon, Switzerland; Argene Biosoft, Viva Diagnostika, Hürth, Germany) and goat anti-mouse immunoglobulin (Ig) G (Dianova, Hamburg, Germany) conjugated with fluorescein isothiocyanate (FITC).

Leukocytes, bronchial aspirate, and urine were investigated by shell vial culture and viral isolation with human embryonic lung fibroblasts. Three days after infection, shell vial cultures were fixed with methanol and analyzed by IFA (anti-CMV immediate early antibody, Argene Biosoft, Viva Diagnostika; FITC-conjugated goat anti-mouse IgG, Dianova). Phase contrast microscopy was used to analyze viral isolation for ≥6 weeks. Cytopathic effects of various viruses were confirmed by using viral typing with IFA and monoclonal antibodies.

At the initial evaluation, the following antibodies were determined semiquantitatively by using ELISA (Medac, Hamburg, Germany): CMV IgG, CMV IgM, and herpes simplex virus (HSV) IgG. Patients with antibody indices >1 were considered antibody positive.

Clinical Data

The following values were regularly recorded: body temperature, heart rate, blood pressure, respiratory rate, need for mechanical ventilation, oxygen supply (FiO_2), urinary output, hemodiafiltration, partial pressure of oxygen in arterial blood, pH, leukocyte count, platelet count, serum bilirubin, aspartate aminotransferase (AST), C-reactive protein, and serum creatinine. The severity of organ failure over time was recorded by monitoring the most relevant organ functions (pulmonary, cardiovascular, hematologic, hepatic) and using the Sepsis-related Organ Failure Assessment Score (SOFA) (*16*). Impairment of the central nervous system was not evaluated (Glasgow Coma Scale) because most patients received sedatives.

Statistics

Statistical analysis was performed by using nonparametric tests (Fisher exact test, Mann-Whitney U test) and GraphPad Prism 3.02 software (GraphPad Software, San Diego, CA, USA). Significance level was set at $p = 0.05$.

Results

Patients

Among 375 patients in ICU, 38 consecutive patients with septic shock were eligible, but 13 were excluded because of CMV seronegativity (n = 5), immunosuppressive therapy (n = 2), or ICU stay <7 days (n = 6). Thus, 25 CMV-seropositive patients with septic shock and an ICU stay ≥7 days were enrolled in the study.

Active CMV Infection

During the first 2 weeks after onset of septic shock, active CMV infection was detected by sensitive quantitative pp65-antigenemia assay in 8 (32%) patients (*15*). Active CMV infection was also detected by shell vial culture in 4 of these patients (in bronchial aspirate for 3 patients and in urine for 1). For 1 patient for whom shell vial culture in bronchial aspirate was positive, shell vial culture was also positive in leukocytes. Initial detection of active CMV infection was delayed when using shell vial culture (detected 1, 11, 20, and 21 days after onset of septic shock) compared with pp65-antigenemia in the same patients (0, 7, 10, and 14 days). Overall, pp65-antigenemia was low (median 3 positive/5×10^5 leukocytes; range 1–17) and became nondetectable with no antiviral therapy (median 26 days after onset of active CMV infection; range 1–61 days). One patient died while CMV infection was still active.

CMV IgM antibodies were found in 2 (25%) of 8 patients with and 2 (12%) of 17 patients without active CMV infection, a difference that was not significant. Also the quantitative levels of CMV IgG and IgM antibodies did not differ between groups with and without active CMV infection (Table).

Characteristics of Patients with and without Active CMV Infection

Patient characteristics such as age, sex, primary disease, and severity of organ failure at time of entry into the study did not differ between patients with and without active CMV infection (Table). Hydrocortisone (200 mg/day) was given to patients in both groups; no differences between groups were noted in body temperature, leukocyte count, platelet count, serum creatinine level, serum bilirubin level, AST level, and C-reactive protein level. Systemic infection by gram-positive and gram-negative microorganisms was detected equally in both

groups, and catecholamine treatment for cardiovascular dysfunction was similar for both groups.

Overall, the severity of sepsis-related failure of multiple organs, determined by SOFA score (*16*), did not differ between patients with and without active CMV infection; however, patients with active CMV infection required mechanical ventilation and ICU therapy for a longer time than did patients without active CMV infection ($p = 0.0025$) (Table). Although mortality rates were not significantly different between patients with and without active CMV infection (63% vs 35%; $p > 0.05$), the deaths occurred later (median 44 days after onset of septic shock, range 24–72 days) for patients with active CMV infection than for patients without (median 21 days, range 14–35 days) ($p = 0.03$).

The clinical course of patients with positive CMV shell vial culture in bronchial aspirate was associated with the longest duration of mechanical ventilation (47, 50, and 80 days) and of ICU stay (50, 71, and 87 days); however, because of the low number of cases, statistical analysis was not performed.

Other Viral Infections

All 25 patients were HSV seropositive; viral isolation in bronchial aspirate showed reactivation of HSV in 8 (32%) patients, thereby showing for the first time that HSV and CMV reactivation were associated ($p = 0.004$, Table) and occurred simultaneously (Figure). Active HSV infections developed without skin or mucosal rash (occult HSV infection). Because of the low number of cases, the clinical outcome of patients with active CMV, HSV, or CMV/HSV coinfection could not be further differentiated. Viral isolation in bronchial aspirate and urine did not detect additional opportunistic viral infections such as polyoma BK virus and exogenous viral infections such as adenovirus, respiratory syncytial virus, and parainfluenzavirus in any patient.

Discussion

While CMV is well known as a cause of serious illness in immunosuppressed patients, it is now being discussed as a pathogen of emerging importance for patients with septic shock. Generally, active CMV infection is not

Table. Characteristics and clinical course of patients with septic shock, with and without active CMV infection*

	Active CMV infection	No active CMV infection	Significance
No. patients	8	17	
CMV IgG, index	12.4 (5.4–14.7)	11.7 (1.5–18.3)	NS†
CMV IgM, index	0.4 (0.28–3.94)	0.28 (0.2–1.8)	NS†
Sex, n			
Male	5	10	NS‡
Female	3	7	NS‡
Age, y	66 (40–78)	60 (44–78)	NS†
Primary condition, n			
Abdominal surgery	1	7	NS‡
Abdominal tumor	1	4	NS‡
Pancreatitis	3	1	NS‡
Trauma	2	5	NS‡
Vascular surgery	1	0	NS‡
Bacteremia, n (%)	4 (50)	10 (59)	NS‡
Candidemia, n (%)	2 (25)	1 (6)	NS‡
SOFA score§	10 (7–13)	10 (7–16)	NS‡
Leukocyte count, g/L§	27 (10.4–53.3)	22.4 (7.2–74.3)	NS†
Platelet count, g/L¶	106 (37–151)	112 (37–385)	NS†
Serum creatinine, µmol/L§	183 (73–345)	160 (72–347)	NS†
Serum bilirubin, µmol/L§	27 (6–279)	54 (4–336)	NS†
Aspartate aminotransferase, U/L§	55 (7–267)	45 (9–229)	NS†
C-reactive protein, mg/L§	258 (16–456)	220 (115–437)	NS†
ICU stay after onset of septic shock, d	42 (16–87)	18 (10–42)	p = 0.0025†
Mechanical ventilation, d	39 (15–80)	16 (5–38)	p = 0.0025†
Receipt of catecholamines, d	7 (4–41)	7 (1–35)	NS†
Mortality rate, n (%)	5 (63)	6 (35)	NS‡
HSV reactivation, n (%)	6 (75)	2 (12)	p = 0.0036‡

*Median (range), unless otherwise indicated; CMV, cytomegalovirus; Ig, immunoglobulin; NS, not significant ($p>0.05$); SOFA, sepsis-related organ failure assessment; ICU, intensive care unit; HSV, herpes simplex virus.
†According to Mann-Whitney U test.
‡According to Fisher exact test.
§Highest values.
¶Lowest values.

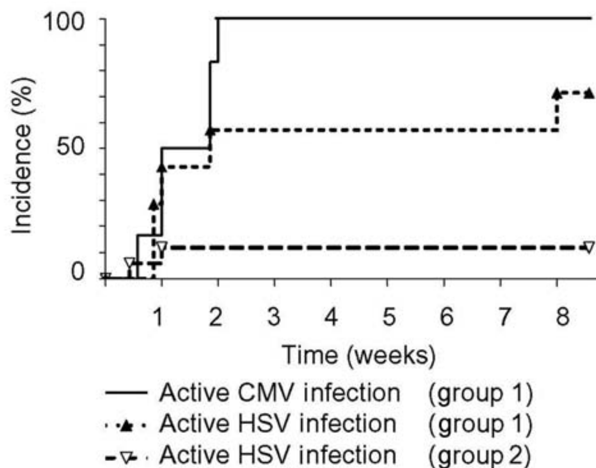

Figure. First detection of cytomegalovirus (CMV) and herpes simplex virus (HSV) reactivation after onset of septic shock. Incidence of active CMV and HSV infection is shown for patients with active CMV infection (group 1; n = 8) and without active CMV infection (group 2; n = 17). CMV reactivation occurred during the first 2 weeks after onset of septic shock (median 7 days) and was associated with HSV reactivation, which occurred during the same period. The incidence of active HSV infection was different between groups 1 and 2 (75% vs 12%; p = 0.004). Active CMV infection was detected first by CMV pp65 antigenemia; active HSV infection was detected by virus isolation of bronchial aspirates.

recognized in such patients because critically ill patients are not routinely monitored for CMV infection.

CMV reactivation developed in one third of our patients within 2 weeks of onset of septic shock, as has been found in studies using a similar prospective study design (*3,11*). Diagnostic assays of different sensitivity, different patient groups, and study designs could account for discrepant results obtained by other groups (*5,10*). Thus, onset of active CMV infection was detected later in the retrospective studies (*4,17,18*).

Active CMV infection in patients with septic shock was characterized by a low viral load and resolved within a few weeks without antiviral therapy. We hypothesize that upon CMV reactivation, patients with septic shock could mount a protective antiviral immune response, which was different from the immune response of most patients after transplantations (*19*); however, this hypothesis remains to be confirmed.

In a previous study we compared different assays for CMV monitoring of patients with organ transplants and demonstrated equal sensitivity of our pp65 antigenemia assay and CMV PCR of blood cells but lower sensitivity of shell vial culture, CMV PCR in plasma, and CMV mRNA detection by nucleic acid sequence-based amplification (*15*). Because of low viral loads, the incidence of active CMV infection could be easily underestimated by less sensitive assays for patients with septic shock, which was shown here in that shell vial culture in blood cells detected only 1 patient with active CMV infection. Less sensitive assays could have been also the problem of studies that failed to detect active CMV infection in critically ill patients (*5–7*). We assume that assays with sensitivity similar to that of our pp65-antigenemia assay (e.g., CMV PCR of blood cells) may be equally used for CMV monitoring of patients with septic shock, considering the results of patients who had received transplants (*3,11,15*).

Shell vial culture was more likely to detect active CMV infection in bronchial aspirate than in urine or blood cells. Pulmonary CMV infection may be relevant for patients with septic shock (*8,20*). Shell vial culture of urine was rarely positive for CMV in patients with septic shock, a finding which differed for patients having received a kidney transplant (*21*).

As expected, quantitative analysis of CMV IgG and IgM antibodies could not discriminate between patients with and without active CMV infection. CMV IgG antibodies were analyzed to identify patients with previous CMV infection (CMV-seropositive patients); however, diagnosis of active CMV infection by detection of CMV IgM antibodies or rising CMV antibody titers are no longer recommended when sensitive CMV monitoring by pp65-antigenemia assay or CMV PCR are available because the information they provide is limited.

The clinical role of active CMV infection in patients with septic shock is an area of ongoing discussion (*4*). We demonstrated that active CMV infection is associated with prolonged ventilation time and ICU stay. Ventilation time and ICU stay were more prolonged in a subgroup of patients for whom shell vial culture in bronchial aspirate was positive. CMV infection was associated with pulmonary disease despite low pp65 antigenemia and self-limiting CMV infection. We suppose that immunopathologic mechanisms could contribute to CMV-associated illness (*22*) in addition to direct cytopathic effects of the infection (*20*). Association of tumor necrosis factor and pulmonary immunopathologic features of active CMV infection was recently confirmed in a mouse model showing murine CMV reactivation after cecal ligation and puncture (*9*).

Deaths occurred later for patients with active CMV infection than for those without active CMV infection. This finding could be the consequence of CMV-associated disease, as has been suggested (*17*). Although our study was not designed to clarify the causality between active CMV infection and increased illness, we argue that active CMV infection increases illness and not vice versa. In the mouse model of CMV reactivation, the causality between sepsis, CMV reactivation, and pulmonary disease has already been shown (*9*).

Recently, reactivations of HSV and human herpesvirus 6 have been reported in critically ill patients (*7,23*). We demonstrated for the first time an association between active HSV and CMV infection (p = 0.004). HSV was isolated from bronchial aspirate in the absence of skin and mucosal lesions, whereas other herpesviruses, such as varicella-zoster virus, could not be isolated. The coincidence of HSV and CMV reactivation during the first 2 weeks of septic shock suggests a common trigger mechanism for herpesvirus reactivations. In future studies, more sensitive assays (e.g., PCR) may be used to analyze the incidence of other occult herpesvirus reactivations. Reactivation of polyoma BK virus, which commonly causes opportunistic infection after transplantation, was not detected by virus isolation. This finding leads to the hypothesis that the conditions that stimulate polyomavirus reactivation and those that stimulate CMV and HSV reactivation may differ. The absence of exogenous viral infection (e.g., adenovirus, respiratory syncytial virus, parainfluenzavirus) strengthens the suggestion that exogenous nosocomial viral infections are uncommon in patients in ICUs (*24*). Thus, monitoring for viral infections could focus on endogenous herpesvirus reactivations in patients with septic shock. Immunosuppression and proinflammatory cytokines, drugs, or combinations are presumed to be involved in herpesvirus reactivations; however, the exact mechanisms are still elusive for patients with septic shock (*13,25*).

After organ transplants, CMV-associated illness and death could be reduced by early antiviral therapy; however, delayed therapy has been less effective (*2*). Anecdotal reports show that critically ill patients with already established CMV organ disease may not benefit from antiviral therapy (*3,4,20*). The effect of preemptive antiviral therapy or antiviral prophylaxis has not been tested so far in patients with septic shock; however, in the mouse model, prophylactic treatment with ganciclovir prevented murine CMV reactivation and CMV-associated pulmonary fibrosis (*9*).

Despite the low patient number in this and previous studies, we suggest that CMV is a pathogen of emerging importance that can no longer be ignored for patients with septic shock. Thus, interventional studies aimed at preventing CMV-associated illness in patients with septic shock are needed.

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Antimicrobial Drugs in the Home, United Kingdom

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A total of 6% of 6,983 households in the United Kingdom had leftover antimicrobial drugs, and 4% had standby antimicrobial drugs. Respondents with leftover drugs were more educated, more knowledgeable about antimicrobial drugs, younger, and female. Of respondents with leftover drugs, 44% kept them in case of future need, and 18% had taken these drugs without medical advice.

Antimicrobial drug resistance is increasing worldwide and is related to use of these drugs (*1*,*2*). Use of leftover drugs may increase antimicrobial drug resistance in the community by exerting selective pressure in the commensal flora (*3*,*4*). Retention and use of leftover antimicrobial drugs are widespread in countries that sell antimicrobial drugs without a prescription (i.e., over-the-counter) (*5*,*6*). In countries in which over-the-counter antimicrobial drug sales are restricted, studies on use of leftover drugs have been few and mostly questionnaire-based, relying on respondent recall (*7*,*8*). A large survey of British household drug practices showed that 2% of medicines were leftover or standby drugs (*9*), but no information was obtained about the households containing them. To inform antimicrobial drug education campaigns (*10*,*11*) and research examining the relationship between antimicrobial drug prescribing and resistance (*12*), we conducted a household survey to identify antimicrobial drugs present and the characteristics of respondents who kept them.

Methods

The survey formed part of the Office for National Statistics' Omnibus Survey in the United Kingdom in February, March, June, and July 2003. A representative sample of households was selected by using previously published methods (*9*). Households were invited by letter to participate in a study about their views and experiences on a variety of subjects. For households with >1 adult member, 1 person ≥16 years of age was selected by a random number tables. At the household visit, respondents were informed that the survey was sponsored by the department of health and was about drugs for treating infections. Respondents were asked to show the interviewer all drugs in the household that had been prescribed for infections, even if the person for whom they were prescribed was not present (temporarily or permanently), and asked whether the drugs were currently being used for an infection for which they were prescribed, had been prescribed for a previous infection (leftover drugs), or were being kept for use in the future (standby drugs). The amount of unused drug was noted and prescription details were recorded and coded by their listing in the British National Formulary. Antimicrobial drugs included all antifungal and antibacterial drugs.

Respondents were asked a series of questions about their use of antimicrobial drugs and whether they agreed or disagreed with a series of statements from education campaigns (*10*,*11*) about the usefulness of these drugs for coughs and colds, viruses, bacteria, and normal flora; the importance of completing courses of drug therapy, and antimicrobial drug resistance. Ethical approval was not required for this ongoing national survey, but respondents were able to refuse participation in any part of the survey at any time and were told that all information was anonymous and strictly confidential.

To enable comparison with nationally prescribed antimicrobial drugs, the number of community-prescribed drugs in England was obtained from prescribing analysis and cost data (PACT) (*13*). Sampling weights based on the 2001 census data for Great Britain were applied to household and respondent data to allow for any oversampling or undersampling by region, Carstair's deprivation quintile,

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age group, and sex. Multivariable logistic regression (ignoring sampling weights) was used to investigate independent associations between respondent characteristics and the presence of leftover antimicrobial drugs prescribed for the respondent.

Results

Of 10,981 selected households, 25% refused to participate and 10% could not be contacted. Respondents in 7,120 (65%) households completed the questionnaire and 6,983 (64%) agreed to the drug survey. A total of 19% of the 6,983 households had an antimicrobial drug leftover (6%), kept for standby use (4%), or currently in use (11%) (Table 1). Six percent of leftover drugs had been prescribed for ≤3 days, 16% for 4–5 days, 61% for ≥6 days, and 17% were to be taken as required. No specific class of oral antimicrobial drugs was kept more often as leftover drugs relative to PACT prescription rates (Figure 1).

Four percent of respondents had leftover antimicrobial drugs that had been prescribed. The most educated respondents were twice as likely as those least educated to have leftover antimicrobial drugs (5.4% with a university degree vs. 2.4% with no formal degree) (Table 2). Respondents most knowledgeable about antimicrobial drugs (6.1% if all 11 questions were answered correctly) were twice as likely as those least knowledgeable (3.1% if ≥5 were answered incorrectly) to keep them (Table 2, Figure 2). Level of education and knowledge of antimicrobial drugs were independently associated with being more likely to have leftover drugs (Table 2). Younger respondents and women were more likely to have leftover drugs (Table 2, Figure 2). Thirty-eight percent of respondents' leftover drugs had been dispensed >1 year earlier, and 48% of these drugs had more than half of the original amount remaining (Table 1). Of those who had kept a leftover antimicrobial drug prescribed in the past year, 44% did so for future need (Table 1). Of those with a leftover drug, 18% had taken antimicrobial drugs without advice compared with 4% of respondents who did not have a leftover drug ($p<0.0005$).

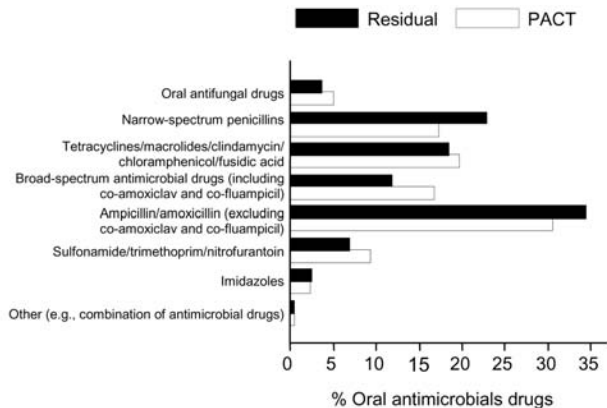

Figure 1. Oral leftover antimicrobial drugs compared with prescriptions issued (p = 0.16), United Kingdom, 2003. PACT, prescribing analysis and cost data.

When directly questioned, 38% of respondents (95% confidence interval 36.4–38.8%) recalled that they had been prescribed a drug in the past year. Younger age in women (50% if 16–24 years of age, 43% if 25–44 years of age, and 44% if 45–54 years of age) and lower level of education (33% with a degree and 40% with no formal qualifications on leaving school at the minimum age [16 years]) were independently associated with being prescribed an antimicrobial drug.

Table 1. Outcomes from household survey of antimicrobial drugs and interviews of an adult member of the household about behavior in relation to these drugs, United Kingdom, 2003*

Data	Antibacterial or antifungal drugs, % (95% CI)	Antibacterial drugs, % (95% CI)	Antifungal drugs, % (95% CI)
Household-based (6,983 households)			
Households with drug	18.9 (18.0–19.9)	13.9 (13.1–14.8)	6.9 (6.3–7.6)
Households with current drug	10.6 (9.9–11.4)	7.9 (7.2–8.5)	3.6 (3.2–4.1)
Households with leftover drug	6.2 (5.7–6.8)	4.5 (4.0–5.0)	2.1 (1.8–2.5)
Households with standby drug	3.7 (3.3–4.2)	2.4 (2.1–2.8)	1.5 (1.2–1.8)
Households with leftover or standby drug	9.6 (8.9–10.3)	6.7 (6.1–7.3)	3.5 (3.1–4.0)
Antimicrobial drug–based (506 leftover, 292 standby)			
Leftover drug >1 y old	38 (33–44)	32 (27–38)	55 (46–63)
Standby drug >1 y old	31 (26–37)	29 (23–37)	36 (28–46)
Leftover drug is more than half left	48 (43–53)	50 (44–56)	44 (36–53)
Respondent-based (7,120 respondents)			
Respondent had a leftover drug that was originally prescribed	3.9 (3.4–4.4)†	2.8 (2.4–3.3)	1.2 (1.0–1.5)
Respondent prescribed a drug in the last year	37.6 (36.4–38.8)		
Respondent kept leftover drug from their most recent prescription	5.9 (5.0–7.0)		
Respondent kept drug in case of future need	44 (35–52)		

*Some prescriptions contained both antibacterial and antifungal drugs and contribute to all 3 columns. CI, confidence interval.
†This outcome is examined in more detail in Table 2.

Table 2. Characteristics of respondents who had a leftover antimicrobial drug, United Kingdom, 2003*

Outcome: respondent had leftover drug originally prescribed for respondent	% (95% CI)	Crude OR† (95% CI)	p value	Adjusted OR† (95% CI)	p value
Age group, y					
16–24	3.8 (2.4–6.0)	0.89 (0.82–0.98)	0.01	0.97 (0.88–1.06)	0.49
25–44	4.5 (3.7–5.4)				
45–54	4.1 (3.0–5.5)				
55–64	3.9 (2.9–5.3)				
65–74	2.9 (2.0–4.3)				
≥75	2.1 (1.3–3.5)				
Sex					
Male	3.2 (2.6–4.0)	1		1	
Female	4.4 (3.8–5.2)	1.38 (1.05–1.82)	0.02	1.22 (0.94–1.57)	0.14
Education					
Degree	5.4 (4.1–7.1)	1	<0.0005	1	0.001
Other	4.3 (3.6–5.1)	0.78 (0.56–1.10)		0.74 (0.54–1.02)	
No formal degree	2.4 (1.8–3.2)	0.43 (0.29–0.65)		0.44 (0.29–0.68)	
No. questions regarding drugs answered incorrectly					
0	6.1 (4.7–7.8)	0.87 (0.79–0.95)	0.002	0.91 (0.84-0.98)	0.02
1	4.2 (3.1–5.5)				
2	3.4 (2.6–4.5)				
3	4.0 (2.9–5.4)				
4	2.5 (1.6–3.8)				
≥5	3.1 (2.1–4.5)				
Carstair deprivation score					
1st quintile (least deprived)	4.4 (3.4–5.9)	1.00 (0.91–1.11)	0.99	1.01 (0.92–1.10)	0.89
2nd quintile	3.9 (2.8–5.5)				
3rd quintile	3.4 (2.6–4.6)				
4th quintile	3.1 (2.3–4.1)				
5th quintile (most deprived)	4.6 (3.6–5.8)				
Prescribed a drug in the past year					
No	2.1 (1.7–2.6)	1		1	
Yes	6.9 (5.9–8.0)	3.51 (2.66–4.65)	<0.0005	3.50 (2.69–4.54)	<0.0005

*CI, confidence interval; OR, odds ratio.
†For age group, no. of questions regarding drugs answered incorrectly used Carstair deprivation score; the odds ratio is between adjacent categories. The corresponding p value is a test for trend.

Discussion

This large survey of household antimicrobial drugs, which was conducted in a country with restricted over-the-counter sales, showed an association between keeping leftover antimicrobial drugs and higher education and knowledge about these drugs, younger age, and being female. The strength of this study is that we asked a large representative sample to show us their antimicrobial drugs rather than relying on respondent memory. However, we may not have been shown all antimicrobial drugs, and some of the leftover drugs may have been misclassified as standby drugs. The characteristics of respondents in households with standby drugs were similar to those of respondents with leftover drugs. The number of antimicrobial drugs found was twice that found by a recent survey in Sweden that relied on respondent recall; in that study, the respondents also reported a lower number of antimicrobial drug prescriptions (*8*).

Persons may keep leftover antimicrobial drugs because too much was prescribed for their initial infection. Most community-acquired infections are respiratory or urinary, for which many prescribed courses of antimicrobial drugs are longer than necessary (*14*). If the standard duration of treatment could be shortened and package size reduced to contain enough drug for 3 to 5 days, the temptation to keep antimicrobial drugs might be diminished. Our finding supports this suggestion because prescriptions for ≥6 days constituted 61% of leftover drugs, whereas prescriptions for ≤3 days constituted 6% of leftover drugs. Evidence shows that repeated treatment with antimicrobial drugs exerts greater selective pressure on normal bacterial flora than a single course of treatment (*14*,*15*). Consequently, persons who use leftover antimicrobial drugs repeatedly are at greater risk for colonization and infection with drug-resistant organisms (*1*,*3*,*4*,*15*).

Our results show that any public education campaign to reduce retention and use of leftover antimicrobial drugs must be directed at well-educated groups rather than the less educated, who are prescribed more antimicrobial drugs. One explanation may be that well-educated people

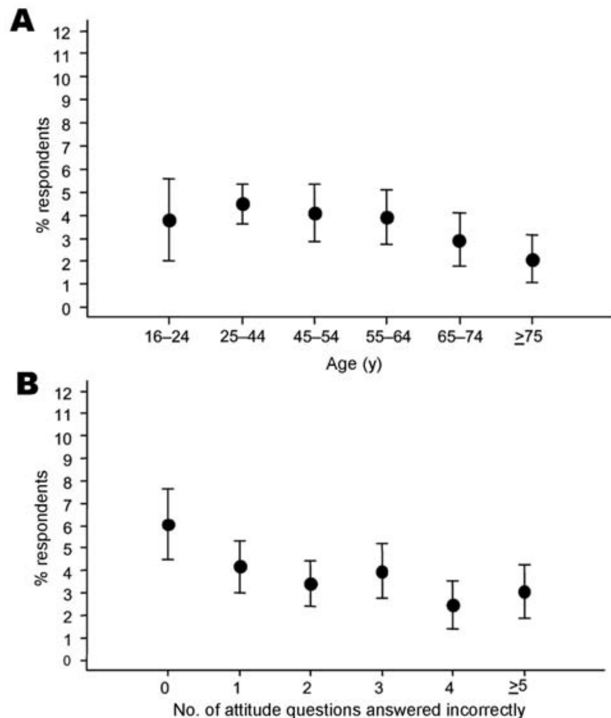

Figure 2. Percentage of respondents with a leftover antimicrobial drug (all antimicrobial drugs, i.e., antibacterial plus antifungal drugs) by A) age (y) of respondent ($p = 0.01$, by test for trend) and B) no. of attitude questions answered incorrectly ($p = 0.002$, by test for trend). Error bars show 95% confidence intervals.

are confident that they can make their own decisions about antimicrobial drug use, and this may be particularly relevant when their infection is less severe or becomes asymptomatic. Furthermore, they do not discard these drugs because 44% of those with leftover antimicrobial drugs admitted keeping them in case of future need. Antimicrobial drug education campaigns should reinforce the message that leftover drugs should be returned to the pharmacist or that these drugs should only be taken after the advice of a health professional.

Our finding of high levels of leftover antimicrobial drugs suggests that prescription does not equate to use. This will be an important source of bias in epidemiologic studies examining risk factors for antimicrobial drug resistance (*12*) that include dose and duration of antimicrobial drug treatment in their analysis of exposure (*1*,*4*).

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Human Prion Disease and Relative Risk Associated with Chronic Wasting Disease

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The transmission of the prion disease bovine spongiform encephalopathy (BSE) to humans raises concern about chronic wasting disease (CWD), a prion disease of deer and elk. In 7 Colorado counties with high CWD prevalence, 75% of state hunting licenses are issued locally, which suggests that residents consume most regionally harvested game. We used Colorado death certificate data from 1979 through 2001 to evaluate rates of death from the human prion disease Creutzfeldt-Jakob disease (CJD). The relative risk (RR) of CJD for CWD-endemic county residents was not significantly increased (RR 0.81, 95% confidence interval [CI] 0.40–1.63), and the rate of CJD did not increase over time (5-year RR 0.92, 95% CI 0.73–1.16). In Colorado, human prion disease resulting from CWD exposure is rare or nonexistent. However, given uncertainties about the incubation period, exposure, and clinical presentation, the possibility that the CWD agent might cause human disease cannot be eliminated.

An emerging wildlife epizootic of chronic wasting disease (CWD) (*1*), a contagious prion disease among mule deer, white-tailed deer, and Rocky Mountain elk, has potential public health implications (*2–5*). CWD is related to other mammalian transmissible spongiform encephalopathies (TSEs), such as Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep. In prion diseases, a normally produced cellular protein accumulates in an abnormal, misfolded, and aggregated form (*6*), which results in neuron destruction and a universally fatal outcome after a prolonged incubation period.

CWD infects wild and captive deer and elk in several US states and Canadian provinces. The highest reported disease prevalence is in a contiguous region, spanning parts of Colorado, Wyoming, and Nebraska (Figure 1), where the estimated disease prevalence is 5% in mule deer, 2% in white-tailed deer, and 0.5% in elk (*7*). CWD was first noted in captive deer at a research station in north-central Colorado near Fort Collins in the 1960s (*1*) and later in a wild elk near Estes Park in 1981 (*8*). No clear epidemiologic connections have been found between original cases and more recent cases, which suggests that unidentified risk factors may be contributing to the relatively wide and unpredictable geographic distribution of CWD (*2–4*).

Humans and animals can acquire TSEs by consuming prion-contaminated food. Outbreaks of prion disease include an epidemic of kuru among the cannibalistic Fore tribe of the New Guinea highlands (*9*) and an epizootic of BSE in the United Kingdom, caused by feeding to cattle protein supplements derived from prion-infected cattle offal (*10*). Food-based prion transmission between species also occurs, although a phenomenon known as the "species barrier" decreases transmission efficiency. In vitro studies (*11*,*12*) indicate that this natural barrier reduces human susceptibility to animal prion diseases, including CWD. As yet, no cases of human prion disease have been linked with CWD (*5*,*13–15*), and natural transmission of CWD to humans or traditional domestic livestock seems unlikely (*2*,*3*,*5*,*12*,*14*,*16*,*17*).

The otherwise reassuring molecular evidence of species barriers is clouded by the disparate experiences with scrapie and BSE as foodborne human pathogens. Scrapie

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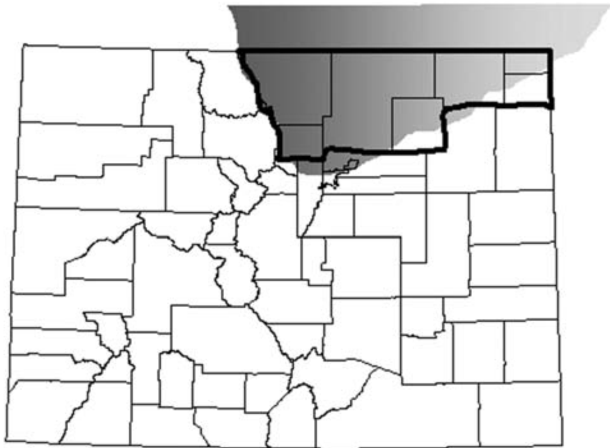

Figure 1. Location of chronic wasting disease (CWD)–endemic area in northeastern Colorado, USA (*7*) (gray shading) in relationship to Colorado counties regarded as CWD counties (bold outline) for purposes of comparing Creutzfeldt-Jakob disease rates and relative risk among resident human populations.

exposure has not been demonstrated to increase CJD risk, despite extensive human exposure (*18*). Conversely, in Britain the consumption of BSE-infected cattle led to an epidemic of variant CJD (vCJD), beginning in the mid-1990s (*19–23*). As of June 2006, however, only 161 cases of vCJD have been identified in the United Kingdom (*24*), despite the dietary exposure of millions of Britons to the BSE agent. In addition, recent studies indicate that large numbers of cases of vCJD are unlikely to occur in Britain in the future (*25*). Because the CWD agent is distinct from the BSE agent (*12*,*26–29*) and the type and degree of human exposure to these 2 agents differ, the risk for CWD transmission to humans cannot be directly extrapolated from the BSE and vCJD epidemics (*30*).

Because no completely reliable experimental animal model exists for testing the potential for CWD to cause CJD (*30*), human case investigations and epidemiologic studies remain valuable tools for assessing the potential risk associated with CWD exposure (*5*). Data that define human CWD exposure from consumption of infected deer or elk do not exist. However, in 7 northeastern Colorado counties (Boulder, Larimer, Logan, Morgan, Phillips, Sedgwick, and Weld) that are considered CWD-endemic areas (*7*) (Figure 1), the Colorado Division of Wildlife (CDOW) hunter license records indicate ≈75% (38,458 of 51,048) of deer and elk hunting licenses purchased from 1995 through 2001 were issued locally (CDOW, unpub. data), which suggests that county residents consume most regionally harvested game. Using Colorado death certificate data from 1979 through 2001, we modeled whether residence in a CWD-endemic county affected the risk-adjusted probability that a death is from CJD. We also examined whether the probability that a death is from CJD increased over time. To account for the possibility that CJD may have been misclassified, we also conducted sensitivity analyses using an expanded definition of event, similar to criteria used by Majeed et al. (*31*).

Materials and Methods

Study Population

Colorado death certificate data from 1979 through 2001 were used. Deaths during 1979–1998 and 1999–2001 were classified by the ICD-9 and ICD-10 codes, respectively. Sporadic CJD is extremely rare in persons <30 years of age (*32*), and vCJD cases have not been reported in patients <12 years (*33*). Therefore, we restricted all analyses to deaths occurring at ≥12 years, which provided 506,335 eligible deaths. We classified deaths as due to CJD if the codes 046.1 (ICD-9) or A81.0 (ICD-10) were listed as either the direct or contributory cause (events = 65).

Additional Colorado death certificate data used included age at death, sex, and marital status. We considered marital status as a predictor, because it may influence whether symptoms are recognized, which subsequently increases the likelihood of diagnosing CJD. Years of education data were not collected before 1989; therefore, this variable was not considered as a predictor. Figure 2 contains individual characteristics for persons who died in Colorado with CJD listed on the death certificate and smoothed population CJD rates (*34*).

In 1998, the Colorado Department of Public Health and Environment (CDPHE) initiated human prion disease surveillance. From 1998 through 2001, CDPHE identified 20 Colorado resident deaths consistent with prion disease (Table 1). For 10 of these 20 deaths, CJD was confirmed by examination of brain tissue from biopsy or autopsy specimens. Three deaths were classified as probable CJD; rapidly progressive dementia clinically consistent with prion disease was supported by nonspecific tests. Seven of the 20 CJD deaths were classified as suspected CJD because the diagnosis was made without autopsy, biopsy, or supportive testing. In 14 of these 20 deaths, the ICD code indicated CJD. Inexplicably, the remaining 6 patients who died (4 with confirmed CJD, 0 with probable CJD, and 2 with suspected CJD) had a medical record consistent with CJD, but the deaths were not coded as such. Three of these deaths (2 with confirmed CJD, 0 with probable CJD, and 1 with suspected CJD) were identified under our expanded definition. CDPHE review of the death certificates for the 6 misclassified deaths found that CJD was not reported as a cause of death and that the ICD-10 codes were consistent with the stated cause of death.

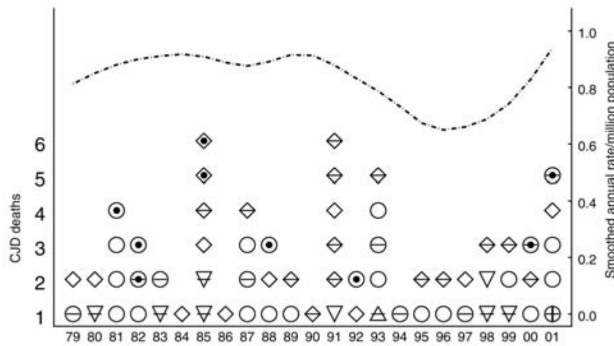

Figure 2. Colorado deaths 1979–2001 (left axis) with Creutzfeldt-Jakob disease (CJD) listed as the direct or contributory cause on the death certificate with age category at death identified by the plotting symbols 12-30 years (△), 31-55 years (▽), 56-70 years (○) and >70 years (◇). Indicators of CWD-endemic county resident (●), female (–), and black (X) or other (I) race are also identified. On the basis of these death certificate data and Colorado demographic data (*34*), we also display a smoothed CJD death rate per million population over time (right axis) (·–·).

Statistical Considerations

A review of Colorado death certificates identified 65 deaths with CJD listed on the death certificate from 1979 through 2001; from all causes, 81,916 and 424,419 persons ≥12 years died in the CWD-endemic and non–CWD-endemic counties, respectively. We were interested in testing whether the relative risk (RR) was greater than 1.0, where RR is the probability of a CJD death, given residence in a CWD-endemic county, divided by the corresponding probability in a non–CWD-endemic county. The RR is approximated by the odds ratio for a rare event such as death from CJD. Assuming a 2-sided χ^2 test with a significance level of 0.05, we had >85% power to detect an unadjusted RR of 2.47. Assuming 65 CJD deaths, this corresponds to 21 (2.56 cases/10,000 deaths) and 44 (1.04 cases/10,000 deaths) deaths in the CWD-endemic and non–CWD-endemic counties, respectively.

We conducted separate analyses for the primary predictors of interest: residence in a CWD-endemic county and death year. The RRs and 95% confidence intervals (CIs) were estimated by using logistic regression in SAS (SAS Institute, Cary, NC, USA). Covariates in the multivariable analysis were death age, sex, ICD classification, and marital status. The CWD county analysis also was adjusted for death year. For death year, ICD classification was considered as an effect modifier.

Results

Characteristics of Persons Who Died

Descriptive characteristics by CWD endemicity of county are presented in Table 2. Due to the large sample size, statistical significance was observed for all covariates, although most differences were relatively small. Those who died in CWD-endemic counties were more likely to be white, >70 years of age, and married or widowed rather than divorced.

Table 1. ICD codes and corresponding event classifications for human prion disease deaths of Colorado residents, 1998–2001*

Death y	CWD-endemic county	Sex	Death age, y	CJD status	ICD-9/ICD-10 codes†	Event classification
1998	No	F	>70	Probable	**046.1**	CJD/expanded
	Yes	M	>70	Confirmed	358.9, 507.0, 799.9	None‡
	No	F	31–55	Confirmed	**046.1**	CJD/expanded
	No	M	31–55	Confirmed	**046.1**	CJD/expanded
1999	No	F	31–55	Confirmed	**A81.0**	CJD/expanded
	No	M	56–70	Probable	**A81.0,** F17.1, I50.0, J44.9	CJD/expanded
	No	F	56–70	Confirmed	**G31.9**	Expanded
	No	F	>70	Suspected	**A81.0,** G20, **R29.8**	CJD/expanded
2000	No	M	56–70	Suspected	**A81.0**	CJD/expanded
	Yes	F	56–70	Confirmed	**A81.0,** E86, **G93.4**	CJD/expanded
	Yes	M	56–70	Confirmed	**G31.9,** J96.9	Expanded
	No	F	>70	Suspect	**A81.0,** J18.9	CJD/expanded
	Yes	F	56–70	Suspected	**G93.4,** F32.9, F41.9, J44.9	Expanded
2001	Yes	F	56–70	Confirmed	**A81.0,** I46.9	CJD/expanded
	No	M	56–70	Probable	**A81.0,** I48, I64	CJD/expanded
	No	F	56–70	Confirmed	**A81.0,** R53, R56.8	CJD/expanded
	No	M	>70	Suspected	**A81.0,** E87.8, **G96.9,** J18.9, N19, R99	CJD/expanded
	No	F	56–70	Confirmed	G20	None‡
	Yes	M	>70	Suspected	I10	None‡
	No	M	56–70	Suspected	A81.0, **I46.9**	CJD/expanded

*CWD, chronic wasting disease; CJD, Creutzfeldt-Jakob disease.
†**Boldface** codes qualify for at least 1 event definition (Appendix Table 1).
‡Codes reported for cases missed on all definitions; 358.9, unspecified myoneural disorder; 507.0, pneumonitis due to solids or liquids, specifically due to inhalation of food or vomit; 799.9, asphyxia, other unknown and unspecified; G20, Parkinson's disease; I10, essential (primary) hypertension.

Table 2. Characteristics of persons who died at ages ≥12 years, Colorado, 1979–2001*

	CWD-endemic counties, N = 81,916 (16.18%); no. (%)	Non–CWD-endemic counties, N = 424,419 (83.82%), no. (%)	p value†
Age at death, y			<0.0001
12–30	3,419 (4.17)	17,868 (4.21)	
31–55	9,367 (11.44)	5,8379 (13.76)	
56–70	16,182 (19.75)	94,684 (22.31)	
>70	52,947 (64.64)	253,476 (59.72)	
Unknown	1 (0.00)	12 (0.00)	
Education, y			<0.0001
Unknown‡	31,788 (38.81)	167,480 (39.46)	
<12	15,432 (18.84)	75613 (17.82)	
12	16,843 (20.56)	95,337 (22.46)	
13–16	13,853 (16.91)	70,115 (16.52)	
>16	4,000 (4.88)	15,874 (3.74)	
Sex			<0.0001
Female	40,665 (49.64)	204,864 (48.27)	
Male	41,251 (50.36)	219,554 (51.73)	
Unknown§		1 (0.00)	
ICD			0.0002
1979–1998 (ICD-9)	68,479 (83.60)	356,978 (84.11)	
1999–2001 (ICD-10)	13,437 (16.40)	67,441 (15.89)	
Marital status			<0.0001
Single	6,701 (8.18)	40,806 (9.61)	
Married	37,430 (45.69)	186,286 (43.89)	
Divorced	7,419 (9.06)	48,062 (11.32)	
Widowed	30,310 (37.00)	148,137 (34.90)	
Unknown	56 (0.07)	1,128 (0.27)	
Race			<0.0001
White	81,229 (99.16)	403,351 (95.04)	
Black	213 (0.26)	16,243 (3.83)	
Other	474 (0.58)	4,825 (1.14)	

*CWD, chronic wasting disease.
†χ^2 test.
‡Not recorded before 1989.
§Excluded from χ^2 test.

Univariate Analyses

Univariate analyses allowed us to describe event characteristics. Table 3 contains the univariate RRs and corresponding 95% CIs for available predictors. CWD-endemic counties contributed 16.18% of total deaths but only 13.85% of deaths with CJD listed on the death certificate (p = 0.61) (Figure 2). This finding corresponds to an unadjusted CJD rate in CWD-endemic counties of 1.10/10,000 deaths; in non–CWD-endemic counties, this rate was 1.32/10,000 deaths. We saw a slight decrease in CJD risk over time (p = 0.54); 43.08% of CJD deaths occurred before 1989. CJD risk decreased with age of death; 46.15% of CJD deaths occurred in persons 56–70 years of age and 40.00% in those >70 years. Given this younger population, predictable changes occurred in the distribution of marital status.

Multivariable Models

Table 4 contains the adjusted RRs for CWD endemicity of county and year of death. An RR >1.0 is consistent with the hypothesis of an increased risk for death from CJD, given residence in a CWD-endemic county. In the multivariable model, residing in a CWD-endemic county did not achieve statistical significance (RR 0.80, 95% CI 0.40–1.62). Death year remained not significant after adjusting for the additional covariates (for every 5-year increase, RR 0.92, 95% CI 0.73–1.16).

Death Rates

In addition to analyzing death certificate data, we computed annual age-standardized CJD death rates per million population (Figure 3) for the CWD-endemic and non–CWD-endemic counties (*34*). These population rates were age-standardized by using the 2001 age distribution for Colorado. Smoothed age–standardized rates were similar to the crude population rates for Colorado shown in Figure 2 (smoothed median 0.88, range 0.65–0.94). As expected, given the smaller population size, more variability was observed for these rates in the CWD-endemic counties (smoothed median 0.67 per million, range 0.11–1.37), than the non–CWD-endemic counties (smoothed median 0.96 per million, range 0.73–1.01). Overall, annual crude

Table 3. Univariate relative risk estimates of available risk factors for Creutzfeldt-Jakob disease, data from Colorado death certificates, 1979–2001*

Covariate	RR (95% CI), N = 506,335, events = 65
Age at death	p = 0.029
Units = 10 y	0.87 (0.78–0.99)
CWD county†	p = 0.61
No	1.0
Yes	0.83 (0.41–1.68)
Death year†	p = 0.54
Units = 5 y	0.95 (0.79–0.13)
Sex	p = 0.90
Male	1.0
Female	0.97 (0.60–1.58)
ICD-10	p = 0.83
No	1.0
Yes	1.07 (0.56–2.05)
Marital status	p = 0.0094
Widowed	1.0
Divorced	0.80 (0.23–2.85)
Married	2.86 (1.51–5.42)
Single	2.19 (0.86–5.57)
Unknown	–‡
Race	p = 0.94
White	1.0
Nonwhite	2.87 (0.40–20.6)

*RR, relative risk; CI, confidence interval; CWD, chronic wasting disease.
†Primary predictor.
‡Insufficient events.

population rates were slightly lower than age-standardized rates in both the disease-endemic counties (smoothed median 0.52 per million, range 0.09–1.29) and non–disease-endemic counties (smoothed median 0.85 per million, range 0.76–1.01) (data not shown).

Expanded Definition Analyses

We considered that if CWD were transmissible to humans, then it might be manifested with different signs and symptoms than typical sporadic CJD, resulting in misdiagnosis or classification under a different ICD code. Therefore, in addition to assessing data for CJD, we conducted sensitivity analyses using an expanded definition (Appendix). This definition increased the number of event codes to 29 ICD-9 and 30 ICD-10 (events 1,911). These codes corresponded to neurodegenerative syndromes in which signs are exhibited that are prominent in some forms of prion disease. To minimize false-positive results, we did not consider death from Alzheimer disease after 55 years of age as an event. In the United Kingdom, most vCJD cases have occurred in persons ≤55 years, with a median age at death of 28 years (range 14–74 years) (*35*). In addition, among patients >55 years, the incidence of age-related neurodegenerative diseases tends to obscure all but dramatic increases in conditions that may be attributable to CWD exposure. Therefore, to increase specificity, we also considered the expanded definition restricted to deaths in persons 12–55 years, which provided 89,033 eligible deaths (events 339). The adjusted expanded definition RRs for CWD endemicity of county and year of death are contained in Table 4. Under the expanded definition, we see a decrease in risk over time (p<0.0001), although significance is lost when the analysis is restricted to deaths of those who died before the age of 55 years.

Discussion

CWD has occurred in free-ranging deer and elk in northeastern Colorado for >25 years (*7*,*8*), so some persons likely have been exposed to the CWD agent. The human risk from exposure to CWD cannot be quantified because identifying exposed persons is not possible. The CDOW records indicate that ≈75% of deer and elk hunting licenses in 7 northeastern Colorado counties with high CWD prevalence are issued locally, which indicates that residents consume most game harvested in this region. Using Colorado death certification data from 1979 through 2001, we modeled the risk for a CJD death with CWD-endemic county residence as the exposure of interest. Similarly, we examined whether CJD deaths have increased overall. Given the possibility of misclassification of CJD and human TSEs, sensitivity analyses were conducted for expanded event definitions.

Human prion disease is rare, and increased risk due to CWD exposure appears to be subtle or nonexistent. No significant difference was found in the proportion of deaths from CJD in CWD-endemic versus non–CWD-endemic counties (adjusted RR 0.81, 95% CI 0.40–1.63). The upper CI value does not exclude an increased risk for CWD-endemic county residents, but it is inconsistent with a dramatic increase in that risk. Clearly, using residence in a CWD-endemic county as a surrogate for exposure has

Table 4. Results for primary predictors from multivariable analyses for CJD and expanded event definitions, data from Colorado death certificates, 1979–2001*

Covariate	CJD, N = 506,335, events = 65, RR (95% CI)	Expanded age 12–55 y, N = 89,033, events = 339, RR (95% CI)	Expanded, N = 506,335, events = 1,911, RR (95% CI)
CWD-endemic county	p = 0.55	p = 0.75	p = 0.48
No	1.0	1.0	1.0
Yes	0.81 (0.40, 1.63)	0.95 (0.69–1.31)	1.05 (0.93–1.18)
Death year	p = 0.48	p = 0.15	p < 0.0001
Units = 5 y	0.92 (0.73–1.16)	0.93 (0.84–1.03)	0.81 (0.77–0.84)

*CJD, Creutzfeldt-Jakob disease; RR, relative risk; CI, confidence interval; CWD, chronic wasting disease.

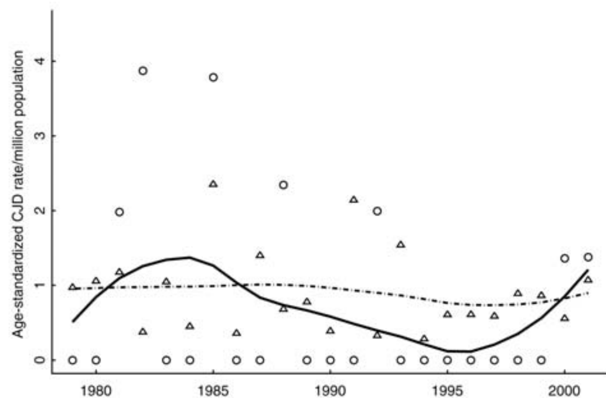

Figure 3. Annual age-standardized Creutzfeldt-Jakob (CJD) death rates per million population were calculated for chronic wasting disease (CWD)-endemic (○) and non-CWD-endemic (△) counties. Population rates were age-standardized to the 2001 age distribution for Colorado (*34*). We also display smoothed rates for the endemic (–) and non-CWD-endemic (·—·) counties.

several limitations. The most obvious is that many persons with no history of hunting or deer and elk consumption are included in the exposed cohort. Conversely, exposed persons may live outside these counties. Given the potentially long incubation periods associated with prion diseases, ample opportunity would exist for infected persons to move from disease-endemic counties before the onset of illness. Moreover, other unrecognized risk factors (i.e., familial CJD or iatrogenic sources of infection) could confound epidemiologic investigations.

When Colorado CJD rates were examined over time, no significant change in CJD deaths was demonstrated (5-year RR 0.92, 95% CI 0.73–1.16). Although finding that risk for deaths from neurologic disease decreased over time under our expanded event definition is reassuring (5-year RR 0.81, 95% CI 0.77–0.84), this analysis should be interpreted with caution. The findings could be influenced by the lack of specificity in the definition and the switch from ICD-9 to ICD-10 codes in 1999. After excluding deaths in persons >55 years of age in the expanded definition, the results became inconclusive.

Although an increase in CJD deaths has not been observed in Colorado, due to the long incubation periods of prion diseases, infected persons may not have had sufficient time for disease to develop or may have left the state before disease onset. Although the prevalence and known range of CWD has increased over time (*2–4*), CWD exposure may be decreasing due to ongoing efforts by the public health and wildlife management agencies (*2–4*). Active education about CWD has been ongoing in northeastern Colorado since 1995. This information campaign includes several specific recommendations to minimize exposure for hunters, meat processors, and taxidermists (*4*). In addition, since 1994, testing has been available for game harvested in CWD-endemic counties, thereby removing a proportion of harvested, CWD-infected deer and elk from the human food chain.

Identifying cases of human prion disease remains a challenge. How human prion disease linked to CWD would be manifested clinically or pathologically is not clear. The probability of CJD being accurately diagnosed is influenced by changes in diagnostic practices; access to medical care, particularly specialized neurologic consultations; and the availability of diagnostic testing, including autopsy and postmortem pathologic examinations. Improved case ascertainment should result from the establishment of the National Prion Disease Pathology Surveillance Center, which offers free diagnostic testing, complemented by increased Colorado surveillance efforts, including classifying human prion diseases as a physician-reportable condition, funding to pay for autopsies, and outreach to neurologists, pathologists, and coroners (*36*). Increased publicity about BSE, CWD, and human TSEs may have led to changes in diagnostic practices or case recognition, particularly in CWD-endemic areas due to a perceived association of CWD with human disease.

Death certificate data undoubtedly underestimate the prevalence of CJD. A limitation of this study is that diagnosed human TSE cases may not be recorded as CJD on the death certificate. Between 1998 and 2001, CDPHE surveillance identified 6 persons who died with a medical history of CJD for whom CJD was not reported on the death certificate; therefore, those deaths were not captured as events in our survey, although 3 of these deaths were identified under our expanded definition. Given that CDPHE surveillance overlapped only the past 4 years of our study, we could not reclassify these additional TSE deaths as CJD without introducing an obvious bias in the analysis of year of death. As a post hoc sensitivity analysis to our primary CJD endpoint in the CWD county analysis, we reclassified these 6 missed cases as events and computed the unadjusted RR. Although including these cases changed the CWD county point estimate from 0.83 (95% CI 0.41–1.68) to 1.16 (95% CI 0.64–2.12), the results remained highly nonsignificant ($p = 0.63$). The results of this sensitivity analysis should be interpreted with caution as increasing awareness of CJD is unlikely to be uniform across a state or country. In our analysis, this heterogeneous distribution may have resulted in an increase in misclassification bias over time, such that reclassifying cases that were not identified on the death certificate led to identifying an excess of CJD that was unrelated to exposure in the CWD-endemic counties.

Despite increased scrutiny, evidence of increased CJD in Colorado has not yet been demonstrated. Smoothed Colorado CJD annual rates based on death certificate data are consistently <1 case per million population (median

0.88, range 0.65–0.94). In the United Kingdom, which arguably has the most comprehensive human prion disease surveillance, the annual crude mortality rates from sporadic CJD per million population were 0.86, 1.08, 0.84, and 0.57 in England, Wales, Scotland, and Northern Ireland, respectively, over the period from 1990 to 2003 (*35*). The overall mortality rate from sporadic CJD from 1999 through 2002 in Australia, Canada, United Kingdom, and 8 additional European countries was estimated to be 1.39 per million population >10 years, although rates were highly variable across countries (0.48–2.23) (*37*). Approximately 84% of Colorado's population is >10 years of age (*38*) such that the comparable median is 1.05 (range 0.77–1.12). Thus Colorado's CJD rates appear comparable to or below other reported rates.

Continued case surveillance remains crucial for identifying and characterizing human prion disease (*5*). Recognition of CWD transmission to humans will likely require the identification of a human TSE patient with a history of exposure to deer or elk, evaluation of the clinical course and pathologic features at autopsy, and characterization of the prion strain in laboratory studies. Additional epidemiologic studies, such as a case-control study or cohort study that compares hunter license data with death certificate data, also should be conducted. Until the health risks from CWD can be fully ascertained, preventative steps to reduce exposure to the CWD agent and other animal prion disease agents (e.g., BSE, scrapie) should continue (*5*,*30*).

CWD has existed in wild deer and elk of northeastern Colorado for well over 2 decades. However, neither the number of CJD deaths in CWD-endemic counties nor the rate of CJD in CWD-endemic counties or in Colorado as a whole have increased. Although our findings are consistent with those of other studies that suggest no connection between CWD and human TSEs (*5*,*12*), we cannot exclude the possibility that an isolated case of human disease associated with the CWD agent has occurred or may yet occur. However, our findings do suggest that death from CJD remains rare in Colorado.

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Appendix Table 1. ICD-9 code classifications for expanded event definition, 1979–1998

Code definition	Age at death, y	ICD-9	Total with code* (12–55 y)†
Slow virus infection of the central nervous system	≥12	046	0 (0)
Creutzfeldt-Jakob disease	≥12	046.1	54 (8)
Other slow virus infection	≥12	046.8	0 (0)
Unspecified slow virus infection	≥12	046.9	0 (0)
Presenile dementia	12–55	290.1	8 (8)
Senile dementia, depressed or paranoid type	12–55	290.2	0 (0)
Cognitive or personality change of other type	≥12	310.1	0 (0)
Other cerebral degenerations	≥12	331	0 (0)
Alzheimer disease	12–55	331.0	18 (18)
Pick disease	≥12	331.1	19 (3)
Senile degeneration of the brain	≥12	331.2	55 (0)
Other cerebral degeneration	≥12	331.8	13 (8)
Other cerebral degeneration, unspecified	≥12	331.9	569 (35)
Other extrapyramidal disease and abnormal movement disorders	≥12	333	0 (0)
Other degenerative diseases of the basal ganglia	≥12	333.0	78 (8)
Myoclonus	≥12	333.2	4 (1)
Other choreas	≥12	333.5	11 (0)
Other and unspecified conditions	≥12	333.9	22 (1)
Spinocerebellar disease	≥12	334	0 (0)
Primary cerebellar degeneration	≥12	334.2	7 (0)
Other cerebellar ataxia	≥12	334.3	18 (1)
Cerebellar ataxia in diseases classified elsewhere	≥12	334.4	0 (0)
Other spinocerebellar disease	≥12	334.8	27 (6)
Unspecified spinocerebellar disease	≥12	334.9	66 (6)
Encephalopathy, unspecified	≥12	348.3	620 (183)
Sleep disturbances	≥12	780.5	7 (1)
Abnormal involuntary movements	≥12	781.0	22 (2)
Abnormality of gait	≥12	781.2	13 (1)
Lack of coordination	≥12	781.3	49 (0)

*Deaths may have more than 1 code reported.
†Total deaths with code for persons ages 12–55 y.

Appendix Table 2. ICD-10 code classifications for expanded event definition, 1999–2001*

Code definition	Age at death, y	ICD-10	Total with code† (12–55 y)‡
Creutzfeldt-Jakob disease	≥12	A81.0	11 (1)
Other atypical virus infection of CNS	≥12	A81.8	0 (0)
Atypical virus infection of CNS, unspecified	≥12	A81.9	0 (0)
Organic amnesic syndrome, not induced by alcohol and other psychoactive substances	≥12	F04	0 (0)
Early-onset cerebellar ataxia	≥12	G11.1	5 (1)
Late-onset cerebellar ataxia	≥12	G11.2	0 (0)
Other specified degenerative diseases of basal ganglia	≥12	G23.8	1 (0)
Degenerative disease of basal ganglia, unspecified	≥12	G23.9	2 (0)
Myoclonus	≥12	G25.3	2 (0)
Other specified extrapyramidal and movement disorders	≥12	G25.8	2 (1)
Extrapyramidal and movement disorder, unspecified	≥12	G25.9	5 (1)
Alzheimer disease with early onset	12–55	G30.0	0 (0)
Alzheimer disease with late onset	12–55	G30.1	0 (0)
Other Alzheimer disease	12–55	G30.8	0 (0)
Alzheimer disease, unspecified	12–55	G30.9	3 (3)
Pick disease	≥12	G31.0	9 (0)
Senile degeneration of brain, not elsewhere classified	≥12	G31.1	2 (0)
Other specified degenerative diseases of nervous system	≥12	G31.8	3 (0)
Degenerative disease of nervous system, unspecified	≥12	G31.9	56 (7)
Disorders of initiating and maintaining sleep [insomnias]	≥12	G47.0	1 (0)
Sleep disorder, unspecified	≥12	G47.9	1 (0)
Multisystem degeneration	≥12	G90.3	15 (1)
Encephalopathy, unspecified	≥12	G93.4	110 (34)
Disorder of central nervous system, unspecified	≥12	G96.9	5 (1)
Other and unspecified abnormal involuntary movements	≥12	R25.8	0 (0)
Ataxic gait	≥12	R26.0	2 (0)
Other and unspecified abnormalities of gait and mobility	≥12	R26.8	13 (0)
Ataxia, unspecified	≥12	R27.0	9 (0)
Other and unspecified lack of coordination	≥12	R27.8	0 (0)
Other and unspecified symptoms and signs involving cognitive functions and awareness	≥12	R41.8	3 (1)

*CNS, central nervous system.
†Deaths may have >1 code reported.
‡Total deaths with code ages 12–55.

the Health Sciences Center. Her primary research interests are the application of biostatistical methods to infectious disease data.

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Human Rotavirus G9 and G3 as Major Cause of Diarrhea in Hospitalized Children, Spain

Alicia Sánchez-Fauquier,* Vanessa Montero,* Silvia Moreno,* Monica Solé,* Javier Colomina,† Miren Iturriza-Gomara,‡ Ana Revilla,* Isabel Wilhelmi,§ Jim Gray,† and Gegavi/VIGESS-Net Group[1]

In Spain, diarrhea remains a major cause of illness among infants and young children. To determine the prevalence of rotavirus genotypes and temporal and geographic differences in strain distribution, a structured surveillance study of hospitalized children <5 years of age with diarrhea was initiated in different regions of Spain during 2005. Rotavirus was detected alone in samples from 362 (55.2%) samples and as a coinfection with other viruses in 41 samples (6.3%). Enteropathogenic bacterial agents were detected in 4.9% of samples; astrovirus and norovirus RNA was detected in 3.2% and 12.0% samples, respectively; and adenovirus antigen was detected in 1.8% samples. Including mixed infections, the most predominant G type was G9 (50.6%), followed by G3 (33.0%) and G1 (20.2%). Infection with multiple rotavirus strains was detected in >11.4% of the samples studied during 2005.

Group A rotaviruses are a major cause of severe diarrhea in infants. In developing countries, severe diarrhea caused by human rotavirus results in an estimated 500,000 to 608,000 childhood deaths annually; worldwide, it results in ≈2 million hospitalizations (*1*,*2*).

Rotaviruses belong to the *Reoviridae* family. Viral particles are nonenveloped, and triple-layered protein capsids enclose the genome of 11 dsRNA segments. The major protein in the central layer of the viral capsid is VP6, which determines 7 different groups of rotaviruses (A–G). The outer layer of the viral capsid is composed of 2 structural proteins, VP4 (encoded by gene 4) and VP7 (encoded by gene 7, 8, or 9, depending on the strain) (*3*). These 2 proteins carry the major antigenic determinants, which elicit neutralizing antibodies and are thought to be type specific. Group A rotaviruses are widespread in humans and animals and are subdivided into distinct genotypes, G and P (*4*). Epidemiologic studies of rotavirus infections are increasingly showing that a great diversity of rotavirus strains are cocirculating in the human population throughout the world. The most common genotypes of group A rotaviruses (≈90%), which cause dehydrating gastroenteritis in infants and young children worldwide, were G1P[8], G2P[4], G3P[8], and G4P[8]; G1P[8] is the most prevalent worldwide (*5*). However, other G genotypes are epidemiologically important, such as G5 in Brazil, (*6*,*7*), G9 and G10 in India (*8*,*9*), and G8 in Malawi (*10*).

In Spain, diarrhea remains an important cause of illness among infants and young children. A study conducted from 1998 through 2002 detected rotavirus in 1,155 (31%) of 3,760 specimens tested. G1 was the predominant genotype detected (53%), followed by G4 (24%), G2 (14%), G9 (6%), and G3 (2%) (*11*). The distribution of genotypes indicated a genotypic shift over time: G4 strains predominated (57%) from 1998 through 2000, whereas G1 gradually increased to account for 75% from 2000 through 2002 (*11*). Similar studies conducted in other regions of Spain indicated similar shifts in the prevalence of rotavirus genotypes (*12*,*13*).

We conducted structured surveillance among children with diarrhea who were hospitalized in 6 hospitals in Spain; our primary goals were to determine the prevalence of rotavirus diarrhea in hospitalized children, the G and P

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types among infecting rotavirus strains, and the temporal and geographic differences in strain distribution throughout the regions.

Materials and Methods

Hospitals and Patients

Stool samples were collected from children attending 6 public hospitals located in different healthcare areas throughout Spain. These hospitals intentionally represented the geographic, climatic, and ethnic diversity of Spain. Their respective catchment areas are shown in Table 1. The study was conducted between January 2005 and January 2006 and included children <5years of age who were hospitalized with acute gastroenteritis and from whom a stool sample was obtained.

Acute gastroenteritis was defined as ≥3 looser-than-normal stools within a 24-hour period or an episode of forceful vomiting and any loose stool. To enable reporting of test results to hospitals, stool specimens were labeled with the date of collection and a unique surveillance identification number. Permission for enrollment in the study was obtained from children's legal guardians, and ethical approval was obtained from the institutional review board of the Hospital de La Ribera.

Specimen Collection and Testing

Whole stool specimens were collected and transported immediately to hospital laboratories and stored at 4ºC until processing. All fecal samples were screened for enteropathogenic bacterial agents by conventional culture methods previously described (*14*).

Each month, specimens were sent to the reference laboratory (Viral Gastroenteritis Unit, National Center for Microbiology, Instituto de Salud Carlos III, Madrid, Spain). A 10% suspension in 0.1 mol/L phosphate-buffered saline (pH 7.2) was prepared and tested by reverse transcription (RT)-PCR for rotavirus, astrovirus, norovirus, and sapovirus (*11*,*15*,*16*) and by an immunochromatographic method for enteric adenoviruses (*14*).

Nucleic Acid Extraction and G/P Rotavirus Typing

Viral RNA was extracted from 250 µL of the 10% fecal suspension by using the guanidine isothiocyanate method and the Rnaid Spin Kit (BIO 101, Anachem Bioscience, Bedfordshire, UK) according to the manufacturer's instructions, with slight modifications (*16*). RNA was eluted in 50 µL of RNase-free distilled water and stored at –20ºC. To determine the G/P type patterns present in children hospitalized from 2005 through 2006, a total of 98 rotavirus strains were P typed. G and P rotavirus genotyping were performed by using RT-PCR methods as previously reported (*11*,*17*).

DNA Sequencing and Analysis

Rotavirus amplicons were genetically characterized by nucleotide sequencing of both strands of the amplified PCR products. These products were purified by using QIAquick PCR Purification kit (Qiagen, Valencia, CA, USA) and then sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an ABI automated sequencer (Applied Biosystems, model 3700). Data analysis was performed by using Clustal for multiple alignments and neighbor-joining and maximum parsimony methods for phylogenetic analysis (Bionumerics, Kortrijk, Belgium). Spanish strains were submitted to GenBank under accession numbers DQ440613 through DQ440624.

Results

Microbiology

A total of 656 hospitalized children were enrolled. Enteropathogenic bacterial strains were detected in 5.0% of samples (Table 2). Astrovirus and norovirus RNA was detected in 3.2% and 12.0% samples, respectively, and adenovirus antigen in 1.8% samples.

A total of 403 rotavirus strains were detected. Rotavirus was found alone in 362 (55.2%) samples but was found in another 41 samples (6.3%) as a coinfection with other viruses. The percentage of children with gastroenteritis

Table 1. Description of catchment area served by each hospital in the study*

Name	Complejo Hospitalario Universitario Albacete	Complejo Hospitalario San Pedro Alcántara	Complejo Asistencial León	Hospital Fuenlabrada	Hospital Severo Ochoa	Hospital La Ribera
Municipality (province)	Albacete	Cáceres	León	Fuenlabrada (Madrid)	Leganés (Madrid)	Alzira (Valencia)
Location within Spain	Southeast	West	Northwest	Center	Center	East
Climate	Continental, semiarid	Mediterranean, continentalized	Mediterranean, continentalized	Continental	Continental	Mediterranean
No. habitants	159,518	89,029	136,414	195,133	181,248	210,637
Birth rate (per 1,000)	10.0	8.05	6.9	11.8	11.9	11.3
No. children <5 y of age	6,362	2,867	3,776	9,210	8,627	9,479

*Predominant ethnic group in each area was Caucasian.

Table 2. Pathogens in children <5 years of age, hospitalized with gastroenteritis, by region, Spain, 2005–2006

Pathogen	No. samples (%)						
	Albacete	Cáceres	León	Fuenlabrada	Leganés	Valencia	Total samples (%)
Rotavirus	79 (55.7)	87 (54.4)	29 (36.7)	29 (50.9)	123 (62.7)	15 (68.2)	362 (55.2)
Astrovirus	0	4 (2.5)	0	1 (1.7)	0	0	5 (0.8)
Adenovirus	3 (2.1)	0	1 (1.3)	0	7 (3.6)	0	11 (1.7)
Norovirus	8 (5.6)	18 (11.3)	5 (6.3)	3 (5.3)	9 (4.6)	0	43 (6.5)
EB*	16 (11.3)	0	4 (5.1)	3 (5.3)	9 (4.6)	0	32 (4.9)
EB + adenovirus	1 (0.7)	0	0	0	0	0	1 (0.1)
Rotavirus + astrovirus	1 (0.7)	1 (0.6)	2 (2.5)	0	5 (2.6)	1 (4.5)	10 (1.5)
Rotavirus + norovirus	2 (1.4)	7 (4.4)	3 (3.8)	2 (3.5)	16 (8.2)	0	30 (4.6)
Norovirus + astrovirus	0	0	0	2 (3.5)	3 (1.5)	0	5 (0.8)
Norovirus + astrovirus + rotavirus	0	0	0	0	1 (0.5)	0	1 (0.1)
Negative specimens	32 (22.5)	43 (26.8)	35 (44.3)	17 (29.8)	23 (11.7)	6 (27.3)	156 (23.8)
Total specimens†	142 (21.6)	160 (24.2)	79 (12.0)	57 (8.7)	196 (29.9)	22 (3.4)	656

*EB, enteropathogenic bacteria.
†Percentages of isolations per total specimens in each region were 77.5, 73.1, 55.7, 70.2, 88.3, 72.7, and 76.2, respectively.

caused by rotavirus as unique agent ranged from 36.7% in Leon to 68.2% in Valencia (Table 2).

Rotavirus Characterization

G typing RT-PCR for rotavirus alone was performed on 362 samples positive for rotavirus but could not be determined in 10 (2.8%) samples. The G types detected, including mixed infections with multiple rotavirus strains, are shown in Table 3. Briefly, the most predominant G type was G9 (50.6%), followed by G3 (33.0%), G1 (20.2%), and G2 (7.1%); the least common G type was G4 (0.6%). G1, previously reported as the most common G type in Spain, was found in only 20.2% of rotavirus infections. With the exceptions of Valencia and Albacete, where G1 and G3, respectively, were the predominant G types, the results from all other regions showed a predominance of G9. However, even in these 2 areas, G9 was the second most common strain detected when cases with coinfection were added (26.7% and 31.6%, respectively).

Common G/P combinations, infrequent patterns, and mixed-infection combinations were all detected (Table 4). G9P[8] (40%) and G3P[8] (31%) were the most common combinations detected, but G types in combination with P[6] and P[9] were also detected.

Using DNA sequencing and phylogenetic analysis of partial sequences of the gene encoding VP7, we compared 2 G3 strains from this study with 9 G3 strains isolated previously in Spain. All G3 strains from Spain shared >99.0% homology and were more closely related to each other than to strains isolated in Italy, United Kingdom, India, and China.

Discussion

Genetically and antigenically diverse rotavirus strains cocirculate in humans. The prevalence of rotavirus genotypes varies according to location and time. Throughout the world, genotyping and serotyping studies have identified common cocirculating rotavirus types, and G1P[8], G2P[4], G3P[8], and G4P[8] are the predominant strains. However, from time to time, other less common genotypes, such as G9P[8], G5P[8], and G8P[6], have been predominant in various countries (*5*).

Table 3. Rotavirus G genotypes in children <5 years of age, hospitalized with gastroenteritis, by region, Spain, 2005–2006

Rotavirus G types	No. samples (%)						Total no. samples (%) N = 352†
	Albacete, n = 79	Cáceres, n = 86	León, n = 23	Fuenlabrada, n = 28	Leganés, n = 121	Valencia, n = 15	
G1*	20 (25.3)	10 (11.6)	4 (17.4)	4 (14.3)	21 (17.4)	11 (73.3)	71 (20.2)
G2*	2 (2.5)	4 (4.7)	2 (8.7)	4 (14.3)	13 (10.7)	0	25 (7.1)
G3*	36 (45.6)	36 (41.9)	5 (21.7)	11 (39.3)	27 (22.3)	0	116 (33.0)
G4*	0	1 (1.2)	0	0	1 (0.8)	0	2 (0.6)
G9*	25 (31.6)	52 (60.5)	13 (56.5)	13 (46.4)	71 (58.7)	2 (13.3)	178 (50.6)
G1 + G2	1 (1.3)	1 (1.2)	0	2 (7.1)	0	0	4 (1.1)
G1 + G9	1 (1.3)	0	0	0	2 (1.7)	1 (6.7)	4 (1.1)
G1 + G3	0 (0.0(	0	0	0	1 (0.8)	0	1 (0.3)
G2 + G9	0	0	0	0	3 (2.5)	0	3 (0.9)
G3 + G9	2 (2.5)	16 (18.6)	1 (4.3)	2 (7.1)	6 (5.0)	1 (6.7)	28 (8.0)

*Includes mixed infections.
†G typing for 10 samples could not be determined.

Table 4. G- and P-type combinations detected in 98 fully characterized strains

Genotype	No. samples	Pattern
G9 P[8]	39	Common (91%)
G3 P[8]	30	
G1 P[8]	15	
G2 P[4]	5	
G2 P[6]	1	Infrequent (3%)
G3 P[9]	1	
G9 P[6]	1	
G1+G9 P[8]	2	Mixed infections (6%)
G2+G9 P[8]	1	
G2+G9 P[4]	1	
G2+G9 and P[4]+P[8]	1	
G3+G9 and P[6]+P[8]	1	

In Spain, previous studies have identified G1P[8] and G4P[8] as the predominant cocirculating strains from 1996 through 2004 (*11*,*17*,*18*) (Table 5). However, in our study, conducted in 2005 and 2006, a major shift in the predominant strains was detected. G9P[8] and G3P[8] have become the predominant genotypes cocirculating in several regions of Spain, and infection with multiple rotavirus strains was detected in 11.4% of the cases studied.

Since its widespread introduction into the human population in 1995, G9P[8] has become one of the predominant viruses worldwide. In 2 separate studies conducted in Thailand (*19*,*20*), this genotype has been reported as the predominant virus circulating from 2000 through 2002 and in Brazil from 1999 through 2002 (*21*). G3P[8] has recently been reported as the predominant strain circulating in the Japanese population (*22*).

Less common G- and P-type combinations were also detected in this study. This finding may suggest either an earlier reassortment between animal and human strains, resulting in the emergence of strains such as G2P[6] and G3P[9], or zoonotic transmission to humans of an animal strain, as possibly occurred with G9P[6]. The VP4-genotypes P[6] and P[9] are reported to be associated with infection in pigs and cats, respectively. Although animal rotavirus strains replicate poorly in humans and person-to-person transmission is rare, the relatively high frequency of multiple infections detected in this study suggests that the opportunity for dual infection of a cell, and therefore reassortments, exists (*23*).

The main limitations of this study are having only 1 year of data, the minimal variations in the sampling schemes in each institution (frequency of sampling, test procedures, motivations of investigators), and the small sample size collected. Although the sampling strategy enabled monitoring for rotavirus in a large number of children, future studies with hospital-based surveillance should be initiated in different areas of Spain, and even Europe, with larger samples.

Morbidity rates worldwide and morbidity and mortality rates caused by diarrhea in developing countries remain high despite efforts to improve sanitary conditions, water quality, and the healthcare infrastructure. These high rates have driven efforts to develop a safe and effective rotavirus vaccine, and the World Health Organization has recognized that developing a vaccine is a priority for reducing infant deaths in developing countries. The level

Table 5. Predominant cocirculating rotavirus strains, Spain, 1996–2006*

Rotavirus G-type(s)	Year, %									Average (%) (n = 1,598)
	1996–1997† (n = 322)	1998–1999‡ (n = 141)	1999–2000‡ (n = 86)	2000–2001‡ (n = 200)	2001–2002‡ (n = 149)	2002–2003§ (n = 102)	2003–2004§ (n = 141)	2004-2005§ (n = 105)	2005–2006 (n = 352)	
G1 alone	68	18	27	70	79	79	79.5	50	17.1	54
G2 alone	0	1	9	23	17	16	1	11	5	9
G3 alone	2	1	12	0	0	0	17	7	24	7
G4 alone	29	68	39	3	1	0	0	26	0.6	19
G9 alone	0	11	13	3	2	3	1	5.4	39.5	9
G1+G2	0	0	0	0	1	0	1.5	0.2	1.1	0
G1+G4	1	1	0	1	0	2	0	0.4	0	1
G1+G9	0	0	0	0	0	0	0	0	1.1	0
G1+G3	0	0	0	0	0	0	0	0	0.3	0
G2+G9	0	0	0	0	0	0	0	0	0.8	0
G3+G9	0	0	0	0	0	0	0	0	7.7	1
Undet.¶	0	0	0	0	0	0	0	0	2.8	0
Total samples	322	141	86	200	149	102	141	105	362	1,608

*χ^2 test showed annual variations in G1, G2, G3, G4, and G9 prevalence rotavirus types and in G3 + G9 mixed infections (χ^2 = 15.50 with 8 degrees of freedom, p>0.95).
†Adapted from Reference *11*.
‡Adapted from Reference *17*.
§Adapted from Reference *18*.
¶Undet., undetermined.

and type of protection in rotavirus disease is poorly understood, although neutralizing antibody responses are thought to be type specific. Because these responses are associated with VP7 and VP4 viral proteins, establishing the G and P genotypes of strains circulating in the human population is important. Currently, 2 candidate rotavirus vaccines are undergoing clinical trials. A multivalent vaccine directed against G1, G2, G3, G4, and P[8] and a monovalent vaccine to G1P[8] have been developed (*24*,*25*). Homotypic protection has been demonstrated for both vaccines, but the degree to which they cross-protect against less common G- and P-type combinations not included in the vaccine formulations has yet to be established, and the importance of genotype-specific protection against rotavirus disease is still under discussion (*26,27*). Considering that G9 rotavirus type has emerged as one of the most common rotavirus genotypes in humans around the world, and it is becoming very prevalent in some countries, future rotavirus vaccine candidates will need to provide adequate protection against disease caused by G9 viruses. Therefore, surveillance of regional networks must be maintained to document rotavirus strain distribution and prevent the appearance of new strains or new variants that could escape immune protection induced by an outdated vaccine.

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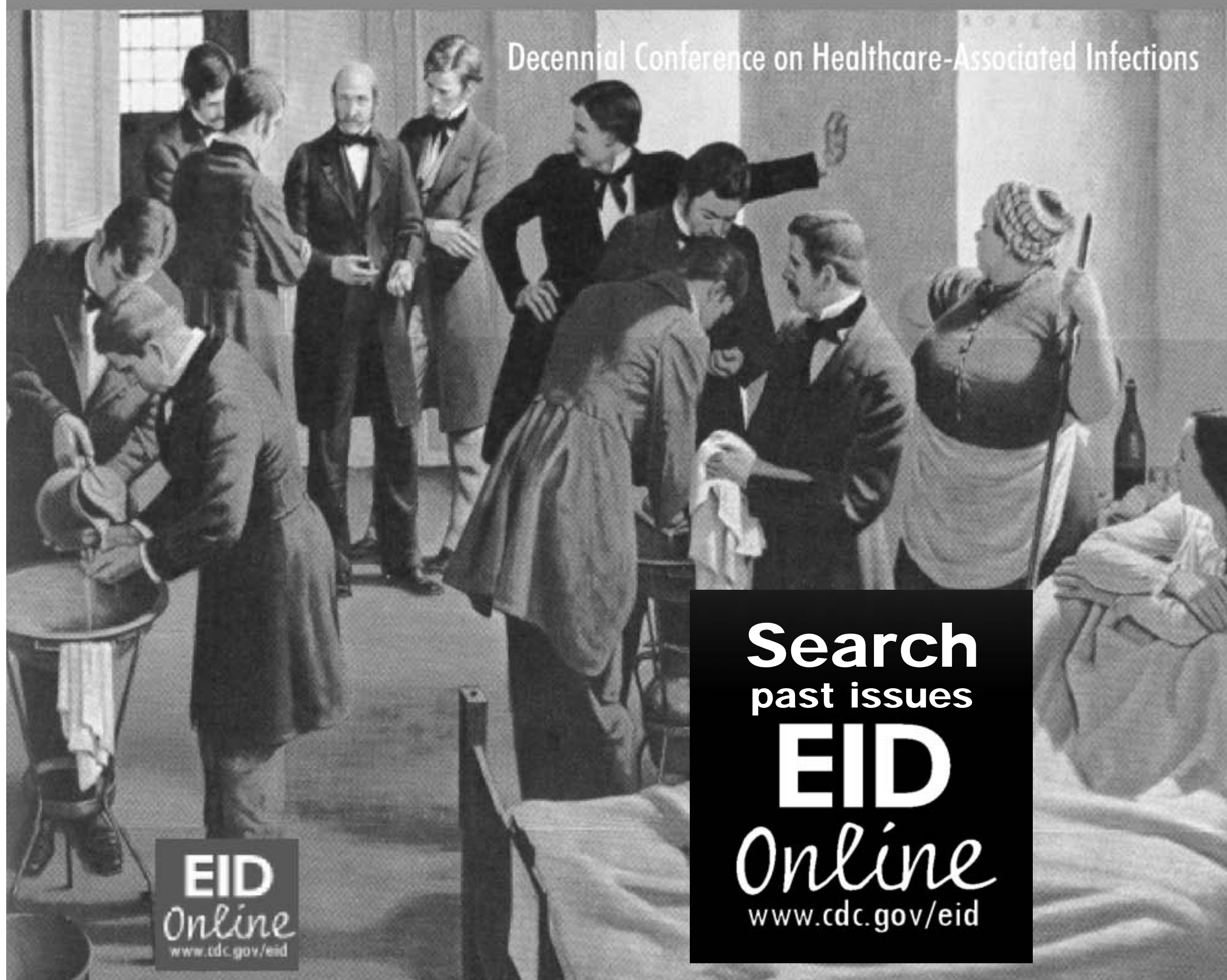

Low Frequency of Poultry-to-Human H5N1 Virus Transmission, Southern Cambodia, 2005

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To understand transmission of avian influenza A (H5N1) virus, we conducted a retrospective survey of poultry deaths and a seroepidemiologic investigation in a Cambodian village where a 28-year-old man was infected with H5N1 virus in March 2005. Poultry surveys were conducted within a 1-km radius of the patient's household. Forty-two household flocks were considered likely to have been infected from January through March 2005 because >60% of the flock died, case-fatality ratio was 100%, and both young and mature birds died within 1 to 2 days. Two sick chickens from a property adjacent to the patient's house tested positive for H5N1 on reverse transcription–PCR. Villagers were asked about poultry exposures in the past year and tested for H5N1 antibodies. Despite frequent, direct contact with poultry suspected of having H5N1 virus infection, none of 351 participants from 93 households had neutralizing antibodies to H5N1. H5N1 virus transmission from poultry to humans remains low in this setting.

From its identification in poultry in the People's Republic of China in 1996 and outbreak among commercial farms and live poultry markets in Hong Kong in 1997 (*1*), highly pathogenic avian influenza A (H5N1) virus has become an unprecedented epizootic and spread to domestic poultry and wild bird populations in Asia (*2*,*3*), the Middle East, Europe, and Africa (*4*). This epizootic has affected farmers and the agricultural industry, claimed human lives, and raised the specter of a global influenza pandemic, perhaps even beyond the scale of the devastating 1918 "Spanish" influenza pandemic (*5*).

In Cambodia, highly pathogenic H5N1 was first reported in poultry in January 2004 (*6*). Of 92 poultry outbreaks that year, 15 were confirmed by isolation of H5N1 viruses (*7*). During the first 4 months of 2005, 4 fatal human H5N1 cases were detected in Kampot Province, southeast Cambodia (*8*). These human cases occurred contemporaneously with unreported outbreaks of high deaths among chicken flocks throughout Kampot Province. However, H5N1 virus was confirmed in both a person and poultry in only 1 area of Kampot Province, a village in Banteay Meas District, ≈20 km from the Vietnam border and 15 km from the household of the first confirmed human H5N1 case-patient in Cambodia.

The patient from Banteay Meas District was a 28-year-old male farmer in whom a low-grade fever and dizziness developed on March 17, 2005. Approximately 1 week before he became sick, chickens at his home suddenly began dying. His family reported that he plucked at least 1 chicken and ate poultry that had died of illness suggestive of H5N1 disease. He may also have collected dead birds. On the third day of his illness, nonproductive cough, shortness of breath, and watery diarrhea developed. Two days later, he was transported to a Phnom Penh hospital. His condition rapidly deteriorated, and he died the next day despite mechanical ventilation and inotropic support. H5N1 virus infection was confirmed by reverse transcriptase (RT)–PCR from blood; tracheal aspirates; and nasopharynx, throat, and rectal swab specimens collected during his hospitalization (Institut Pasteur – Cambodia, unpub. data).

The farmer's rural village provided a setting in which we could study the epidemiologic features of H5N1 virus

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in poultry and humans. We report results of a retrospective study of poultry deaths and an H5N1 antibody seroepidemiologic investigation among residents of this village in Banteay Meas District, Kampot Province, Cambodia.

Methods

Retrospective Poultry Death Survey

We conducted an immediate investigation in response to notification of the confirmed human H5N1 case in Banteay Meas District. From March 25 through 27, 2005, all households located within a radius of 1 km from the H5N1 case-patient's household were mapped and positioned with a hand-held global positioning system (Garmin, Olathe, KS, USA). We collected information on illness suggestive of H5N1 among animals in each household by interviewing the head of the family with a standardized questionnaire. Households where the head of the family was not at home or could not be found were omitted.

A household chicken flock was considered likely to have been infected by H5N1 virus during the previous 6 months if all of the following characteristics were reported: flock death >60%, 100% case-fatality ratio, and sudden death of young and mature birds within 1 or 2 days of becoming sick. We collected sick poultry and carcasses for H5N1 virus testing. Cloacal swabs of 10 to 14 randomly selected, live, healthy poultry were also collected from each household where birds remained.

Seroepidemiologic Investigation

We conducted a seroepidemiologic investigation June 3–7, 2005, ≈2 months after the village reported high poultry deaths. It consisted of interviews of household members with a standardized 39-question questionnaire on demographic information and data on specific exposures to animals and the environment during the last 12 months; a 5-mL venous blood specimen was also collected from participants. Four investigation teams of 3 members each visited all households in 4 different directions, starting from the household of the confirmed human case-patient, until 300 participants were enrolled in the study. Each household was visited once, and no further attempts were made to interview absent adult household members. The sample size was estimated to have a 95% chance of detecting ≥1 seropositive person, if one assumes a 2% prevalence of H5N1 antibodies in the village. Written informed consent was obtained from adults or from a parent or guardian for children <18 years of age. The study was approved by the Cambodian Ethics Committee.

Laboratory Methods

All animal samples were placed into viral transport medium in sterile tubes in the field, kept cold, and transported daily to the National Animal Health Laboratory of the Ministry of Agriculture, Forestry and Fisheries in Phnom Penh. Cloacal specimens and organ samples from sick and dying poultry were tested for influenza A with an indirect fluorescent antibody assay. Positive results were forwarded to the virology unit of the Institut Pasteur in Cambodia for confirmation with real-time RT-PCR to detect H5 viral RNA. Human blood specimens were centrifuged, and sera were aliquoted and frozen at –80°C. Sera were shipped on dry ice to the Hong Kong Government Virus Unit Laboratory for detection of H5N1 neutralizing antibodies by microneutralization assay and confirmatory Western blot assay. Serologic evidence of H5N1 virus infection was defined as an H5N1 neutralizing antibody titer ≥80 with a confirmatory Western blot assay (*9*).

Statistical Analyses

The position codes of all surveyed households were entered into ArcGIS version 9.0 (ESRI Systems, Redlands, CA, USA). We used the space-time scan statistic to determine the cluster of households most likely to have been affected by H5N1 virus in the previous 6 months. Analysis was performed with SaTScan version 5.1.3 (*10*); cases were assumed to follow a Poisson distribution. The space-time statistic is defined by a cylindrical window with a circular geographic base and height pertaining to time. The window is moved in space and time for each geographic location and size. We obtained several overlapping cylinders of different sizes and shapes covering the study area; each cylindrical window reflected a possible cluster. The most likely cluster is the one least likely to have occurred by chance, according to the maximum likelihood ratio test statistic. Individual and household data were entered into EpiData version 3.02 (EpiData Association, Odense, Denmark) and validated with a duplicate data file. STATA version 8.0 (StataCorp LP, College Station, TX, USA) was used for all statistical analyses. Odds ratios were estimated with bivariate logistic regression. Independent associations between demographic and behavioral data and households that were likely to be affected by H5N1 in poultry were also analyzed by logistic regression models. We accounted for the cluster effect of households with STATA's "cluster" option for logistic regression, which specifies that observations are independent across households but not within households. For multivariate analysis, variables with a p value ≤0.1 were retained in the models. Selected variables whose correlation coefficient was >0.4, which indicates colinearity between these variables, were not included in the logistic regression model.

These investigations were conducted as a collaborative effort between the Cambodian Ministry of Health and Ministry of Agriculture, Forestry and Fisheries, Institut Pasteur in Cambodia, the World Health Organization,

Hong Kong Public Health Department, the Centers for Disease Control and Prevention, and the Food and Agricultural Organization of the United Nations.

Results

Poultry Deaths in the Village

Of 194 households located within 1 km of the H5N1 patient's house, 163 (84%) had occupants who were home at the time of the survey. No household refused to participate.

Among interviewed households, 155 (95%) raised chickens (median 20, range 1–80 per household) and 52 (32%) raised ducks (median 4, range 1–50). Fifty households (31%) reared both ducks and chickens. Sixty-three households owned pigs (range 1–4 animals). From January 1 through March 26, 2005, 102 (66%) of 155 households reported deaths of chickens. Of these households, 73 (72%) recorded deaths during the last 4 weeks of this 3-month period (Figures 1 and 2). The median poultry flock death ratio was 90% (range 4%–100%). According to our definition, 42 households were likely to have had an outbreak of H5N1, for an overall attack rate of 27% among households with chickens. Flock death ratio was >80% in 31 of these households. The initial mean flock size in households likely to have had H5N1 in chickens was significantly larger than in households without chicken deaths (31 vs. 20, p = 0.003).

Eleven households with a high likelihood of H5N1 (35%) in chickens also owned ducks, although only 2 of these described simultaneous deaths of ducks: 1 reported a duck flock death ratio of 80%, the other a ratio of 100%. Seven other households reported high levels of death among duck flocks (>60%), but the number of deaths among chickens did not suggest H5N1. Overall, raising ducks with chickens was not associated with deaths in chickens (p = 0.57).

Cloacal swabs were collected from 28 chickens and 14 ducks. Specimens from 2 sick chickens were positive for H5N1 by RT-PCR. These chickens belonged to a household located ≈50 m from the household of the confirmed human H5N1 case-patient. The owner of the sick chickens reported that the farmer with H5N1 virus infection spent daylight hours in his compound.

The space-time scan statistic detected a significant cluster of 25 (60%) households with an overall relative risk of 7.9 (log likelihood ratio 34.1, p = 0.001) (Figure 1). The cluster was confined to the period from February 25 through March 26, with a radius of 444 m, which contained both the household of the confirmed H5N1 case-patient and the house with the 2 H5N1-infected chickens.

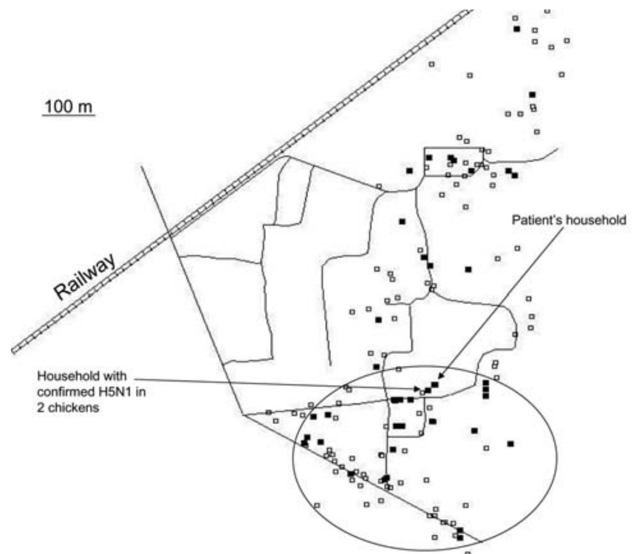

Figure 1. Clustering of 25 households with a high likelihood of avian influenza H5N1 (35%) in chickens, February 27–March 26, 2005, southern Cambodia. White squares indicate visited households without chicken deaths, and black squares indicate households with a chicken flock that was probably infected with H5N1 virus. The cluster is indicated by the circle.

Seroepidemiologic Survey Findings

Among 93 households that were surveyed, 351 persons participated and 3 refused. An average of 4 people resided in each household, the median age was 23 years (range 1 month–81 years), and 150 (42.7%) of the sample were male; 207 (59%) were farmers of both crops and livestock. The rest of the participants were students (29.3%), had no stated occupation (18.8%), or were construction or factory workers (0.9%). Reflecting the rural setting and the common means of livelihood, ownership of animals, including poultry, was high (Table 1). The number of households with chickens decreased by 17.5% (p<0.001) after the outbreak, while the number of households that possessed ducks and pigs remained similar (–15.7% and 5.7%, p = 0.43 and 0.74, respectively).

Because many households owned poultry or pigs, a substantial proportion of the surveyed population had regular, high-intensity contact with these animals in the 12 months before the survey; this contact included collecting, processing, and eating sick birds or birds that had recently died when H5N1 viruses were thought to be circulating among flocks in the village. Despite this finding, none of the villagers interviewed reported having a febrile or respiratory illness during the same period, and none of the 351 participants had neutralizing antibodies suggestive of H5N1 virus infection on microneutralization assay.

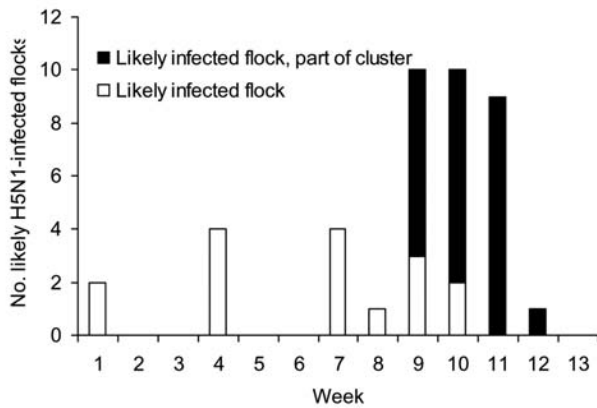

Figure 2. Infected flocks detected by week of reporting period, January 1–March 26, 2005, southern Cambodia. Cluster refers to households within the circle on Figure 1.

We compared exposures of residents from households with a high probability of having had an outbreak of H5N1 in their chicken flock (n = 96) with occupants from households where no chickens died (n = 166) (Table 1). Bivariate analysis showed that households that purchased live poultry in the preceding year were almost 4× more likely to have had H5N1 in their flock than households that did not buy live chickens. In contrast, certain behavior by household members appeared to reduce the risk for H5N1 virus infection in their household flock by half (and the difference remained significant after controlling for poultry purchasing): cleaning cages or stalls, cleaning up poultry feathers, and handling live poultry (Table 1). Slaughtering chickens was not a significant risk factor after controlling for exposures that were significant on multivariate analysis (Table 2).

Discussion

The primary finding of our investigations is that transmission of H5N1 viruses from infected poultry to humans appears to have been low in a rural Cambodian population with confirmed and suspected H5N1 poultry outbreaks, and where a human H5N1 case occurred during 2005. This finding is consistent with other studies that have described low frequency of H5N1 neutralizing antibody among healthcare workers and household contacts since 2004 (*11–13*). Moreover, our findings suggest that asymptomatic and mild H5N1 virus infections had not occurred in the population we investigated. Although H5N1 virus was only isolated from birds in 1 household, evidence suggested an H5N1 outbreak among numerous chicken flocks in the village beginning ≈6 weeks before the human H5N1 case was confirmed. Given that direct contact with poultry and poultry products was common among people in this village, a high proportion of villagers were presumably exposed to H5N1 virus. Genetic analysis of H5N1 virus isolates from the infected farmer and 2 chickens confirmed that no reassortment with elements of human influenza A viral genome had occurred (*2*). We cannot say why illness developed in 1 person when family, neighbors, and many other villagers who reported similar poultry exposures did not have any evidence of H5N1 virus infection.

The seroprevalence of H5N1 antibody in the Cambodian population surveyed was substantially lower than was found in poultry workers in Hong Kong in 1997 with the same microneutralization assay (*13*). Although

Table 1. Comparison of animal exposures in households in which the likelihood of H5N1 outbreak among chickens was high (n = 96) and in households in which no chickens died (n = 166)*

Exposure	Likely H5N1 outbreak households, n (%)	No chicken death households, n (%)	OR	p value	95% CI
Handle live poultry	56 (58.3)	125 (75.3)	0.46	0.025	0.23–0.91
Feed poultry	63 (65.6)	130 (78.3)	0.53	0.093	0.25–1.11
Clean poultry cages and stalls	32 (33.3)	85 (51.2)	0.48	0.015	0.26–0.87
Collect sick poultry	53 (55.2)	86 (51.8)	1.15	0.623	0.66–1.98
Collect dead poultry	55 (57.3)	92 (55.4)	1.08	0.769	0.64–1.82
Pluck feathers from dead poultry	43 (44.8)	88 (53.0)	0.72	0.290	0.39–1.32
Handle poultry organs	51 (53.1)	107 (64.5)	0.62	0.100	0.36–1.09
Transport live poultry	3 (3.1)	7 (4.2)	0.73	0.647	0.19–2.77
Collect or transport feces	37 (38.5)	78 (47.0)	0.71	0.162	0.44–1.15
Raise hatchlings	1 (1.0)	0	NA		
Collect and sell eggs	36 (37.5)	69 (41.6)	0.84	0.585	0.46–1.55
Clean up poultry feathers	31 (32.3)	82 (49.4)	0.49	0.013	0.28–0.86
Clean up poultry feces	36 (37.5)	80 (48.2)	0.67	0.291	0.32–1.41
Slaughter chickens	30 (31.3)	68 (41.0)	0.40	0.017	0.18–0.87
Slaughter ducks	17 (17.7)	40 (24.1)	0.52	0.638	0.03–8.52
Attend cockfight	9 (9.4)	17 (10.2)	0.65	0.474	0.19–2.15
Purchase live poultry	11 (11.5)	6 (3.6)	3.45	0.028	1.14–10.44
Purchase killed poultry	2 (2.1)	0	NA		

*OR, odds ratio; CI, confidence interval; NA, not applicable.

Table 2. Unconditional logistic regression models comparing households in which the likelihood of H5N1 outbreak among chickens was high and households in which no chickens died (n = 262)

Variable	Adjusted odds ratio	p value	Adjusted for variable nos.
1. Clean up cages/stalls	0.5	0.02	4, 5
2. Feed poultry	0.5	0.11	4, 5
3. Handle live poultry	0.4	0.03	4, 5
4. Purchase live poultry	4.5–4.9	<0.01	5 and (1 or 2 or 6)
5. Slaughter chickens	0.7–0.9	0.23–0.58	4 and (1 or 2 or 6)
6. Clean up poultry feathers	0.5	0.01	4, 5

this assay is a highly specific and strain-dependent test and may not detect neutralizing antibody to antigenically distinct H5N1 virus strains, the 2004 Vietnam clade 1 H5N1 virus strain used in our investigation was antigenically identical and genetically similar to H5N1 viruses circulating among poultry in Cambodia in 2005 (*2*). Nonetheless, a small chance exists that previous H5N1 virus infection might have been missed if levels of H5N1 neutralizing antibodies had declined; for example, some human influenza virus infections do not invariably result in a detectable serum antibody response (*14*). However, the kinetics of the H5N1 antibody response in humans is similar to that of human influenza A virus (*15*). In addition, recent evidence shows that the H5N1 virus results in a systemic infection likely to produce a neutralizing antibody response (*16*,*17*). When these results are considered with our findings from a sample of >350 people, H5N1 virus infection was not likely to have occurred without any circulating immunoglobulin G, even 2 months after symptom onset. H5N1 virus transmission to humans may be rare because it only occurs in exposed persons with unique host susceptibilities and a predisposition to an abnormal inflammatory response that results in severe and fatal outcomes, rather than causing a broad spectrum of illness with mild disease and subclinical infections. Nevertheless, further research is needed to better understand the immune response to H5N1 virus infection in humans.

Our investigations also found that some animal-handling practices, such as handling poultry, cleaning poultry stalls and cages, and collecting poultry feathers appeared to reduce the chance that a flock would be infected by H5N1 virus. This finding is in contrast to findings that handling dead or sick poultry is a risk factor for (individual) human H5N1 illness (*18*,*19*). We speculate that some practices that encourage backyard birds to stay close to the house, such as handling poultry, may be protective by reducing the distance healthy fowl need to roam to forage for food, thereby reducing interactions with wild and other domestic birds and contact with contaminated environments. Cleaning poultry stalls and cages and collecting poultry feathers may indicate a better level of general hygiene practices and may also decrease the risk by removing potentially infectious materials. These findings may highlight the value of educating farmers about hygienic animal-handling practices.

Other behavior appeared to modify the risk for H5N1 in domestic fowl. Purchasing live poultry increased risk. The introduction of new birds that may be harboring disease is an obvious threat to a flock. Anecdotal evidence suggests that farmers in Kampot Province had responded to the culling without compensation control measures by attempting to sell birds at the first sign of sickness during outbreaks in 2005 (World Health Organization, unpub. data). Additionally, poultry trade with Vietnamese farmers was common and persisted despite the introduction of laws prohibiting such cross-border trafficking. Southern Vietnam has, like Cambodia, experienced mass H5N1 outbreaks among domestic fowl in the last few years (World Health Organization, unpub. data), and the village examined in this survey was 20 km from the border.

Our results need to be interpreted in the context of several limitations. The interview process involved a recall period of 12 months and did not document more temporally relevant exposures immediately before or during the outbreak. The long recall period may increase the probability of exposure to potential risk factors, making households with and without suspected H5N1 virus infection in flocks more similar. In addition, the temporal association between behavioral risk factors and H5N1 virus infection in poultry was difficult to establish. On the basis of our counterintuitive findings, further risk factor studies should address this potential bias. The classification of households as likely having H5N1 in their flocks was based on a case definition suggested by Food and Agriculture Organization veterinarians. Without confirmation of H5N1 virus infection, we do not know the sensitivity and specificity of this definition and cannot quantify the degree of misclassification, if any. The study did not collect information about the origins of each flock, how long birds had belonged to each household, or internal movements of individual birds and flocks within the village. This limitation hindered mapping the likely circulation of H5N1 virus among poultry in the village, and other factors related to poultry transmission may have been missed. Although we did not record and map households we did not visit, this bias is likely to have been nondifferential because the proportions of nonvisited households were similar for all 4

investigation teams that surveyed in 4 different directions. Finally, specimen collection had limitations. Higher concentrations of virus exist in the trachea of infected birds rather than in the cloacae (*20*), but tracheal sampling was not performed in this survey because it was not acceptable to local farmers.

This study provides evidence of the low transmissibility of the H5N1 virus from infected poultry to humans, even in circumstances in which human-poultry interactions are regular and intense. In this instance, human H5N1 virus infection manifested as a single case of severe illness without any evidence that the virus could cause either mild disease or asymptomatic infection. However, our findings are limited to the investigation period of 2005. As H5N1 viruses continue to circulate and evolve among poultry, poultry-to-human transmission of H5N1 viruses could increase. Extensive investigations should be routinely conducted for all H5N1 outbreaks among humans and animals to monitor the nature and extent of bird-to-human or human-to-human transmission of H5N1 viruses. Additional seroepidemiologic investigations should be conducted to assess the ongoing risk for bird-to-human transmission of H5N1 among rural and other human populations.

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Health Benefits, Risks, and Cost-Effectiveness of Influenza Vaccination of Children

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We estimated cost-effectiveness of annually vaccinating children not at high risk with inactivated influenza vaccine (IIV) to range from US $12,000 per quality-adjusted life year (QALY) saved for children ages 6–23 months to $119,000 per QALY saved for children ages 12–17 years. For children at high risk (preexisting medical conditions) ages 6–35 months, vaccination with IIV was cost saving. For children at high risk ages 3–17 years, vaccination cost $1,000–$10,000 per QALY. Among children not at high risk ages 5–17 years, live, attenuated influenza vaccine had a similar cost-effectiveness as IIV. Risk status was more important than age in determining the economic effects of annual vaccination, and vaccination was less cost-effective as the child's age increased. Thus, routine vaccination of all children is likely less cost-effective than vaccination of all children ages 6–23 months plus all other children at high risk.

The risks of influenza, both annual epidemic and pandemic, have caused national policymakers to ask whether routine influenza vaccination should be expanded to healthy children and adults of all ages. During the 2003–04 influenza season, reports of >150 influenza-associated deaths among children and unprecedented demand for influenza vaccine highlighted the need to reevaluate the nation's influenza vaccination policies regarding children (*1–3*). The Advisory Committee on Immunization Practices (ACIP) and the American Academy of Pediatrics Red Book Committee have recommended that all children 6–23 months of age and their household contacts should receive annual influenza vaccination, and this policy has been widely adopted (*4*,*5*). In February 2006, the ACIP recommended expanding routine influenza vaccination to children 24–59 months old (L. Pickering, pers. comm.). However, a vote to recommend routine influenza vaccination for all children and adults failed. ACIP members requested more information on the projected health benefits, cost-effectiveness, and logistical issues regarding expanding influenza recommendations to other age groups.

Should influenza vaccine be routinely used in older children without high-risk conditions? This question is especially relevant, given the introduction of live, attenuated (intranasal) influenza vaccine (LAIV) for healthy persons ages 5–49 years, which has a higher list price than the inactivated (injected) vaccine but is also potentially more effective (*6*,*7*). Previous studies have examined the cost-effectiveness of influenza vaccination in various age groups (*8–10*). However, these studies may have been overly optimistic regarding vaccination because they assumed high influenza attack rates, low estimates for vaccination costs, or both, thereby limiting their use in policy decisions. Further, no studies have been published that compare the cost-effectiveness of live attenuated influenza vaccines with that of inactivated influenza vaccines.

Our objective in this study was to evaluate the cost-effectiveness of routine annual influenza vaccination, comparing live attenuated with inactivated vaccines, for children in varying age and risk groups from 6 months to 17 years. This is the first study to include measures of health preferences that allow results to be calculated in quality-adjusted life years (QALYs).

Methods

Using standard software (TreeAge Pro 2004 Software, release 6, Treeage Software, Williamstown, MA, USA),

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we created a mathematical model (decision tree) to estimate the effect of influenza vaccination on outcomes and costs among children. The decision tree evaluated 3 options: 1) no vaccination; 2) inactivated influenza vaccine (IIV); and 3) live, attenuated influenza vaccine (LAIV). It estimated costs and outcomes for influenza-related illness for children stratified into 10 subgroups by age (6–23 months, 2 years [24–35 months], 3–4 years, 5–11 years, 12–17 years) and risk status (high risk or not at high risk). Children were defined as being at high risk for influenza-related complications due to preexisting medical conditions (*4*). Since most costs and consequences related to influenza occur during a single influenza season, the time horizon of the decision tree was 1 year. Costs and effects of long-term outcomes (death, long-term sequelae of influenza-related hospitalization or vaccine adverse events), however, were also included in the model. A simplified schematic of the decision tree is shown in Figure 1. Input parameters for probabilities, costs, and outcomes were described by using probability distributions (Tables 1–3).

Natural History of Influenza

Influenza-related outcomes included in the decision tree were episodes of influenza illness (medically attended or not), otitis media, mild pneumonia (and other complications treated on an outpatient basis), hospitalizations (with and without long term sequelae), and deaths. Event rates, by age and risk group, were derived from the published literature and were supplemented by expert opinion where data were limited or unavailable (Table 1) (*11–15*) (see Online Appendix for full list of references; available from http://www.cdc.gov/ncidod/eid/vol12no10/05-1015.htm).

Vaccine Effectiveness

Inactivated vaccine was considered for all 10 subgroups, and LAIV was considered only for children not at high risk. Children 6 months to 4 years were included as a theoretical intended population for LAIV, although LAIV is currently licensed in the United States only for children 5–17 years. The most likely estimate for vaccine effectiveness against symptomatic influenza illness was lower for IIV (0.690) than the most likely estimate for LAIV (0.838) (Table 1) (*16*,*17*).

Vaccination-related Adverse Events

Adverse events attributable to influenza vaccination included in the decision tree were medically-attended episodes of injection site reactions, systemic reactions (defined as fever within 2–7 days of vaccination), anaphylaxis, and Guillain-Barré syndrome (Table 1). Probabilities of medically-attended vaccine adverse events were highest for the youngest age group and declined as age increased.

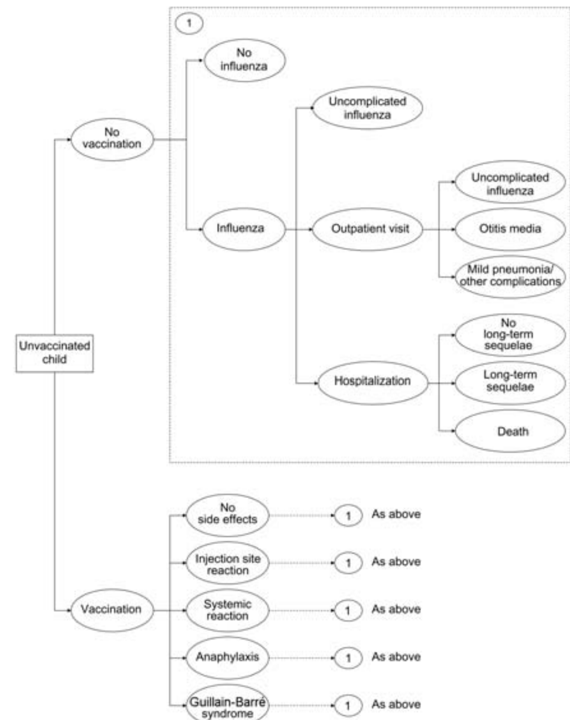

Figure 1. Influenza cost-effectiveness model. Each health state in the model is associated with a cost and quality adjustment from Table 1.

Costs

Costs included direct medical costs (physician visits, over-the-counter remedies, prescription drugs, diagnostic tests, and hospitalizations) and opportunity costs (parent time costs) for physician visits (Table 2). All costs were adjusted to 2003 dollars by using the medical cost component of the Consumer Price Index (available from http://data.bls.cgi-bin/surveymost?cu). Costs of physician visits for influenza illness, influenza-related hospitalizations, and vaccination-related adverse events were calculated by using a large database that reported payments for health insurance companies in the mid-Atlantic states of the United States (The Medstat Group, Ann Arbor, MI, USA). Vaccination costs included vaccine dose costs, administration costs, medical attention for vaccine adverse events, and, if an additional visit was required, parent time costs (*18*,*19*).

It is recommended that first-time recipients aged 6 months through 8 years receive 2 doses of influenza vaccine (*4*). Some children will also require additional office visits to be vaccinated with either 1 or 2 doses. The mean number of additional office visits needed to deliver the recommended number of doses ranged from 1.07 for children ages 6–23 months to 0.75 for children ages 5–17 years (Table 2) (*20*).

Table 1. Model inputs and assumptions for children ages 6 months to 17 years*†

Variable	Most likely estimate	Range for sensitivity analysis
Influenza illness attack rate (annual)		
6-23 mo	0.157	0.02–0.35
2 y	0.155	0.02–0.35
3–4 y	0.155	0.01–0.35
5–11 y	0.08	0.01–0.18
12–17 y	0.06	0.01–0.14
Probability of outpatient visit for child with influenza illness‡		
6–23 mo	0.5	0.17–0.83
2 y	0.47	0.15–0.81
3–4 y	0.43	0.12–0.78
5–11 y	0.28	0.11–0.5
12–17 y	0.24	0.06–0.5
Probability of otitis media for child with medically attended influenza illness		
6–23 months	0.63	0.33–0.8
2 y	0.58	0.27–0.8
3–4 y	0.39	0.17–0.6
5–11 y	0.23	0.05–0.5
12–17 y	0.15	0.01–0.4
Probability of nonhospitalized pneumonia or other outpatient complication for child with medically attended influenza illness§		
6–23 mo	0.2	0.04–0.5
2 y	0.15	0.02–0.4
3–4 y	0.15	0.02–0.4
5–11 y	0.11	0.02–0.3
12–17 y	0.08	0.01–0.2
Hospitalizations for pneumonia or other respiratory conditions due to influenza/10,000 children not at high risk¶		
6-23 mo	28.3	1.9–80.0
2 y	17.1	0–56.8
3–4 y	8.0	0–35.4
5–11 y	3.1	0–16.0
12–17 y	3.1	0–14.9
Probability of long-term sequelae following influenza-related hospitalization‡	0.01	0.001–0.03
Probability of death during influenza-related hospitalization	0.0009	0–0.002
Vaccine effectiveness in preventing influenza illness#		
IIV	0.69	0.4–0.9
LAIV	0.838	0.6–0.96
Probability of medically attended vaccination-related adverse events		
Injection site reaction		
6-23 mo	0.008	0.002–0.017
2 y	0.003	0.001–0.006
3–4 y	0.002	0.0004–0.003
5–11 y	0.001	0.0002–0.002
12–17 y	0.0003	0.0001–0.001
Systemic reaction (fever)**		
6–23 mo	0.013	0.001–0.025
2 y	0.011	0.0008–0.020
3–4 y	0.009	0.0007–0.016
5–11 y	0.004	0.0003–0.008
12–17 y	0.003	0.0002–0.005
Anaphylaxis	0.00000025	0–0.000001
Guillain-Barré syndrome	0.000001	0–0.00001

*IIV, inactivated influenza vaccine; LAIV, live, attenuated influenza vaccine.
†Refer to online appendix (available at http://www.cdc.gov/ncidod/EID/vol12no10/05-1015.htm) for list of references used to derive model inputs.
‡Estimates for children not at high risk are shown. Probabilities are estimated to be twice as high for children at high risk for influenza-related complications.
§Estimates for healthy children shown. Probabilities are estimated to be ≤5 times as high for children at high risk for influenza-related complications. Most likely estimates for children at high risk are 1.6 times as high as for healthy children.
¶Children at high-risk are estimated to be hospitalized at 3–6 times the rate of children not at high risk.
#Assumes vaccine is poorly matched with circulating virus 1 in 10 years (i.e., vaccine effectiveness is assumed to be 0 years with a poor match).
**Definitions and follow-up for incidence of fever following vaccination vary by study. Rates are 2x higher for children at high risk.

Table 2. US $ cost inputs for children ages 6 months to 17 years*

Cost input	Most likely estimate	Range for sensitivity analysis
Influenza-related costs		
OTC medications†	$3	
Physician visit, uncomplicated influenza‡	$27	$10–$78
Physician visit, otitis media§	$78	$23–$197
Physician visit, non-hospitalized pneumonia§	$179	$62–$715
Hospitalization¶	$4,300	$1,300–$34,500
Long-term sequelae following influenza-related hospitalization#	$625,000	$0–$1,000,000
Vaccination costs		
Per dose, IIV** (children <3 y)	$9.56††	1×–4× base case
Per dose, IIV** (children ≥3 y)	$6.86††	
Per dose, LAIV**	$12.89††	$10–$25
Administration costs (0–2 visits) ‡‡	$25	$10–$40
Parent time costs, per visit§§	$32	$0–$64
Total vaccination costs¶¶		$30–$110
6-23 mo	$79	
2 y	$66	
3-4 y	$59	
5-11 y	$49	
12-17 y	$49	
Vaccination-related adverse events		
Physician visit for injection site reaction##	$61	$30–$683
Anaphylaxis***	$2,700	$52–$13,754
Guillain-Barré syndrome†††	$23,360	$6,700–$78,900

*OTC, over the counter; IIV, inactivated influenza vaccine; LAIV, live, attenuated influenza vaccine.
†Vary by age, calculated by costing out recommended dose of acetaminophen for average weight in each age group.
‡Only a proportion of children with influenza illness are assumed to make a physician visit. ICD-9 codes: 487 and 487.0.
§Costs of physician visits for otitis media and nonhospitalized pneumonia vary by age group and include prescription medications and laboratory tests. Costs shown are for children 6–23 mo. See online appendix (available from http://www.cdc.gov/ncidod/EID/vol12no10/05-1015-app.htm) for full list of costs by age.
¶ICD-9 codes: 460-466, 471-474, 477, 478, 480-483, 490-496, 506-508, 510, 511, 514, 518, 519.
#Includes costs of lifetime care and special education.
**2 doses assumed for children <5 y receiving their first influenza vaccination.
††Vaccine dose costs are based on 2004 CDC-negotiated prices. Cost for children <3 y assumes thimerosal-free vaccine is used.
‡‡Current Procedural Terminology (CPT) codes: 99211 for an additional visit ($19.95) and 90471 for a vaccination at an existing visit ($10.37).
§§Each physician visit is assumed to take 2 hours of parent time valued at an average hourly wage rate of $15.54.
¶¶Proportion of children requiring 2 doses is 1 for 6–23 mo, 0.5 for 2 y, and 0.33 for 3–4 y. No. of additional visits needed to administer recommended number of vaccine doses is 1.07 for 6–23 mo, 0.91 for 2 y, and 0.84 for 3–4 y, and 0.75 for 5-17 y. See online appendix for more details. Total vaccination costs in Table 1 exclude average costs for vaccination-related adverse events of $0.18–$2.05 per child, depending on age and risk status.
##5-minute visit, CPT code 99211.
***ICD-9 codes: 999.4, 995.0, 995.6x.
†††ICD-9 code: 357.0.

Health Outcomes

The model projected 4 different outcomes that were averted through vaccination: influenza episodes, hospitalizations, deaths, and QALYs. The QALY is a measure of net health effects that takes into account the health benefits of averted influenza cases as well as the health costs of vaccination-related adverse events. We obtained QALY valuations for each health event in the model from 2 studies (Table 3) (*21*,*22*). In these studies, adult respondents were asked for the amount of time that they were willing to give up from the end of their life to prevent a specific temporary health state in a hypothetical child. We explicitly asked respondents to include a parent's reduction in quality of life associated with a child's illness and any time lost from work to care for a sick child in the time-tradeoff valuation; therefore, time-tradeoff amounts could exceed the length of the event. QALYs lost due to severely disabling long-term sequelae after influenza hospitalization, such as acute necrotizing encephalopathy with irreversible neurologic damage, were also included (*23*,*24*). An influenza-related death was assumed to result in the loss of 1 QALY for each year of life lost.

Analysis Plan

The primary outcome measure was the incremental cost-effectiveness ratio in dollars per QALY. Secondary measures included costs and events averted per 1,000 vaccinated children, dollars per influenza-related event avoided, dollars per hospitalization avoided, and dollars per death averted. One-way sensitivity analyses were conducted on all variables, in which the impact on the average $/QALY saved was examined by altering each variable

Table 3. Quality adjustments for influenza-related illness and vaccination-related adverse events (decrease in utility)*†

Event	Most likely estimate	Range for sensitivity analysis
Episode of influenza	0.005	0.002-0.009
Otitis media	0.042	0.023-0.065
Nonhospitalized complications (pneumonia)	0.046	0.027-0.071
Hospitalization, pneumonia	0.076	0.054-0.100
Anaphylaxis	0.020	0.006-0.041
Guillain-Barré syndrome	0.141	0.092-0.199

*Quality adjustments are included in model as a one-time decrement in utility for each temporary health state. For example, an episode of influenza results in a 1-time loss of 0.005 quality-adjusted life years (QALYs). Utility losses were calculated by dividing discounted time-traded off by respondent's discounted life expectancy. See online appendix for references.
†Average life span used to calculate total QALYs lost due to lifelong sequelae and death was 77.9–78.2 years, depending on child's current age.

within the range of given values (Table 1). Two-way sensitivity analyses were conducted on variables for which the results were most sensitive in 1-way sensitivity analysis. A scenario analysis examined the effect of excluding parent time costs. Another scenario analysis evaluated the effect of using an alternative calculation for quality adjustments, which used the duration of the health state in the child as the denominator instead of respondent's life expectancy. To evaluate the effects of parameter uncertainty, a probabilistic sensitivity analysis was conducted. For the probabilistic sensitivity analysis, each variable was assigned a distribution of possible values, assuming β distributions for probabilities and quality adjustments and log-normal distributions for costs (a technical appendix listing details of all distributions is available online at http://www.cdc.gov/ncidod/eid/vol12no10/05-1015.htm). For each run in the probabilistic sensitivity analysis, the model randomly picked a different value for each variable from its associated distribution. The model was run 10,000 times for each age-risk and vaccine combination separately. Cost-effectiveness acceptability curves show the cumulative probabilities of the cost-effectiveness ratio, from $0 to $250,000/QALY, due to vaccinating children against influenza (i.e., the curves display the probability of the cost-effectiveness being less than or equal to a given $/QALY amount), by using the results from the Monte Carlo analysis.

Results

Health Benefits, Risks, and Costs

Influenza vaccination with IIV was projected to be cost saving for children ages 6–35 months at high risk and to require a net investment for all other age and risk groups. The projected benefits of vaccination decreased as age increased (Table 4). For example, routine influenza vaccination with IIV of children 6–23 months old not at high risk was projected to avert 108 influenza events per 1,000, while vaccination of 5- to 11-year-old children was projected to averted 55 influenza events per 1,000. Among the 5- to 11-year-olds not at high risk, the projected number of influenza-related hospitalizations and deaths averted by influenza vaccination with IIV was only one tenth the number averted among 6- to 23-month-old children not at high risk. For children not at high-risk age ≥5 years, the number of projected influenza events averted was similar for LAIV and IIV.

QALYs and Cost-Effectiveness

All vaccination strategies had net positive QALYs gained, which indicated that the health benefits of vaccination outweighed the risks (Table 4). For children not at-high risk, the QALYs gained by IIV use were highest for 6- to 23-month-olds at 3.0 QALYs gained per 1,000 children vaccinated, compared with 2.4 per 1,000 children vaccinated for 2-year-olds and 1.7 per 1,000 children vaccinated for 3- to 4-year-olds. For children at high risk, the QALYs gained by IIV use ranged from 1.3 to 7.2 per 1,000 children vaccinated, depending on age group. For children 5–17 years old not at high risk, LAIV use would result in slightly higher QALYs gained because of the vaccine's higher effectiveness at 0.5 to 3.7 per 1,000 children vaccinated.

IIV use was cost saving among children at high risk ages 6 months to 2 years (Table 5). For children <5 years not at high risk as well as children at high risk in all age groups, IIV use had mean cost-effectiveness ratios of <$30,000 per QALY saved. Cost-effectiveness ratios based on dollars per influenza episode averted yielded patterns similar to the ratios of dollars per QALY saved, ranging from cost savings for children at high risk ages ≤2 years to $1,070 per influenza case averted for healthy 12- to 17-year-olds (Table 5).

Using base-case vaccine purchase prices for LAIV and IIV (Table 2), LAIV for children ages 5–17 years not at high risk had higher mean net costs and yielded greater mean health benefits than IIV. The cost-effectiveness ratios for LAIV were $72,000 per QALY gained for 5- to 11-year-olds and $109,000 per QALY gained for 12- to 17-year-olds (Table 5).

Sensitivity Analyses

Probabilistic sensitivity analysis provided confidence intervals for projected costs and events averted and quasi-confidence intervals for cost-effectiveness ratios. By using

Table 4. Health benefits, risks, and costs of influenza vaccination of varying age and risk groups per 1,000 children vaccinated, means* (95% CI†)

	Net costs, $‡	Influenza events averted (all)	Influenza hospitalizations averted	Deaths averted	Vaccine adverse events incurred§	QALYs gained
Using inactivated influenza vaccine						
Non-high risk						
6–23 mo	37,000 (–119,000 to 98,000)	108 (16–276)	2 (0.2–6)	0.002 (0–0.007)	21 (8–47)	3.0 (0.4–9.0)
2 y	43,000 (–40,000 to 83,000)	107 (15–276)	1.2 (0.1–4.2)	0.001 (0–0.005)	14 (5–30)	2.4 (0.3–7.3)
3–4 y	47,000 (2,000–78,000)	107 (15–276)	0.6 (0–2.3)	0.0005 (0–0.0025)	10 (3–24)	1.7 (0.2–5.2)
5–11 y	44,000 (21,000–68,000)	55 (8–142)	0.2 (0–0.7)	0.0002 (0–0.0008)	5 (2–11)	0.6 (0.1–1.7)
12–17 y	44,000 (22,000–68,000)	41 (6–104)	0.2 (0–0.6)	0.0002 (0–0.0008)	3 (1–8)	0.4 (0–1.1)
High risk						
6–23 mo	–74,000 (–552,000 to 83,000)	108 (16–276)	5.5 (0.5–6.5)	0.005 (0–0.020)	32 (11–56)	7.2 (0.8–23.2)
2 y	–22,000 (–292,000 to 72,000)	107 (15–276)	3.5 (0.2–11.4)	0.003 (0–0.013)	25 (7–44)	5.4 (0.6–17.2)
3–4 y	2,000 (–212,000 to 70,000)	107 (15–276)	2.2 (0.1–9.1)	0.002 (0–0.010)	19 (5–37)	4.0 (0.4–13.1)
5–11 y	12,000 (–125,000 to 59,000)	55 (8–142)	1.3 (0.1–3.9)	0.001 (0–0.005)	9 (3–24)	1.6 (0.2–5.6)
12–17 y	13,000 (–120,000 to 59,000)	41 (6–104)	1.3 (0.1–3.9)	0.001 (0–0.005)	6 (1–15)	1.3 (0.1–4.5)
Using LAIV¶						
Non-high risk						
6–23 mo	*32,000 (–155,000 to 99,000)*	*132 (20–319)*	*2.4 (0.3–7.2)*	*0.002 (0–0.009)*	*13 (3–32)*	*3.7 (0.5–10.5)*
2 y	*42,000 (–59,000 to 85,000)*	*130 (20–322)*	*1.4 (0.1–4.9)*	*0.001 (0–0.005)*	*11 (2–26)*	*2.9 (0.4–8.5)*
3–4 y	*50,000 (–3,000 to 83,000)*	*130 (20–322)*	*0.7 (0–2.7)*	*0.0006 (0–0.0029)*	*9 (2–23)*	*2.1 (0.3–6.1)*
5–11 y	48,000 (22,000–73,000)	67 (10–166)	0.3 (0–0.8)	0.0002 (0–0.0010)	4 (1–10)	0.7 (0.1–1.9)
12–17 y	49,000 (23,000–73,000)	50 (8–120)	0.3 (0–0.7)	0.0002 (0–0.0010)	3 (0–7)	0.5 (0.1–1.3)

*CI, confidence interval; QALYs, quality-adjusted life years; LAIV, live, attenuated influenza vaccine.
†Bootstrapped.
‡Net costs = costs of vaccination minus savings from disease averted.
§Includes medically attended injection site reactions, systemic reactions, anaphylaxis, and Guillain-Barré syndrome.
¶Italics indicate that LAIV is not licensed for children <5 y.

base case assumptions, results for LAIV are slightly more favorable than IIV (compared to no vaccination), and vaccination with LAIV is the preferred strategy. However, probabilistic sensitivity analysis indicated projected results were similar for IIV and LAIV.

Cost-effectiveness acceptability curves generated through probabilistic sensitivity analysis are very similar for IIV and LAIV (Figures 2A and 2C). The probability that the cost-effectiveness of IIV would be ≤\$30,000/QALY ranged from 51% to 89% for all children ages 6–23 months and 2 years (Figure 2). For children of any age not at high risk, the probability that IIV would be cost saving was ≤10% (Figure 2A). For children aged ≥5 years not at high risk, the probability that the cost-effectiveness of LAIV, compared with no vaccination, would be ≤\$30,000 per QALY gained was 5%–13% (Figure 2C).

In 1-way sensitivity analyses, cost-effectiveness ratios were most sensitive to changes in influenza illness attack rate, hospitalization rates, total vaccination costs, and vaccine effectiveness (Figure 3). Cost-effectiveness ratios varied notably with total costs of vaccination for IIV. For example, if total costs of vaccination were doubled for children ages 6–23 months who were not at high risk, cost-effectiveness ratios increased (worsened) by a factor of 3. We included costs for parent time associated with taking a child to the physician's office to receive influenza vaccination, which accounted for 41%–66% of total vaccination costs (Table 1). Excluding these time costs resulted in cost-effectiveness ratios approximately half of those reported in Table 5 and Figure 2. Using an alternative calculation for quality adjustments resulted in higher estimates of the projected number of QALYs gained through vaccination. For

Table 5. Incremental cost-effectiveness ratios for use of inactivated and live attenuated influenza vaccination in varying age and risk groups compared to no vaccination, mean (2.5% and 97.5% bootstrapped percentiles)*

	Using inactivated influenza vaccine		Using live, attenuated influenza vaccine†
Age group	Children not at high risk	Children at high risk	Children not at high risk
$ per influenza episode averted‡			
6–23 mo	340 (CS–4,690)	CS (CS–4,090)	*240 (CS–3,890)*
2 y	400 (CS–3,990)	CS (CS–3,620)	*330 (CS–3,340)*
3–4 y	440 (10–3,590)	20 (CS–3,410)	*440 (CS–3,170)*
5–11 y	800 (180–5,850)	210 (CS–5,560)	720 (170–5,290)
12–17 y	1,070 (250–7,780)	310 (CS–7,360)	980 (240–7,070)
$ per hospitalization averted‡			
6-23 mo	19,000 (CS–350,000)	CS (CS–132,000)	*14,000 (CS–287,000)*
2 y	37,000 (CS–633,000)	CS (CS–232,000)	*30,000 (CS–522,000)*
3–4 y	84,000 (1,000–2,587,000)	1,000 (CS–750,000)	74,000 (CS–2,227,000)
5–11 y	202,000 (38,000–1,929,000)	9,000 (CS–310,000)	184,000 (35,000–1,629,000)
12–17 y	206,000 (43,000–1,768,000)	10,000 (CS–304,000)	188,000 (40,000–1,575,000)
$ per death averted‡			
6–23 mo	22 m (CS–1,109 m)	CS (CS–342 m)	*16 m (CS–880 m)*
2 y	42 m (CS–1,762 m)	CS (CS–591 m)	*34 m (CS–1,435 m)*
3–4 y	98 m (1 m–6,840 m)	1 m (CS–1,873 m)	*86 m (CS–5,991 m)*
5–11 y	234 m (32 m–5,993 m)	10 m (CS–876 m)	212 m (32 m; 5,331 m)
12–17 y	238 m (37 m–5,607 m)	12 m (CS–892 m)	217 m (34 m; 5,007 m)
$ per quality-adjusted life-year saved			
6–23 mo	12,000 (CS–208,000)	CS (CS–85,000)	*9,000 (CS–167,000)*
2 y	18,000 (CS–217,000)	CS (CS–100,000)	*15,000 (CS–180,000)*
3–4 y	28,000 (1,000–290,000)	1,000 (CS–130,000)	*25,000 (CS–236,000)*
5–11 y	79,000 (15,000–682,000)	7,000 (CS–260,000)	72,000 (14,000; 592,000)
12–17 y	119,000 (24,000–1,040,000)	10,000 (CS–367,000)	109,000 (22,000; 888,000)

*CS, cost savings; m, million.
†Numerator does not include productivity losses.
‡Italics indicate that live, attenuated influenza vaccine is not licensed for children <5 y.

example, projected gains in QALYs for children not at high risk were 12%–37% higher than in the base case.

Two-way sensitivity analysis on influenza illness rate and vaccine effectiveness (IIV) resulted in changes in the cost-effectiveness ratio from a decrease in 11% for a season with a high influenza illness rate (35%) and high vaccine effectiveness (IIV) to an increase of more than a factor of 30 for seasons with a low influenza illness rate and low vaccine effectiveness (IIV). Combining a high influenza illness rate (35%) with low vaccine effectiveness (IIV) resulted in cost-effectiveness ratio ≈3 times base case results ($43,000/QALY) for children not at high risk ages 6–23 months. Two-way sensitivity analyses on influenza illness rate and total vaccination costs (IIV) had similar results, ranging from a decrease in 6% in the cost-effectiveness ratio to an increase 25 times as high as the base case for seasons with low influenza illness rate and high vaccination costs (IIV). Two-way sensitivity analyses for vaccination costs and effectiveness yielded a 20% lower cost-effectiveness ratio for low costs and high effectiveness of vaccination (IIV) to 7 times the base case for high costs and low vaccine effectiveness (IIV).

Discussion

Major Findings

We found that influenza vaccination of children, both those at high risk and those not at high risk, in all age groups would have health benefits that outweigh vaccine adverse events as measured by QALYs for both IIV and LAIV. For children not at high risk ages 6 months–4 years, we estimated that influenza vaccination with IIV would cost ≤$25,000 per QALY saved. In comparison, other routinely-used preventive interventions, such as pneumococcal conjugate vaccination, cost an average of $7,000/QALY for children <2 years (*22*,*25*); driver-side air bags cost $30,000/QALY (*26*), and costs of other vaccinations range from cost-savings to $150,000/QALY (*27–30*).

Live, attenuated influenza vaccine is currently approved for children ≥5 years of age who are not at high risk, but not for children <5 years or for children at high risk. At a price per dose <$20, its cost-effectiveness ratios are similar to those for IIV. This analysis likely presents a relatively conservative estimate of the potential benefits of LAIV, because we did not include its potentially greater effectiveness against antigenically drifted strains or likely higher effectiveness with 1 dose of vaccine in previously unvaccinated children <9 years (*4*,*6*).

The sensitivity of the results to the influenza illness attack rate (which varies from season to season and from community to community) and to vaccine effectiveness indicates that the cost-effectiveness of influenza vaccination can vary considerably from year to year. In seasons with a low influenza attack rate, the cost-effectiveness of vaccination with IIV would be dramatically higher than in the base case (Figure 3). The 2-way sensitivity analyses

demonstrate even less favorable cost-effectiveness for a scenario that assumes a low influenza illness rate and low level of vaccine effectiveness. In addition, the sensitivity of these results to the total costs of vaccination highlights the potential for delivering vaccinations in settings that have lower costs and reduce the time required for vaccination.

Comparisons with Previous Studies

Our study contributes valuable new information because it incorporates survey-based health state preferences for influenza-related illness and vaccine adverse events. These preferences, which are expressed as QALYs saved, are important for 2 reasons. First, we were able to evaluate the net health benefits of vaccination by subtracting the QALYs lost due to vaccine adverse events from the QALYs gained due to averted influenza cases. The results suggest that vaccination of all children is desirable from a health standpoint. Second, the outcome measure of dollars per QALY saved allows policymakers to compare the cost-effectiveness of influenza vaccination of children with other potential investments in preventive health services.

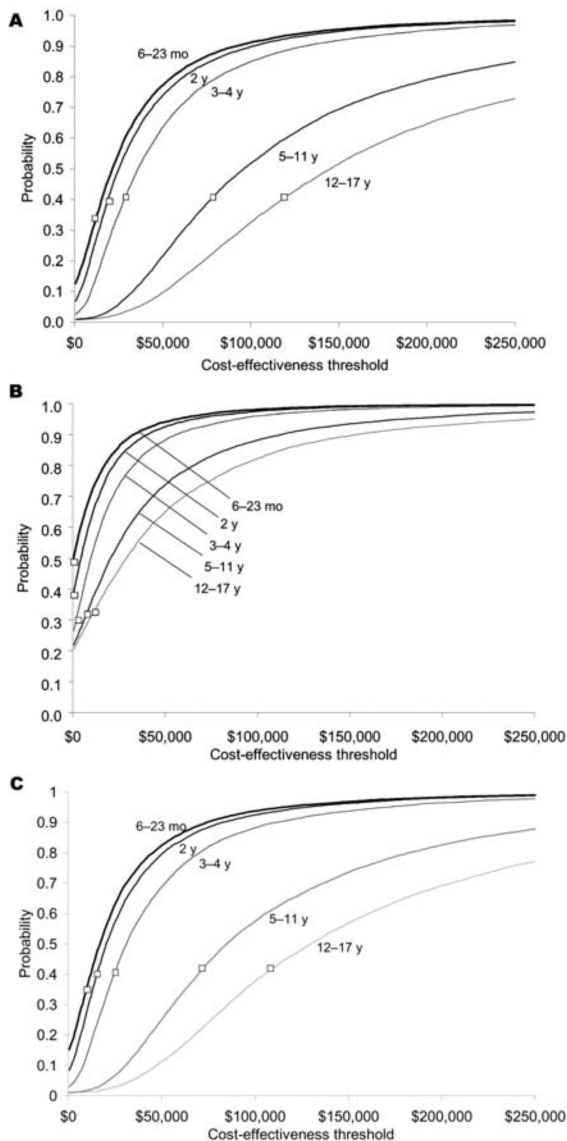

Figure 2. Cost-effectiveness acceptability curves for inactivated influenza vaccine compared with no vaccination (A, children not a high risk; B, children at high risk). Cost-effective acceptability curves for live, attenuated vaccine compared with no vaccine (C, children not at high risk only). Box indicates the mean cost-effectiveness ratio.

Authors of other economic analyses of influenza vaccination in children concluded that vaccination was more cost-effective than we found in our study (*8*,*9*,*31*). However, in these studies, the authors either did not separate children at high risk from those not at risk (*31*) or did not allow for sufficient variability in key variables (*8*,*9*). These studies assumed substantially higher influenza attack rates (*8*,*9*), higher levels of vaccine effectiveness (*8*,*9*), and lower total costs of vaccination (*8*–*10*), all of which would favor vaccination. However, we believe that it is more accurate to include variation in both incidence of influenza-related clinical illnesses and rates of influenza-related health outcomes. Neither our study nor the previous studies included potential benefits of herd immunity.

In a recent study that used cost-benefit analysis to evaluate the economics of influenza vaccination in children, Meltzer et al. arrived at conclusions similar to this analysis for many of the age/risk groups under consideration (*32*). Meltzer et al. found that annual vaccination of children not at high risk was unlikely to be cost-saving and that annual vaccination of children 6–23 months at high risk was likely to generate cost savings. For older children at high risk, they estimated median cost savings, but this analysis projects net costs of influenza vaccination for similar-age risk groups.

Limitations

Some studies that used mathematical models have suggested substantial community herd immunity effects from vaccinating school-aged children (*33*). Although one study demonstrated herd immunity with vaccination rates of >80% among school-aged children during the 1968 pandemic (*34*), a recent study by Pisu et al. (*35*) reported that vaccinating 20%–25% of children <5 years of age in a Texan community did not generate any measurable herd immunity in persons <35 years. Additionally, no field studies have assessed the impact of pediatric vaccination on hospitalization and deaths in adults. Thus, we made the conservative decision to not include herd immunity effects in our analysis. If herd immunity effects had been included in our analysis, the findings would likely have been more favorable for vaccination. Future analyses should evaluate the cost-effectiveness of expanding routine influenza

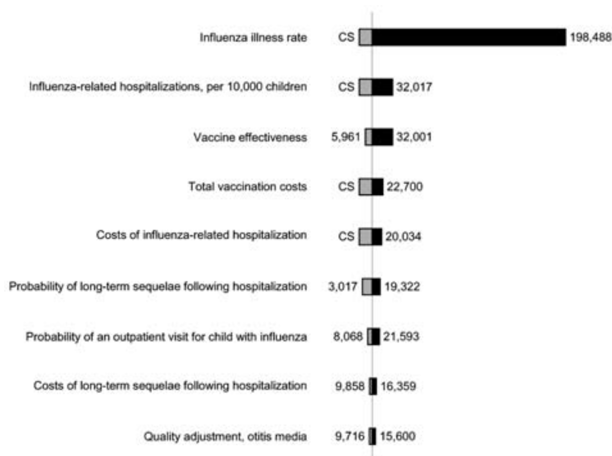

Figure 3. One-way sensitivity analyses on selected variables for children ages 6–23 months not at high risk, in dollars per quality-adjusted life years gained. This figure reports variables to which the results were most sensitive. Variables not reported here had less effect on results than those included above. Base case=$12,300

immunization under different assumptions for vaccine coverage rates and the costs of achieving these rates.

A recent randomized trial suggests that influenza vaccination has little, if any, effect on otitis media in children (*36*), while previous trials have found that influenza vaccination reduces otitis media (*6*,*37*). Our model assumes that only a small proportion of otitis media is preventable by influenza vaccination, and our findings are consistent with estimates of otitis media reduction from influenza vaccination reported in all of these studies. Our model is conservative in that it only includes the effect of reduced incidence of otitis media (or other complications) due to reduced incidence of influenza illness and does not consider any other benefits of vaccination, such as whether vaccinated patients with influenza illness may have a lower probability of experiencing otitis media (or other complications).

The time-tradeoff questions we used to elicit preferences for health states differ from that commonly used for adult illnesses because the loss of quality of life for both parent and child are explicitly included. In addition, parents were asked to include the value of productivity losses to paid or unpaid work for caring for a child with influenza in the time-tradeoff amount; therefore, productivity losses were included in the health state quality adjustments, whereas parent time costs for vaccination were included as dollar costs. As a result, the time-tradeoff amounts presented here are not directly comparable to utility values from generic utility instruments for measuring reductions in quality-of-life for chronic health states, such as the Health Utilities Index (*38*) or the EQ-5D (*39*). The sample sizes for the time-tradeoff studies were small.

Recent data show that some influenza-related deaths in children may occur outside the medical setting (*2*). Only deaths that occurred after an influenza-related hospitalization have been included in this analysis. However, even a 10-fold increase in influenza-related deaths did not appreciably change the cost-effectiveness results since the total number of deaths remains small.

Few data are available to guide assumptions on what proportion of children who experience mild systemic symptoms after vaccination, such as fever or respiratory symptoms, will see a physician. In the absence of reliable data, we selected an assumption that would be more likely to bias against vaccination rather than for and assumed it would be the same as the proportion of children who would visit a physician due to influenza illness. If the number of medically attended, vaccination-related adverse events were lower, the cost-effectiveness ratios would also be slightly lower, but cost-effectiveness results are not very sensitive to this parameter. We did not include any quality adjustment for vaccination itself aside from negative effects of vaccination-related adverse events. If vaccination itself were associated with a decrease in quality of life, cost-effectiveness ratios would be less favorable than in the current analysis. Previous analyses of other vaccinations, which included a quality adjustment for fever and fussiness following vaccination, were not sensitive to this parameter (*22*,*40*).

Conclusions

Routine annual influenza vaccination using IIV for children age ≥2 years not at high risk is likely to result in net health benefits, but cost-effectiveness ratios are likely to be less favorable than for children ages 6–23 months and children of any age with a high-risk condition. Cost-effectiveness among children decreases with increasing age, although risk status is more important than age in determining the economic impact of annual influenza vaccination. Further work is needed to assess the potential impact of herd immunity on the cost-effectiveness of expanding influenza vaccine recommendations.

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Dr Prosser is an assistant professor in the Department of Ambulatory Care and Prevention at Harvard Medical School and Harvard Pilgrim Health Care. Her research interests include conducting economic evaluations of health interventions and improving methods for valuing children's health benefits for cost-effectiveness analysis. Her current research focuses on the cost-effectiveness of childhood interventions, including newborn screening for metabolic disorders, influenza vaccination, and child maltreatment.

Technical Appendix is available at http://www. cdc.gov/ ncidod/eid/vol12no10/05-1015.htm

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West Nile Virus in Horses

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West Nile Virus Isolation from Equines in Argentina, 2006

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West Nile virus (WNV) was isolated from the brains of 3 horses that died from encephalitis in February 2006. The horses were from different farms in central Argentina and had not traveled outside the country. This is the first isolation of WNV in South America.

Since West Nile virus (WNV) was detected in the Western Hemisphere in 1999 (*1*), the National Service of Animal Health (SENASA) has restricted the entry of WNV-susceptible species into the country, and the National Reference Center for Dengue and Arboviral Diagnosis of Argentina, Instituto Nacional de Enfermedades Virales Humanas (INEVH "Dr. Julio I. Maiztegui") incorporated new laboratory techniques, performed multidisciplinary training, and implemented laboratory WNV surveillance for birds, equines, and humans.

The Study

In late February 2006, two horses died after encephalitis developed at 2 stud farms near San Antonio de Areco, 110 km north of the city of Buenos Aires (Figure 1). Clinical signs included circling and acute onset ataxia, hypersensitivity to noise, hyperexcitability, and recumbency. Both mares (Eq001 and Eq002) died within 48 to 72 hours of onset of signs. Although each farm contained ≈300 horses, no other horses had clinical signs.

In March, another horse death was reported at the Jockey Club horse training center in San Isidro, province of Buenos Aires, within 48 hours after its arrival from a polo horse farm near Victoria (Entre Rios Province) (Figure 1). Clinical symptoms of the polo mare (Eq003) were weakness, paralysis, and recumbency.

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Brain tissues from the 3 horses were extracted for virologic studies. Diagnostic tests for rabies virus (Dirección de Laboratorios y Control Técnico, SENASA) and for equine herpesvirus 1 (INTA Laboratory, Castelar, Argentina) were performed, with negative results. At INEVH, virus was isolated in African green monkey (Vero) cells, as described (*2*). Briefly, 20% homogenates of brain tissues were prepared and cultured in Vero cells for 14 days. Cultures were examined daily for evidence of viral cytopathic effect (CPE). Infected cells that showed CPEs were harvested and evaluated by immunofluorescent assay (IFA) (*3*) for flavivirus antigen, by using fluorescein isothiocyanate–labeled flavivirus polyclonal antisera (Centers for Disease Control and Prevention, Puerto Rico), and for WNV antigen by using the WNV-specific monoclonal immunoglobulin M (IgM) antibody H5-46 and IgG neutralizing monoclonal antibody 7H2. Cultures that did not show CPEs on the original isolation were blind passaged 1 more time onto fresh Vero monolayers. Three viral isolates were obtained. Two cultures (ArEq001 and ArEq003) showed CPEs on day 7 postinoculation of the original isolation; in the third culture (ArEq002), CPEs were observed only after the blind passage on day 7 after inoculation. WNV antigen was demonstrated when the 3 culture isolates were examined by IFA. Negative results

Figure 1. Locations of dead horses reported from February to March 2006 in central Argentina.

were obtained for Saint Louis encephalitis virus by using the monoclonal antibody 6B5A-2. Negative results were also obtained for the alphaviruses Western equine encephalitis, Eastern equine encephalitis, and Venezuelan equine encephalitis by using mouse hyperimmune ascitic fluid. Titration of the supernatants by plaque assay gave titers of 10^5 to 10^6 PFU/µL.

For molecular identification of the virus isolates, viral RNA was extracted from 140 µL of infected Vero cell culture supernatant by using QIAamp viral RNA extraction kit (Qiagen, Inc., Valencia, CA, USA). Nested reverse transcription (nRT)–PCR assay was performed as described by Shi et al. (*4*). A DNA band of the correct size was visualized by gel electrophoreses of the RT-PCR product. Using the nRT-PCR previously described by Johnson et al. (*5*), the INTA laboratory also detected WNV RNA directly from brain tissue from the 3 necropsy specimens. In this case, total RNA was extracted from 50 to 100 mg of tissue by using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). WNV NY99 RNA lysate was employed as positive control. INEVH and INTA laboratories did not have infectious WNV in their virus collections.

DNA fragments from C/preM and NS5 genes amplified by RT-PCR assays employing primers WN212/WN619c (5′-TTGTGTTGGCTCTCTTGGCGTTCTT-3′/5′-CAGCCGACAGCACTGGACATTCATA-3′) and WN9483/WN9794c (5′-CACCTACGCCCTAAACACTTTCACC-3′/5′-GGAACCTGCTGCCAATCATACCATC-3′) were sequenced. DNA products were analyzed on a 2% agarose gel, and the bands of the correct predicted size were excised and purified with the Gene Clean Kit (BIO 101, Carlsbad, CA, USA). The nucleotide sequences were determined by automatic dideoxy cycle sequencing techniques (Applied Biosystems, Foster City, CA, USA). Alignments were performed with BioEdit program by the Clustal Wallis method (available from http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic maximum parsimony trees were generated with the TNT program (*6*). Jackknife resampling with 1,000 replicates was performed to evaluate the obtained trees. Phylogenetic analysis of NS5 fragments placed the Argentinean sequences in the North American cluster of lineage IA (Figure 2A). Sequences ArEq001 and ArEq002 showed a 100% nucleotide (nt) identity with hny1999 and differed by only 1 nt from sequence ArEq003. Phylogenetic analysis of the C/preM fragments also placed the Argentinean sequences in lineage IA (Figure 2B). Once again, ArEq001 was identical to ArEq002 but showed 9 nt differences from ArEq003. ArEq001 and ArEq002 grouped together because of 3 point mutations at nt 172, 208, and 245 in our fragments (corresponding to positions 390, 426, and 463 of WNV hny1999 strain. Whereas the first 2 mutations are silent, at position 463 the substitution of G for A results in a change of valine to isoleucine. Correct placement of ArEq003 is not clear because of several nucleotide changes.

Conclusions

Since WNV was first detected in New York in the summer of 1999, it spread from the Atlantic Coast to the Pacific Coast in the United States and has affected Canada, Mexico, and some locations in the Caribbean basin, Central America, and northern Colombia (*7,8*). Our results are further evidence of the expanding geographic distribution of WNV.

WNV was isolated and detected by molecular techniques in Argentina from 3 horses that had no record of traveling outside the country during the preceding 4 months and that had not received vaccines against WNV. San Antonio de Areco may provide ideal conditions for detecting an enzootic cycle, given that >9,000 horses are

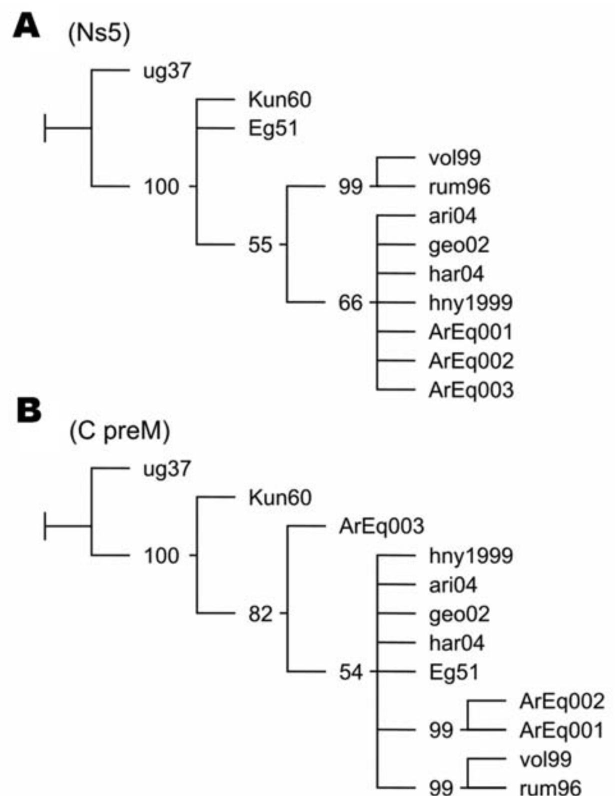

Figure 2. Phylogenetic trees of West Nile virus (WNV) nucleotide sequences. Maximum parsimony trees were obtained with TNT software (*6*). Values of jackknifing support are indicated at nodes. GenBank accession nos. ArEq001, ArEq002, and ArEq003: DQ537383, DQ537385, and DQ811782 (fragments NS5), DQ537382, DQ537384, and DQ811783 (fragments c/prM); ug37: M12294; vol99: AF317203, ari04: DQ164201; geo02: DQ164196; har04: DQ164206; hny1999: AF202541; Kun60: D00246; rum96: AF260969; Eg51:AF260968. A) NS5 fragment. B) C/preM fragment.

raised there. Horses are probably "dead-end" or incidental hosts in the WNV transmission cycle; 10%–20% of the infections result in clinical disease, and the mortality rate in equines varies from 28% to 45% (*9*). Avian deaths were not observed in the affected places. Victoria, a potential site of infection for 1 of the studied equines, is 100 km southeast of Paraná City, where human flavivirus encephalitis cases have been diagnosed since February 2006. Cross-neutralization studies with a panel of flaviviruses are being conducted to identify the specific etiologic agent.

Analysis of the genomic sequences places the 3 Argentinean isolates in WNV lineage IA. Phylogenetic analysis of the NS5 fragment place them in the North American cluster, although an additional resolution of this cluster was not achieved because of high conservation levels and shortage of parsimony informative sites. A clear resolution of lineage IA clades was not obtained from the nucleotide sequences of the C/preM fragments. Nevertheless, a different location for Argentinean sequences was observed according to geographic origin. The substitution of valine to isoleucine, detected in sequences from San Antonio de Areco, was not observed in any sequence available from GenBank database. Additional investigation is necessary to establish possible implications for this change in terms of virulence and tropism of WNV.

WNV could be detected in southern South America because of the great economic value of the horses, which are subjected to continual veterinary evaluations. Animals of less economic value might have become infected and died without being noticed. The results reported here emphasize the need for an integrated surveillance system for WNV in Argentina with greater diagnostic capacity and the need for development of control strategies.

Acknowledgments

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Nemating Symbiont for *Photorhabdus asymbiotica*

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Photorhabdus asymbiotica is an emerging bacterial pathogen that causes locally invasive soft tissue and disseminated bacteremic infections in the United States and Australia. Although the source of infection was previously unknown, we report that the bacterium is found in a symbiotic association with an insect-pathogenic soil nematode of the genus *Heterorhabditis*.

Most newly recognized human pathogens are zoonotic (i.e., able to infect nonhuman animal species) (*1*). Although it is well established that vertebrates are associated with emerging human infectious disease, the role of invertebrates, which constitute >95% of known animal species, has received far less attention.

The genomics era, however, has resulted in a dawning recognition of the importance of invertebrates in the emergence of human infection (*2*). For example, the virulent insect pathogen *Bacillus thuringiensis* is genetically closely related to the human pathogen *Bacillus anthracis*, the cause of anthrax (*3*). *Yersinia pestis*, the cause of plague, contains insecticidal toxins, which may have been laterally transferred from the insect pathogen *Photorhabdus luminescens* (*4*).

Photorhabdus organisms are γ-proteobacteria that display the curious property of bioluminescence (they glow in the dark); 3 species are currently recognized: *P. asymbiotica*, *P. luminescens*, and *P. temperata* (*5*). The latter 2 species have been intensively studied by entomologists because they are virulent insect pathogens. They form a symbiotic relationship with nematodes (*Heterorhabditis* sp.) that invade the larvae of insects. The nematodes regurgitate the bacteria, which kill the insects and provide a food source for the nematodes. Insect-pathogenic nematodes are thought to be harmless to vertebrates and are used in horticulture for biologic control of insects (*6*).

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P. asymbiotica is a human pathogen, the source of which has not previously been identified. First described in 1989 by Farmer et al. (*7*), *P. asymbiotica* has been associated with invasive soft tissue and disseminated bacteremic infections in the United States and Australia. Multifocal skin and soft tissue abscesses are characteristic. Reported predominantly from Texas and the eastern coast of Australia, *P. asymbiotica* infections have been associated with outdoor activity during the warm summer months (*8*). Because this bacterium was not believed to be associated with nematodes, it was given the name *asymbiotica* (not a symbiont) in 1999 (*5*).

The organism can be isolated from soft tissue or blood samples and grows readily on conventional bacterial culture media. However, because clinical microbiology laboratories may misidentify *P. asymbiotica*, the true frequency of human infection is uncertain (*9*).

The Study

We report *Photorhabdus* infection in a 49-year-old Australian man who had fever and soft tissue infections of his right hand and left thigh in February 2006 (Figure 1). The patient had been digging fence post holes in the soft sandy soil at his house in Kingscliff (New South Wales), using his right hand as a scoop. The work caused some minor trauma to the skin of the dorsum of his hand. In the ensuing days, he experienced fever, and a severe local infection developed in his right hand. A secondary abscess developed in his left thigh ≈1 week later. *Photorhabdus* sp. was isolated in pure culture from pus collected from the patient's right hand. Blood cultures were negative for *Photorhabdus* sp.

The patient was initially treated with intravenous cephalosporins and his hand was subjected to debridement and reconstructive surgery. He was switched to a 5-week course of oral ciprofloxacin when the pathogen was identified, and he improved steadily.

We hypothesized that the *Photorhabdus* infection was transmitted by a previously unidentified insect-pathogenic nematode. To prove this hypothesis, seven 650-mL sandy soil samples were collected from the fence post holes dug by the patient and from the surrounding area. To each of these soil samples, 5 insect larvae (*Tenebrio mollitor*) were added as bait. Dead insects were removed from the containers 5 days later. Two of these insects were visibly luminescent.

Photorhabdus sp. was isolated from the luminescent insect hemolymph. Nematodes emerged from the insect cadavers within 14 days. These nematodes were surface sterilized, crushed individually in 100 µL Luria broth with a motorized mortar and pestle, and plated on Luria broth and NBTA agar (nutrient agar supplemented with bromothymol blue and triphenyltetrazolium chloride).

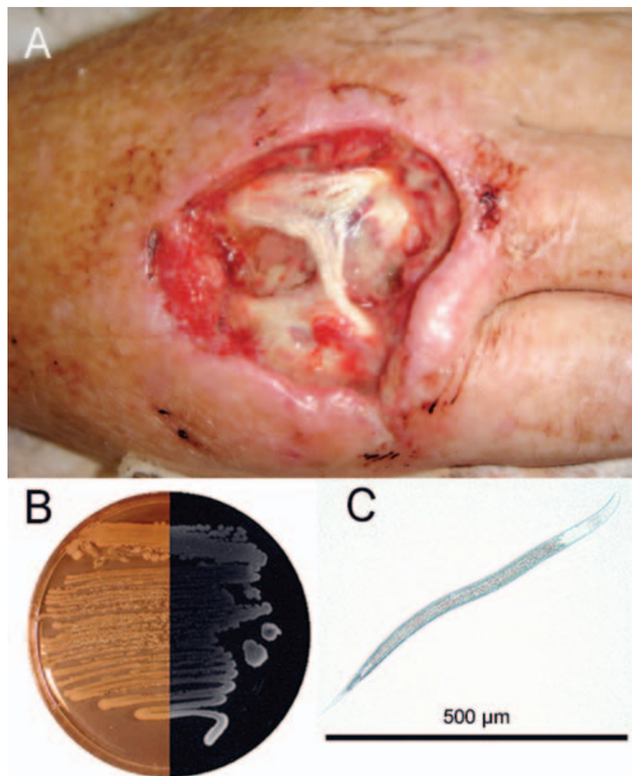

Figure 1. A) Hand of the patient infected with *Photorhabdus asymbiotica* after debridement. B) Composite photograph of a culture of *P. asymbiotica* taken in visible light and in darkness to demonstrate bioluminescence (Luria-Bertani medium). C) Soil nematode from which *P. asymbiotica* was isolated.

Bacteria released from the intestine were bioluminescent and were confirmed to be *Photorhabdus* sp.

We tested whether the nematode-associated *Photorhabdus* from the infected insects was the same strain as that from the infected patient. The *Photorhabdus* isolates recovered from nematodes and from the patient's hand were compared on the basis of nucleotide sequences of 2 housekeeping genes, *glnA* and *gyrB*. The same gene fragments were also sequenced from a sample of 50 diverse *Photorhabdus* strains, including *P. asymbiotica* that had been isolated from patients in Australia and the United States. Multilocus sequencing is a powerful technique for typing and epidemiologic surveillance of many human pathogens (*10*,*11*). Phylogenetic analysis of these data confirmed that the human- and nematode-derived isolates of *Photorhabdus* were the same strain (referred to as *P. asymbiotica* Kingscliff). This strain clusters with other *P. asymbiotica* strains isolated from Australia (Figure 2).

Nematodes containing the Kingscliff bacteria were analyzed by amplifying the internal transcribed spacer region of crushed infective juvenile nematodes and of chromosomal DNA isolated from the nematodes (*13*). The PCR products were then purified and sequenced. BLAST (National Center for Biotechnology Information, Bethesda, MD, USA) searches showed that the Kingscliff nematode is a member of the genus *Heterorhabditis* and is most closely related to *H. indica* (98% identity) and to other members of this tropical group of isolates. Morphologic analysis and molecular analysis of coding DNA regions and mitochondrial DNA are currently underway.

Using the White-trap method (*14*) we have serially infected insects in vitro with the *P. asymbiotica* Kingscliff–*Heterorhabditis* complex, confirming that insects provide suitable prey. One feature of *Heterorhabditis* species is their specificity of association with their own species of bacterium. The 10 described nematode species do not grow and develop on bacteria from another species of nematode. Bacteria isolated from the human wound, the infected insect, and the nematode, as well as bacteria isolated from all 3 recognized species of *Photorhabdus* (*P. luminescens* TT01, *P. temperate* K122, and *P. asymbiotica* USA) and *Escherichia coli*, were tested with the Kingscliff nematode for growth in vitro on lipid agar media. Only Kingscliff bacteria from the wound, insect, and nematode supported growth and development

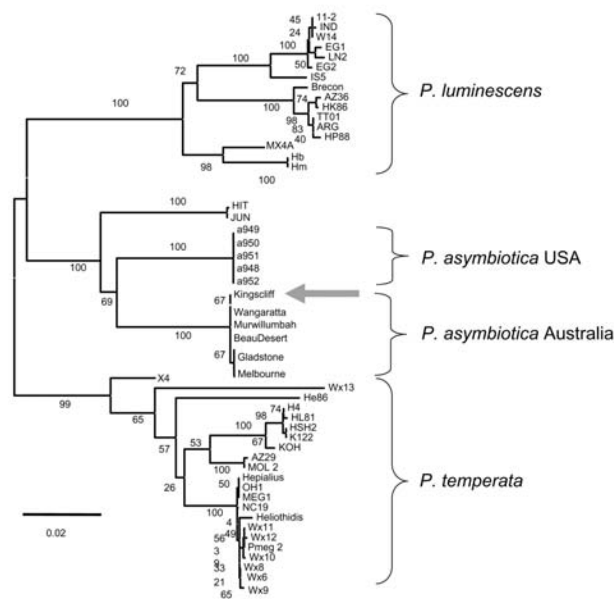

Figure 2. Phylogenetic tree of concatenated sequences of fragments of the *glnA* gene (474 bp) and the *gyrB* gene (576 bp) in 52 *Photorhabdus* isolates representing known diversity across the genus. The tree was constructed with the neighbor-joining algorithm and the K2-P method of distance estimation as implemented in MEGA version 3.0 (*12*). A total of 1,000 bootstrap replicates were performed, and the percentage of bootstrap trees supporting each node are given. The Kingscliff isolate (arrow) clusters with *P. asymbiotica* isolates from Australia, both in the concatenated tree (bootstrap score = 100%) and in individual gene trees (not shown). The scale bar shows percentage relatedness.

of the Kingscliff nematode and the Kingscliff bacteria were retained by this nematode. In all other instances, infective juvenile nematodes failed to recover. *H. bacteriophora* nematodes failed to recover on Kingscliff bacteria from all 3 sources and on *Escherichia coli*. These data indicate that the Kingscliff bacterium is required for the growth and reproduction of the Kingscliff nematode, and lack of development or growth on any other strain indicates the specificity of this association, a characteristic of *Photorhabdus-Heterorhabditis* associations. *Photorhabdus asymbiotica* has been shown to be a nematode symbiont; the specific epithet is a misnomer.

Conclusions

P. asymbiotica is not the first bacterial symbiont of nematodes to be associated with human disease. *Wolbachia*, an intracellular bacterial symbiont of the nematodes *Onchocerca volvulus* and *Brugia malayi*, has been implicated in the pathogenesis of 2 major human infectious diseases, river blindness and lymphatic filariasis (*15*). However, unlike *Wolbachia*, *P. asymbiotica* appears to actively reproduce in its human host. *O. volvulus* and *B. malayi* nematodes are borne by an insect vector. The insect-pathogenic nematode bearing *P. asymbiotica* does not appear to have been borne by an insect vector. Whether this nematode is able to penetrate intact human skin is unclear, although direct skin penetration by nematodes is well recognized (e.g., hookworm, *Strongyloides stercoralis*). Although the patient described here had a history of minor skin trauma, previous case reports suggest infection beginning in uninjured skin.

With continued population growth and movement, changes in human behavior, and changes in the environment, new human infectious diseases can be expected to continue to cross the species barrier. Given the dominance of invertebrate animal species in the biosphere, more invertebrate pathogens will likely emerge as agents of human infection.

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Chikungunya Infection in Travelers

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The largest described outbreak of chikungunya virus has been occurring on the islands of the southwest Indian Ocean since March 2005. We describe the manifestations of chikungunya virus infection in travelers returning from these islands, with focus on skin manifestations.

Chikungunya virus is an arthropodborne virus (genus *Alphavirus*, family *Togaviridae*) (*1*). It was first isolated in Tanzania in 1953 (*2*). Since then, chikungunya outbreaks have been reported in Africa and Asia. Transmission to humans occurs through bites of *Aedes* (mainly *Aedes aegypti* and *A. albopictus*) mosquitoes (*3–7*).

An outbreak of chikungunya occurred in the Comoro Islands in early 2005; since then, the virus has circulated to other islands in the Indian Ocean. The first case of chikungunya infection was identified in the Indian Ocean island of Reunion in March 2005. After a period of lower transmission during winter, case numbers increased dramatically with the arrival of the Southern Hemisphere summer in December (*8*). The Reunion epidemic is the largest ever described; ≈150,000 cases were reported from March 2005 through February 2006. We describe skin manifestations of this chikungunya infection in travelers returning from the area.

The Study

All adult travelers who came to our tropical diseases unit from March 2005 through February 2006 were prospectively included in this study if they had symptoms of chikungunya virus infection after traveling to any island in the southwest Indian Ocean (Comoro, Mauritius, Mayotte, Reunion, Seychelles). Children <5 years of age were not included because no pediatricians practiced in our hospital. Chikungunya virus was suspected if fever, joint pain, or skin eruption was present. Diagnosis of chikungunya infection was confirmed by an immunocapture ELISA derived from a yellow fever test by using a goat anti-human immunoglobulin (Ig) M antibody (Sigma, Saint Louis, MO, USA), an inactivated cell-culture–grown chikungunya virus and a mouse anti-chikungunya hyperimmune ascitic fluid (Institut Pasteur, Lyon, France), and a horseradish peroxidase–labeled antimouse IgG conjugate (Sigma) (*9*). Other blood tests were performed according to the discretion of the physician in charge of the patient.

During the study period, chikungunya infection was confirmed serologically in 22 patients (14 female, 8 male); median age was 47 years (25–72 years). Seventeen patients had returned from Reunion (77%), 3 from Comoro (14%), and 2 from Mauritius (9%). Twelve (55%) patients were tourists, 8 (36%) were islanders settled in France but returning from visiting their relatives, and 2 (21%) were French residents in these islands. The median duration of stay (residents excluded) was 21 days (2–90 days). Symptoms appeared in 19 (86%) patients while they were abroad and within 3 days of return in 3 patients (14%). The median lag time between onset of symptoms and consultation was 13.5 days (range 2 days–9 months).

Fever and joint pain were noted in all patients. The median duration of fever was 4 days (range 2–7 days), and joint pain was mainly distal and symmetric, involving wrist (18 patients, 81%), ankles (17 cases, 77%), phalanx (16 patients, 73%), knees (14 patients, 64%), and elbows and shoulders (4 patients each, 18%). The other extradermatologic signs were asthenia in 17 patients (77%), headache in 13 (59%), muscle pain in 12 (55%), swelling in 10 (45%), peripheral lymphadenopathy in 9 (41%), bleeding from the nose or gums in 4 (18%), nausea or vomiting in 3 (14%), and eyesight trouble in 1 (4%). In the 6 patients seen during the first week of symptoms (acutely ill patients), lymphopenia was present in 67%, thrombocytopenia in 50%, and increase of alanine aminotransferase/aspartate aminotransferase in 67%.

Skin manifestations occurred in 17 patients (77%). Because skin eruption occurs during the first week of the disease, however, only 6 (27%) patients with these signs were seen at the time of consultation. All 6 had a generalized exanthema consisting of noncoalescent macular or papular lesions (Figure). The elementary cutaneous element was an erythematous macule in 5 patients and maculopapules in 1. The number of macules was >50, and the anatomic location of the rash was abdomen, thorax, back, and limbs. No lesions were seen on the face; palms and soles were involved in 3 patients. Islands of normal skin were seen in 5 patients. An aphthoid lesion was seen in 1 patient. Pruritus of the skin or a burning sensation was reported in 3 patients. Bleeding from the nose or gum, purpura, petechiae, ecchymosis, and bullous lesions were not observed. Erythema followed the onset of fever by 1 to 2 days, lasted 3–7 days, and disappeared without scaling in all cases, regardless of the patient's skin color.

The main complaint after the first week was persistent arthralgia. The treatment was exclusively symptomatic:

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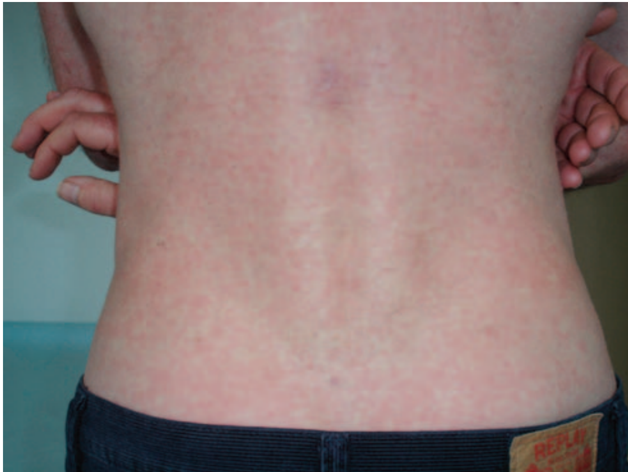

Figure: Erythematous maculopapuar exanthema with islands of normal skin on the back.

pain killers and nonsteroidal antiinflammatory medications.

The dramatic ongoing epidemic of chikungunya infection in the southwestern part of the Indian Ocean, particularly Reunion Island, provided the opportunity to describe the skin manifestations of this infection among travelers. The main characteristic of the skin eruption was a generalized macular erythematous exanthema, often with islands of normal skin and a sensation of pruritus. The infection was also characterized by a short incubation period (1–12 days). Most patients had fever, arthralgia, exanthema, asthenia, myalgia, and headache.

Conclusions

Among travelers returning from the tropics, febrile exanthema associated with arthralgia is not specific to chikungunya and may be observed with several other viral and bacterial infections and adverse cutaneous reactions (*10*). Chikungunya and dengue infections are probably the most difficult to differentiate. The viruses are transmitted by the same mosquito species; disease-endemic areas are nearly the same in Asia, Africa, and the Indian Ocean; and clinical symptoms are similar. In addition, simultaneous coinfection with chikungunya and dengue viruses has been reported (*5,11*). In the only published study comparing chikungunya and dengue clinical manifestations in Thailand, the onset of symptoms was more abrupt; the febrile course was shorter; and maculopapular rashes, conjunctival injection, and arthralgia were significantly more frequent than in dengue (*12*). Shock and gastrointestinal hemorrhages occurred only in dengue patients, but the tourniquet test did not help to differentiate chikungunya from dengue (*12*). The skin manifestations reported in our study are similar to those described for classic dengue fever infection, which is characterized by a pruritic, macular or a maculopapular rash in which small islands of normal skin are spared (*12*).

The *Alphavirus* genus consists of 30 species of arthropodborne viruses (*1,13*). Of these, 6 mosquitoborne viruses are associated with fever, arthralgia, and rash: the Ross River and Barmah Forest viruses in the South Pacific, o'nyong-nyong and Sindbis viruses in tropical Africa, chikungunya virus in Africa and Asia, and Mayaro virus in South America. Symptoms are usually of short duration, ≈1 week, and complete recovery is the rule, except in some cases of chikungunya infection, in which arthralgia may last for months (*14,15*). Indeed, 7 patients in our study had at least a 1-month duration of arthralgia; 1 patient consulted us 9 months after the onset of disease. The clinical manifestations reported in this study illustrate the difficulty in differentiating chikungunya infection from classical dengue fever but may help to differentiate this infection from other causes of febrile exanthema in travelers.

Dr Hochedez is a dermatologist in the Infectious and Tropical Diseases Unit, Hôpital Pitié-Salpêtrière, Paris, France. His work focuses on diagnosis, epidemiology, and treatment of travel-associated diseases.

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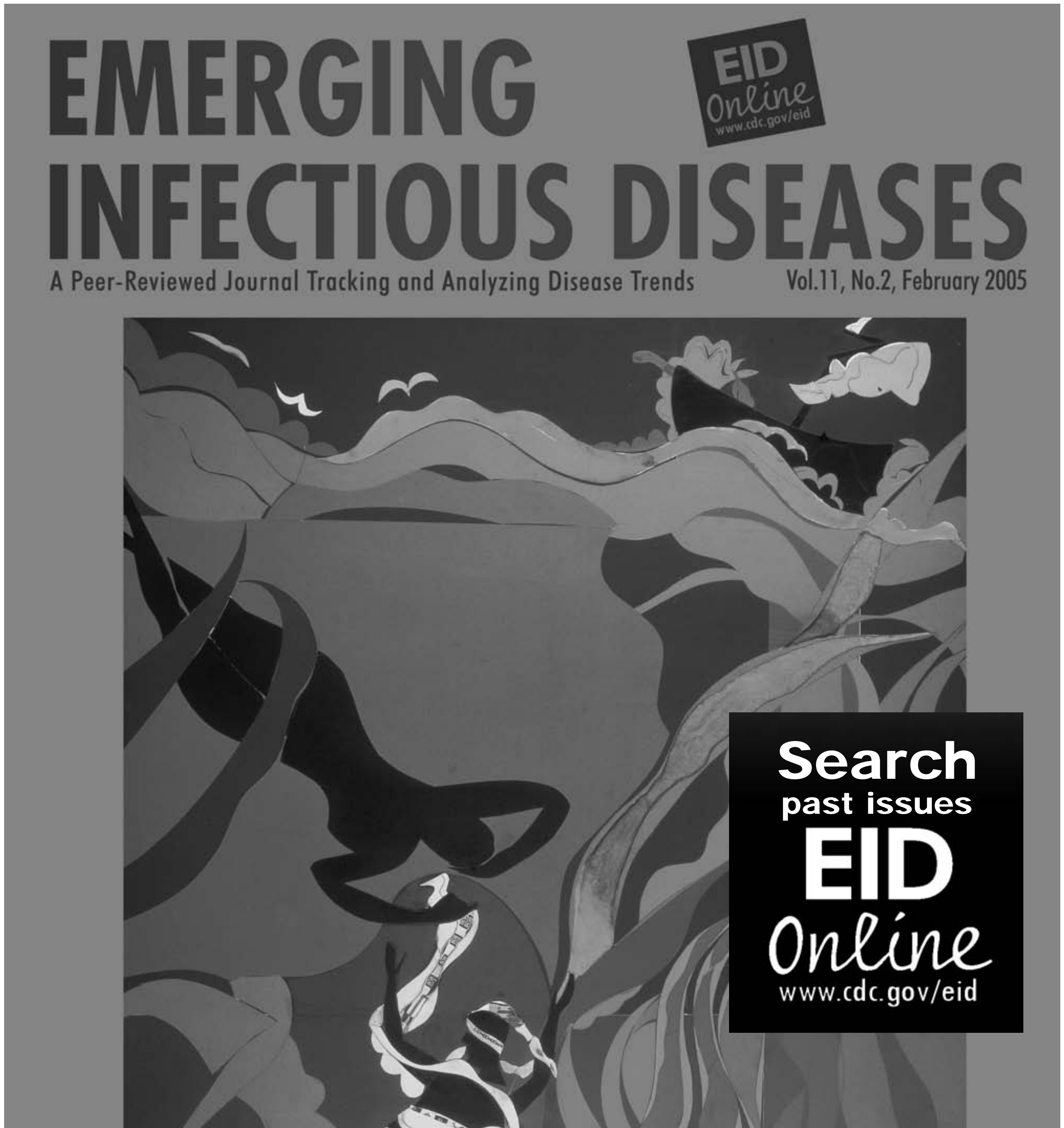

Siberian Subtype Tickborne Encephalitis Virus, Finland

Anu E. Jääskeläinen,* Tapani Tikkakoski,† Nathalie Y. Uzcátegui,* Andrey N. Alekseev,‡ Antti Vaheri,*§ and Olli Vapalahti*§¶

We isolated 11 Siberian subtype tickborne encephalitis virus (TBEV) strains from *Ixodes persulcatus* ticks from a TBEV-endemic focus in the Kokkola Archipelago, western Finland. Thus *I. persulcatus* and the Siberian TBEV are reported in a focus considerably northwest of their previously known range in eastern Europe and Siberia.

Tickborne encephalitis (TBE) is a disease endemic in a zone extending from central and eastern Europe to Siberia and Japan. Three subtypes of the causative agent tickborne encephalitis virus (TBEV) are known: the European, Siberian, and Far Eastern (*1*,*2*). The main vector for the European subtype is *Ixodes ricinus,* and for the other 2 subtypes, *I. persulcatus* (*1*,*3*–*5*). *I. ricinus* is found in Europe and Middle East (*6*), and *I. persulcatus* ranges from eastern Europe to China and Japan. The boundary between their distribution lies at the Russian side of the Finnish-Russian border (*1*,*7*). The distribution areas of both tick species overlap in eastern Europe (*4*,*5*) (Figure 1). *I. persulcatus* has not been reported from northern or western Europe except for an engorged nymph on a willow warbler (*Phylloscopus trochilus*) in northeastern Sweden in May 1992 (*6*).

In Finland, the TBE-endemic areas are mostly in the coastal regions: two thirds of cases come from the Åland Islands. Other TBE-endemic regions include the Archipelago of Turku, a focus in Isosaari (an island outside Helsinki), the Lappeenranta region in southeastern Finland, and the Archipelago of Kokkola in western Finland (*9*). The virus was found in the same areas (except for Isosaari) as early as the 1960s by screening antibodies to TBEV from cattle sera (*10*).

The TBE focus in the Archipelago of Kokkola (63°50'N, 23°10'E), ≈300 km south of the Arctic Circle, has a peculiar location; it is an isolated focus, far from other TBE-endemic areas, and is the northernmost TBE-endemic area known. Furthermore, the recent TBE cases have been severe with sequelae (*11*,*12*). A cluster of cases in 2002 led us to study ticks in the Kokkola Archipelago for TBEV.

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The Study

A total of 1,181 ticks were collected by flagging in the Archipelago of Kokkola in June 2004 (Table 1). In the tick-collecting areas 1–7, TBE patients had reported tick bites, and in areas 8–10, no TBE cases have been found. All the locations were islands or peninsulas within 20 km of each other.

The ticks were homogenized in pools of ≈10 with Dulbecco phosphate-buffered saline plus 0.2% bovine serum albumin (D-PBS-BSA) and sand to 122 pools. RNA was isolated from 100 μL of the pools by TriPure Isolation Reagent (Roche Diagnostics, Espoo, Finland). The RNA was dissolved in 20 μL diethyl pyrocarbonate–treated water, and 10 μL was used for nested reverse transcription (RT)–PCR, amplifying a 252-nt sequence from the TBEV-NS5 gene to detect TBEV-RNA according to Puchhammer-Stöckl et al. (*13*), except that the outer forward primer used was 5′-GGAGGCTGAACAACTGCAC-3′. TBEV-RNA was detected in 13 pools (each consisted of 10 adult ticks) (Table 1). Assuming that only

Figure 1. The known distribution of tickborne encephalitis (TBE)–virus endemic areas and *Ixodes* ticks in northern Europe. Yellow: TBE-endemic areas, adapted from International Scientific Working Group on Tick-Borne Encephalitis (*8*). To the south and west from the solid line, *Ixodes ricinus* distribution; to the east from the dashed line, *I. persulcatus* distribution; Lpr, Lappeenranta; EST, Estonia; LV, Latvia; LT, Lithuania.

Table 1. Tick collection sites in Kokkola Archipelago, June 2004*

Location	TBE case-patient, sex/age/y	No. ticks (n/f/m)	No. tick pools	Tick pools positive in RT-PCR†	Tick pools positive in virus isolation†
1	M/48/2004 (*12*)	184 (19/87/78)	19	4, 8, 9	8, 9
2	M/21/2002 (*11*)	80 (24/25/31)	8	25, 26	25, 26
3	F/24/2002 (*11*)	158 (2/87/69)	16	39	39
4	F/12/2002 (*11*)	474 (16/227/231)	48	79, 81, 84, 85, 86, 102, 118	79, 81, 84, 86, 102, 118
5	F/73/2002 (*11*)	41 (2/22/17)	5	–	ND
6	M/6/2003	166 (6/83/77)	17	–	ND
7	M/7/2003	6 (0/3/3)	1	–	ND
8	No known cases	55 (1/25/29)	6	–	ND
9	No known cases	9 (2/5/2)	1	–	ND
10	No known cases	8 (0/6/2)	1	–	ND

*TBE, tickborne encephalitis; RT-PCR, reverse transcription–polymerase chain reaction; n, nymphs; f, adult females; m, adult males; ND, not determined.
†Nos. indicate the tick pool numbers, the same as used in the phylogenetic tree.

1 tick in a positive pool was positive for TBEV RNA, the overall TBEV prevalence was 1.1%.

To isolate TBEV strains from the RT-PCR–positive tick pools, 20 µL of the supernatant of the pools diluted 1:1 in D-PBS-BSA was injected intracerebrally into suckling NMRI mice. One litter of suckling mice was used for each pool. The mice were followed for 14 days or until symptoms of illness appeared, and then they were killed. From 200 µL of the homogenized mouse brains, diluted 1:5 with D-PBS-BSA, RNA was extracted by TriPure, and RT-PCR for the partial TBEV-E gene was performed. The cDNA was produced with the reverse primer 5′-CCYCCAGCCARGAGRAAGC-3′ by M-MuLV-RT enzyme (Fermentas, Vilnius, Lithuania), and subsequent PCR was performed with this and a forward primer 5′-AACAGGGAYTTTGTCACTGGYACTC-3′ by *Taq* DNA polymerase (Fermentas) (detailed RT-PCR protocol available from the authors upon request).

A region of 205 nt from the NS5 gene from the RT-PCR–positive tick pools and 1,225-nt stretch from the E gene from the brains of the infected suckling mice were sequenced (GenBank accession nos. in Table 2). Unexpectedly, based on the partial NS5 sequences from the RT-PCR–positive tick pools (data not shown), the TBEV strains in Kokkola belonged to the Siberian subtype of TBEV. A phylogenetic tree based on the partial E gene sequences (1,076 nt) obtained from the TBEV isolates was prepared by the maximum likelihood method (Figure 2, scripts and datasets available from the authors upon request). Within the 1,076-nt stretch of the E gene, the Kokkola strains were ≥99.6% identical to each other. The closest relatives were Latvia-1-96 (97% identical) and the Estonian strains Est54, Est3535, and EK328 (95%–96%). Consequently, the Siberian subtype strains isolated from Finland and nearby Baltic states form a lineage together within the Siberian subtype. Other Siberian subtype strains Vasilchenko, Aina, Zausaev, and TBEV228 showed 92%–94% identity, and the European and Far Eastern subtypes showed 84%–86% identity. However, the vector tick species for Siberian-type TBEV, *I. persulcatus,* was not known to exist in Finland. This knowledge led us to study the tick species more carefully. DNA was isolated from 20 tick pools by TriPure and resuspended in 100 µL of TE (Tris-HCl 10 mmol/L, EDTA 1 mmol/L, pH ≈8). The tick species was determined as *I. persulcatus* by amplifying an average of 339 bp from mitochondrial 16S RNA gene by PCR and subsequent sequencing according to Caporale et al. (*14*). Because the ticks were pooled and homogenized before species identification, 30 adult ticks from the same region collected later in the summer were examined microscopically. All these specimens were *I. persulcatus* by morphologic criteria.

Conclusions

A TBE focus has existed in the Kokkola Archipelago at least since the 1960s when TBEV antibodies were detected in cattle (*10*), but the local TBE viruses have not been characterized. A cluster of severe human cases in the beginning of the 2000s prompted us to carry out the present study. In June 2004, we collected 1,181 ticks from the Archipelago of Kokkola and detected TBEV-RNA in 13 pools. Eleven were also positive in virus isolation. The sequences showed that the strains belonged to the Siberian subtype, whereas in the other Finnish TBE-endemic foci, only European subtype TBEV closely related to the central European strains has previously been detected (*15*). Furthermore, the tick species was *I. persulcatus*. Our results show that both *I. persulcatus* and the Siberian type TBEV are occurring several hundreds of kilometers further to northwest than what has been known previously. Because the 2 tick species are similar to the naked eye and in their behavior, and published data on distribution of *Ixodes* ticks in Finland are sparse and outdated, we cannot exclude the possibility that *I. persulcatus* also exists unnoticed elsewhere in Finland. More tick surveys and epidemiologic studies are needed to map the distribution areas of the 2 vector species and of the different TBEV subtypes in Finland. However, in our recent tick collections from

Åland and southern (Isosaari, 60°N, 25°E) and eastern (Lappeenranta, 61°N, 28°E, and Joensuu, 62°N, 29°E) Finland, all ticks were *I. ricinus*.

Some researchers have found indications that the Siberian TBEV might cause more severe or more persistent forms of TBE than the European subtype (*4*), and 3 of 5 recent human TBE cases in Kokkola have been severe (*11*,*12*). However, the number of cases studied from Kokkola is too small for firm conclusions on the severity of the local disease.

Table 2. TBE virus strains compared by sequence analysis*

Strain	Geographic origin	GenBank accession no.
Kokkola 4	Location 1, Kokkola	DQ451297†
Kokkola 8	Location 1, Kokkola	DQ451298,† DQ451286‡
Kokkola 9	Location 1, Kokkola	DQ451299,† DQ451287‡
Kokkola 25	Location 2, Kokkola	DQ451300,† DQ451288‡
Kokkola 26	Location 2, Kokkola	DQ451301,† DQ451289‡
Kokkola 39	Location 3, Kokkola	DQ451302,† DQ451290‡
Kokkola 79	Location 4, Kokkola	DQ451303† DQ451291‡
Kokkola 81	Location 4, Kokkola	DQ451304,† DQ451292‡
Kokkola 84	Location 4, Kokkola	DQ451305,† DQ451293‡
Kokkola 85	Location 4, Kokkola	DQ451306†
Kokkola 86	Location 4, Kokkola	DQ451307,† DQ451294‡
Kokkola 102	Location 4, Kokkola	DQ451308,† DQ451295‡
Kokkola 118	Location 4, Kokkola	DQ451309,† DQ451296‡
Iso40	Isosaari, Finland	AJ298323
Kumlinge A 52	Åland, Finland	X60286
Est54	Estonia	DQ393773
Est3535	Estonia	DQ393774
Est2546	Estonia	DQ393779
Est3476	Estonia	DQ393776
Latvia 1–96	Latvia	AJ415565
RK1424	Latvia	AF091016
Neudoerfl	Austria	U27495
Hypr	Czech Republic	U39292
263	Czech Republic	U27491
Zausaev	Siberia, Russia	AF527415
Vasilchenko	Novosibirsk, Russia	L40361
Aina	Irkutsk, Russia	AF091006
EK-328	Estonia	DQ486861
TBEV228	Novosibirsk region, Russia	DQ385498
TBEV1467	Novosibirsk region, Russia	AY753582
Sofjin-HO	Primorskii Kray, Russia	AB062064
Oshima 5–10	Hokkaido, Japan	AB062063
LIV	United Kingdom	NC 001809
OHFV, strain Bogoluvovska	Russia	AY193805
Langat	Malaysia	AF253419
Powassan, LB strain	United States	NC 003687

*TBE, tickborne encephalitis; LIV, Louping ill virus; OHFV, Omsk hemorrhagic fever virus.
†Accession no. for the partial NS5 gene sequence.
‡Accession no. for the partial E gene sequence.

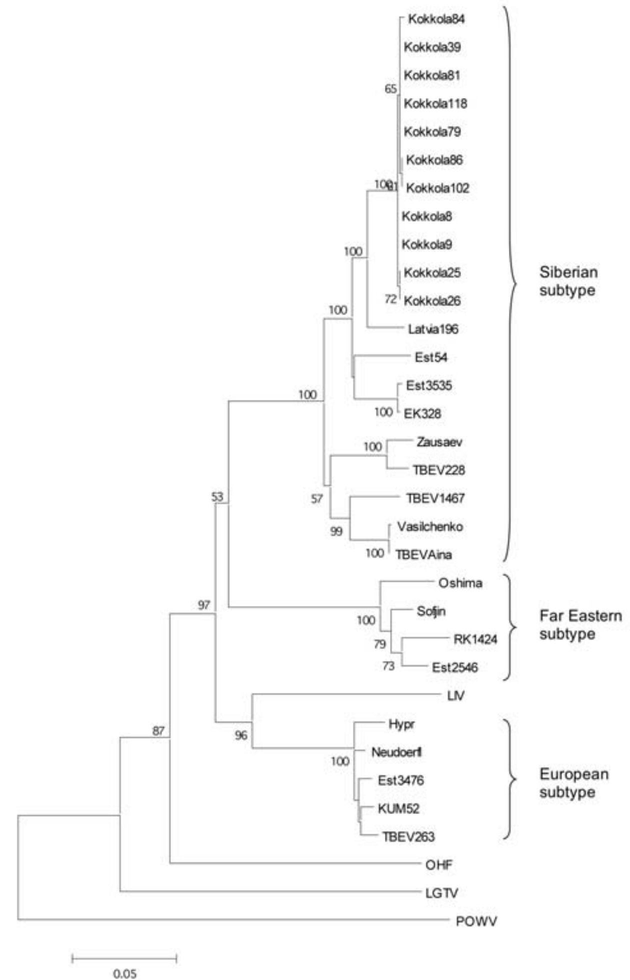

Figure 2. Maximum likelihood phylogenetic tree of partial E gene (1,076 nt). The bar below indicates the nucleotide substitutions per site. The accession nos. of the strains used can be seen in Table 2. The bootstrap support values <50 are not shown.

Acknowledgments

We thank all who participated in the tick collecting in Kokkola Archipelago in June 2004: Anna Katz, Tytti Manni, Niina Putkuri, Auli Saarinen, Pirjo Sarjakivi, Elina Tikkakoski, and Mikko Tikkakoski. We also thank Simo Räisänen for delivering more tick material from the region; Pirjo Sarjakivi and Tytti Manni for excellent technical assistance; and Lorenza Beati, Thomas G. T. Jaenson, and Juhani Terhivuo for help in studying the morphology of ticks.

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Ms Jääskeläinen is a PhD candidate at Helsinki Biomedical Graduate School and conducts research in the Department of Virology, University of Helsinki. Besides the molecular epidemiology of tickborne encephalitis virus, her interests include developing recombinant antigens for flavivirus diagnostics.

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Fourth Human Parechovirus Serotype

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We identified a novel human parechovirus (HPeV) type (K251176-02) from a neonate with fever. Analysis of the complete genome showed K251176-02 to be a new HPeV genotype. Since K251176-02 could not be neutralized with antibodies against known HPeV serotypes 1–3, it should be classified as a fourth HPeV serotype.

Infections with human parechoviruses (HPeVs) are commonly associated with mild gastrointestinal and respiratory symptoms in young children (*1–3*), but more severe conditions, such as flaccid paralysis (*4*) and encephalitis (*5*), have also been described. Recently, a new serotype (HPeV3) has been isolated, which has been associated with transient paralysis (*6*) and neonatal sepsis (*7*).

HPeV1 and HPeV2 were previously known as the enteroviruses echovirus 22 and 23 but were reclassified into a new genus within the family *Picornaviridae* after phylogenetic analysis showed that parechoviruses were distinct from other picornaviruses (*1–3*,*8–11*). HPeVs have predominantly been isolated from young children, and increasing evidence shows that HPeV can cause serious illness in these patients.

We recently showed that infection with HPeV3 is associated with younger age and more severe disease than is infection with HPeV1 (*12*). During the screening of patient samples, we identified 1 aberrant HPeV type. Phylogenetic analysis of the full-length sequence and viral neutralization assays showed that the isolate designated K251176-02 is a new HPeV genotype and serotype.

The Study

Viral culture of the stool of a 6-day-old patient with a 2-day history of high fever and poor feeding and no history of gastrointestinal or respiratory symptoms showed enterovirus cytopathic effects. However, PCR targeted at the 5′ untranslated region (UTR) of enterovirus (*13*) was negative, whereas a 5′ UTR PCR specific for HPeV (*12*) was positive.

Results of sequencing the VP1 region (*12*) suggested that K251176-02 was a novel HPeV genotype. Therefore, the full-length sequence was determined. Combinations of consensus primers were used to generate partially overlapping amplicons that covered the complete genome. Amplicons were sequenced according to a primer walking strategy. The 5′ UTR was amplified by using the 5′ RACE System (Invitrogen, Carlsbad, CA, USA). Because a primer composed of the first 22 nucleotides (nt) of published consensus parechovirus sequences was used to amplify the 5′ UTR proximal end, these 22 nt could not be determined with absolute certainty (*8*). The 3′ UTR end was amplified with a tagged oligo-dT primer.

The complete genome of K251176-02 was 7,348 nt long, containing a 5′ UTR of 708 nt, a large single open reading frame (ORF) of 6,549 nt, and a 3′ UTR of 91 nt followed by a poly(A) tract. The full-length sequence of K251176-02 has been deposited in GenBank under accession no. DQ315670.

We found a best-match nucleotide identity (*14*) of 72.2% in the VP1 gene with HPeV2 CT86-6760, which suggests that K251176-02 is most closely related to HPeV2 CT86-6760. Indeed, phylogenetic analysis of the capsid nucleotide sequence based on Jukes and Cantor distances showed K251176-02 to cluster with HPeV2 CT86-6760 (Figure 1A). However, the genetic distance was considerable (0.327) and comparable to the genetic distance between HPeV1 Harris and HPeV2 Williamson (0.332). Phylogenetic analysis of the nonstructural region showed that K251176-02 clustered with the HPeV3 prototypes A308-99 and Can82853-01 (Figure 1B).

To identify recombination events between the different HPeV prototypes, a SimPlot analysis was performed on the known full-length nucleotide HPeV genomes against K251176-02. The SimPlot analysis (Figure 2) showed the differential similarity of K251176-02 with HPeV2 CT86-6760 in the highly variable P1 region and with HPeV3 in the more conserved P2–P3 region. This finding may be the result of a recombination event.

The secondary structure of the 5′ UTR of K251176-02, determined by the *Mfold* program of Zuker and Turner (http://mfold2.wustl.edu), was predicted to be highly structured and was characterized by a stable hairpin at the proximal end that was also found in known HPeV prototypes (*8*,*11*, data not shown). The predicted secondary structure of the 3′ UTR of K251176-02 contained the same 1-stem loop organization as the HPeV prototypes and was similar to the secondary structure of HPeV1 Harris and HPeV2 Williamson and CT86-6760 (*15*).

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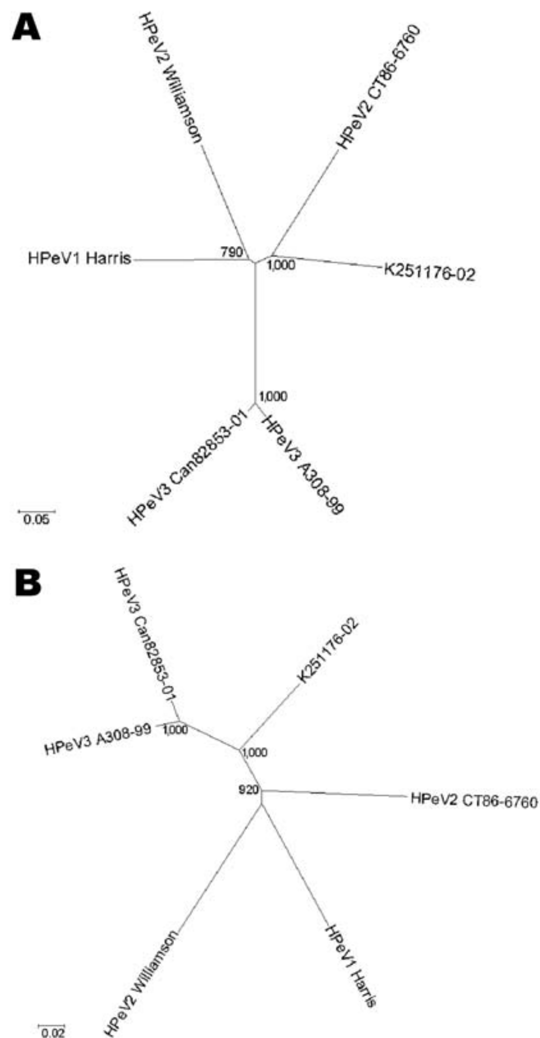

Figure 1. Unrooted phylogenetic trees showing the relationship between K251176-02 (DQ315670) and the prototype strains human parechovirus serotype 1 (HPeV1) Harris (S45208), HPeV2 Williamson (AJ005695), HPeV2 CT86-6760 (AF055846), HPeV3 A308-99 (AB084913), and Can82853-01 (AJ889918) based on nucleotide Jukes and Cantor substitution model for the capsid region (A) and the nonstructural region (B). The tree was constructed by the neighbor-joining method as implemented in MEGA version 3.1. Gaps introduced for optimal alignment were not considered informative and were excluded from the analyses by complete deletion. Numbers represent the frequency of occurrence of nodes in 1,000 bootstrap replicas. The use of other evolution models did not influence the tree topology.

A comparison of the complete ORF of K251176-02 with the HPeV prototypes showed an amino acid identity of 86.9% to 90.1% (Table 1). This amount is in the same range of amino acid identity as observed between known HPeV protoypes. For the VP1 gene, the greatest amino acid identity was observed with HPeV2 CT86-6760 (80.4%). In the nonstructural region, identity was greater to HPeV3, with 98.1% identity in the polymerase gene ($3D^{pol}$).

Comparison of the deduced amino acid sequence in the capsid region of K251176-02 with the HPeV prototypes showed that the sequences that are predicted to be part of the β-barrel structure (*6*,*10*,*11*) are well conserved in K251176-02. Like HPeV1 and HPeV2, K251176-02 also contained an RGD motif at the C-terminal end of the VP1 gene, which was absent in HPeV3 (*6*,*7*,*15*). K251176-02 also contained the common motifs X_2GXGK(S/T) and DDLXQ (2C gene), which are predicted to have a helicase function. The active-site cysteine of the protease 3C is in the context of GXCG, and the active site of polymerase $3D^{pol}$ contains the conserved sequence YGDD. The well-conserved motifs within the $3D^{pol}$ gene (KDELR, PSG, and FLKR) were also found in K251176-02 (*6*,*9*,*11*).

In summary, K251176-02 represents a new genotype in the genus *Parechovirus*. To confirm that K251176-02 is also a new serotype, a neutralization assay was performed. Table 2 shows that K251176-02 could not be neutralized by antisera directed against HPeV1 Harris, HPeV2 Williamson, and HPeV3 A308-99, which confirms that K251176-02 is a new genotype that can be classified as a fourth HPeV serotype.

Conclusions

HPeVs are classified in the genus *Parechovirus* in the family *Picornaviridae*. The recently identified HPeV3 has been associated with severe illness in young children in several studies (*6*,*7*,*12*). This association has increased

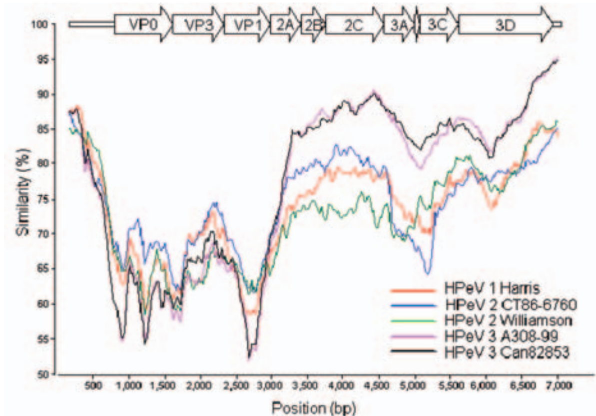

Figure 2. Similarity plot of human parechovirus serotype 1 (HPeV1) Harris (S45208), HPeV2 Williamson (AJ005695), HPeV2 CT86-6760 (AF055846), HPeV3 A308-99 (AB084913), and Can82853-01 (AJ889918) against K251176-02. Each curve is a comparison between the K251176-02 genome and an HPeV prototype. Each point represents the percentage identity within a sliding window 600 bp wide, centered on the position plotted, with a step size of 20 bp. Positions containing gaps were excluded from the comparison by gap stripping, and Jukes and Cantor correction was applied. Similarity plots of the full-length sequences of the HPeV prototypes were generated by using SimPlot version 2.5.

Table 1. Amino acid identity matrix of all known human parechovirus (HPeV) full-length sequences*

Target	HPeV1 (H)	HPeV2 (W)	HPeV2 (CT)	HPeV3 (A308)	HPeV3 (Can)
ORF					
K251176-02	88.7	86.9	90.1	89.1	89.3
HPeV1 (H)	–	88.6	88.1	87.2	87.3
HPeV2 (W)	–	–	85.8	85.1	85.0
HPeV2 (CT)	–	–	–	86.7	86.9
HPeV3 (A308)	–	–	–	–	98.2
HPeV3 (Can)	–	–	–	–	–
P1					
K251176-02	78.3	78.6	81.3	74.7	75.1
HPeV1 (H)	–	81.6	76.4	74.9	75.3
HPeV2 (W)	–	–	74.2	73.9	73.9
HPeV2 (CT)	–	–	–	73.0	73.5
HPeV3 (A308)	–	–	–	–	97.4
HPeV3 (Can)	–	–	–	–	–
P2-P3					
K251176-02	94.3	91.5	94.9	97.0	97.1
HPeV1 (H)	–	92.4	94.5	93.8	93.9
HPeV2 (W)	–	–	92.2	91.2	91.1
HPeV2 (CT)	–	–	–	94.2	94.2
HPeV3 (A308)	–	–	–	–	98.6
HPeV3 (Can)	–	–	–	–	–

*Amino acid identities for the open reading frame (ORF), capsid region (P1), and nonstructural region (P2-P3) are based on p-distance models between K251176-02 (DQ315670) and the HPeV prototypes, HPeV1 (H) (Harris, S45208), HPeV2 (W) (Williamson, AJ005695), HPeV2 (CT) (CT86-6760, AF055846), HPeV3 (A308) (A308-99, AB084913), and HPeV3 (Can) (Can82853-01, AJ889918). The full-length sequence of K251176-02 was aligned with the HPeV prototypes by using ClustalW, included in the Vector NTI Advance 10 software package (Invitrogen, Carlsbad, CA, USA). Alignment was edited by using GeneDoc software (version 2.6.02). The matrix was constructed by using MEGA version 3.1. The 5′ untranslated region (UTR) and 3′ UTR are excluded from the analysis because the regions are noncoding.

the awareness of HPeVs as relevant pathogens in young children.

We identified a new HPeV genotype in a stool specimen from a neonate with high fever. Since classification criteria based on genotyping have not been defined for HPeVs, we used the criteria proposed by Oberste et al. (*14*) for the classification of new enteroviral genotypes. According to these criteria, a new genotype is defined when a best-match nucleotide identity of <70% is found in the VP1 gene. A 70%–75% best-match nucleotide identity indicates further characterization is needed. Therefore, neutralization assays were conducted; these assays showed that K251176-02 did not neutralize with antisera directed against the 3 known HPeV serotypes. This finding indicates that K251176-02 is a new genotype that can be classified as a fourth HPeV serotype.

The patient from whom K251176-02 was isolated had high fever but no signs of neonatal sepsis, as has been found in infections with HPeV3 (*6*,*7*,*12*). Previous data suggest differences in severity of disease between the different HPeV serotypes (*12*); however, more data are needed to elucidate epidemiologic and pathogenic features of the different HPeV serotypes, including K251176-02.

HPeV2 CT86–6760 was genotypically as distinct from HPeV2 Williamson as from other HPeV types (Table 1). The existence of 2 genotypically divergent HPeV serotypes 2 is surprising and needs to be elucidated further. This finding, however, argues in favor of a universal typing method that is based on molecular characteristics (genotyping) instead of serotyping, provided classification criteria are well defined.

Table 2. Neutralization assay with LLcMk2 cells*

Virus	Antiserum			Viral controls
	α-HPeV1 (Harris)	α-HPeV2 (Williamson)	α-HPeV3 (A308-99)	
HPeV1 Harris	–	++++	++++	++++
HPeV2 Williamson	++++	–	++++	++++
K251181-02 (HPeV3)	++++	++++	–	++++
K251176-02	++++	++++	++++	++++

*Culture isolates of K251176-02, human parechovirus 1 (HPeV1, echovirus 22) and HPeV2 (echovirus 23) from a reference panel (National Institute for Public Health and the Environment, Bilthoven, the Netherlands) and K251181-02 that was previously genotyped as HPeV3 (*12*) were incubated with antisera (20 U/mL in Eagle minimal essential medium) directed against HPeV1 Harris, HPeV2 Williamson, and HPeV3 A308-99. The antisera to HPeV1 and HPeV2 were raised in horses. The antiserum to HPeV3 was raised in guinea pigs. Neutralization is done on a 96-microtiter plate containing a monolayer of LLcMk2 cells that have been incubated for 3 days. The assay was determined after viral controls (no antisera used) of the 4 culture isolates showed cytopathic effects >50% (++++).

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ICD-9 Codes and Surveillance for *Clostridium difficile*–associated Disease

Erik R. Dubberke,* Kimberly A. Reske,* L. Clifford McDonald,† and Victoria J. Fraser*‡

We conducted a retrospective cohort study to compare *Clostridium difficile*–associated disease rates determined by *C. difficile*–toxin assays and International Classification of Diseases, 9th Revision (ICD-9) codes. The correlation between toxin assay results and ICD-9 codes was good (κ = 0.72, p<0.01). The sensitivity of the ICD-9 codes was 78% and the specificity was 99.7%.

Clostridium difficile–associated disease (CDAD) is the most common infectious cause of healthcare-associated diarrhea (*1*). Recent studies suggest both the incidence and severity of CDAD may be increasing (*2–9*), but no national surveillance system exists to track CDAD rates. Some studies have used International Classification of Diseases, 9th Revision (ICD-9) codes of hospital discharges to study CDAD rates (*4*,*10*,*11*). The validity of this method has not been studied. We compared CDAD rates determined by ICD-9 codes to rates determined by *C. difficile*–toxin assays at a tertiary-care hospital to determine the sensitivity and specificity of ICD-9 code–based CDAD surveillance.

The Study

Data were collected electronically on a retrospective cohort of patients admitted to Barnes-Jewish Hospital in Saint Louis from January 1, 2003, through December 31, 2003. Patients who had only 1 admission of <48 hours and neonates were excluded. Electronic charts were reviewed for patients who had a positive *C. difficile*–toxin assay or the ICD-9 code indicating *C. difficile*–associated disease (008.45) (Appendix).

A case of CDAD was defined as a patient with a positive *C. difficile*–toxin assay (Tech Laboratory *C. difficile* tox A/B II toxin assay [Tech Laboratory, Blacksburg, VA, USA]) or pseudomembranes seen on colonoscopy. Because the hospital laboratory performs a *C. difficile*–toxin assay only on unformed stool samples and stool toxin testing is ordered based on clinical suspicion of CDAD, all patients with a positive toxin assay were considered CDAD case-patients.

Data were analyzed with SPSS 12.0 for Windows (SPSS, Inc., Chicago, IL, USA). Statistical analyses included κ, χ^2, and Mann-Whitney U tests. A 2-sided p value of 0.05 was considered significant. This study was approved by the Washington University Human Studies Committee.

A total of 45,486 admissions among 28,417 unique patients were included in the analysis (Figure 1). A *C. difficile*–toxin assay was ordered during hospitalization for 3,630 (8%) of these admissions. Toxin assays were positive (CDTA+) in 662 (18%) admissions. The *C. difficile* ICD-9 code was assigned to 745 admissions (ICD9+). The breakdown of admissions, according to toxin assay and ICD-9 status, was as follows: 506 had both a positive toxin assay and received the ICD-9 code (concordant; CDTA+/ICD9+), 156 had a positive toxin assay but no ICD-9 code (CDTA+/ICD9–), and 239 received the ICD-9 code but did not have a positive toxin assay (CDTA–/ICD9+) (Figure 1). The concordance between toxin assays and ICD-9 codes was good (κ = 0.72, p<0.01). The overall mean CDAD rate by ICD-9 codes (16.4/1,000 admissions) was significantly higher then the mean rate by toxin assays (14.6/1,000 admissions) (Figure 2; rate ratio [RR] 1.13, 95% confidence interval [CI] 1.01–1.25).

The median number of days from admission to stool collection was greater in admissions with a positive toxin assay but no ICD-9 code (CDTA+/ICD9–) than in concordant (CDTA+/ICD9+) admissions (6.0 days vs 3.0 days, p<0.01) (Table 1). The first positive stool sample was collected within 48 hours of discharge for 68 (44%) of admissions with a positive toxin assay only (CDTA+/ICD9–) admissions, compared with 72 (14%) of concordant (CDTA+/ICD9+) admissions (p<0.01).

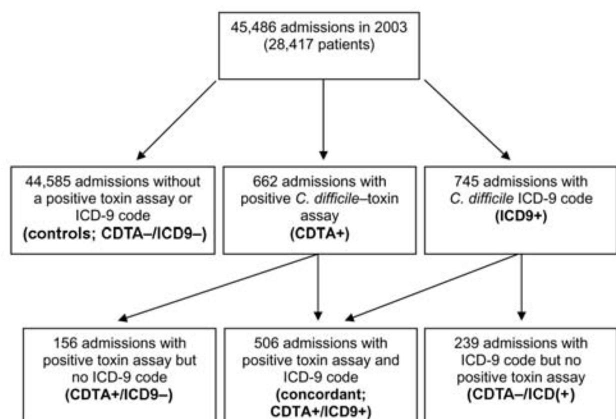

Figure 1. Flowchart of admission groups.

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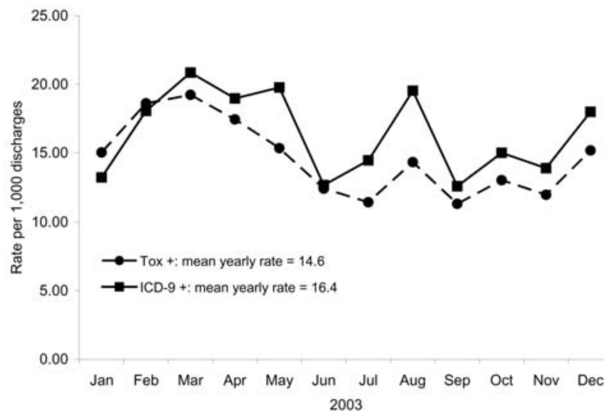

Figure 2. Monthly rates of *Clostridium difficile*–associated disease by diagnosis type.

Upon chart review, documentation of a previous history of CDAD was evident in 142 (59%) of the ICD-9 code only (CDTA–/ICD9+) admissions. A *C. difficile*–toxin assay had been ordered in 137 (57%) of all ICD-9 code only (CDTA-/ICD9+) admissions. One-hundred thirty (54%) had at least 1 stool negative for *C. difficile* toxin.

Overall, 92% of patients with positive toxin assay (CDTA+) and 90% of patients with ICD-9 code only (CDTA–/ICD9+) received antimicrobial drug treatment for CDAD (Table 2). Metronidazole was prescribed in 187 (78%) of the ICD-9 code only (CDTA–/ICD9+) admissions, compared with 591 (89%) of patients with a positive toxin assay (CDTA+) ($p<0.01$). For 75 (31%) of the ICD-9 code only (CDTA–/ICD9+) admissions, oral vancomycin was prescribed, compared with 130 (20%) of patients with a positive toxin assay (CDTA+) ($p<0.01$).

Thirty-four cases of CDAD were missed by *C. difficile*–toxin assay results and subsequently identified through chart review (3 missed positive toxins, 26 CDAD patients transferred from other facilities, 3 positive outpatient toxin assays, and 2 diagnosed by colonoscopy), which brought the total number of cases with positive CDAD diagnostics to 696. ICD-9 codes correctly identified 540 of these cases and correctly classified 44,741 admissions as non-CDAD admissions (sensitivity 78%, specificity 99.7%). When the CDAD rate by toxin assays was adjusted for the additional cases, the adjusted CDAD rate was 15.3/1,000 admissions. This rate was not significantly different from the unadjusted CDAD rate by toxin assay results (RR 0.95, 95% CI 0.86–1.06) or the rate by ICD-9 codes alone (RR 1.07, 95% CI 0.97–1.19).

Conclusions

Overall, there was good correlation between *C. difficile*–toxin assay results and ICD-9 codes. Initially, the CDAD rate by ICD-9 codes appeared higher than the rate by toxin assays. However, once the additional CDAD cases identified through chart review were added, this difference was not significant.

Admissions with only a positive *C. difficile*–toxin assay and no *C. difficile* ICD-9 code (CDTA+/ICD9–) were more likely than concordant (CDTA+/ICD9+) admissions to have their first positive toxin assay within 48 hours of discharge. For these admissions, toxin assay results may not yet have been back at the time of discharge or CDAD may not have been considered a primary diagnosis by the physician and therefore not captured by the medical coders.

Antimicrobial drug treatment patterns suggest ICD-9 code only (CDTA–/ICD9+) admissions were patients who were more likely to have a history of CDAD. Metronidazole is first-line therapy for CDAD at our institution. Oral vancomycin is reserved for recurrent or severe cases. The observation that more ICD-9 code only (CDTA–/ICD9+) patients received oral vancomycin indicates that recurrent CDAD may have been suspected in these patients. In these patients, CDAD appears to have been diagnosed on the basis of the patient's history and symptoms instead of by a positive *C. difficile*–toxin assay. This pattern has been previously reported (*12*).

True CDAD cases may have been misclassified among the controls. A patient who did not have a positive *C. difficile*–toxin assay, who was not assigned the CDAD ICD-9 code, and whose diagnosis was made by colonoscopy would have been missed. However, misclassification is unlikely for two reasons. First, after charts were reviewed, only 2 additional patients were identified whose diagnosis

Table 1. Demographic characteristics of study population by *Clostridium difficile*–toxin assay (CDTA) and ICD-9 status*

Characteristic	Controls, n = 44,585 (%)	CDTA+/ICD-9–, n = 156 (%)	CDTA+/ICD-9+, n = 506 (%)	CDTA–/ICD-9+, n = 239 (%)
Age (median y)	55	64	67	66
Length of hospitalization (median d)	4	13	12	6
Female	25,869 (58)	68 (44)	267 (53)	158 (66)
White	28,071 (63)	110 (71)	347 (69)	170 (71)
Time from admission to stool collection (median d)	NA	6.0	3.0	NA
First positive stool collected within 48 h of discharge	NA	68 (44)	72 (14)	NA

*NA, not available.

Table 2. Comparison of antimicrobial treatment for *Clostridium difficile*–associated disease among patient admissions with a positive *C. difficile*–toxin assay (CDTA+) and patients without a positive toxin assay but with ICD-9 code for *C. difficile* disease (CDTA–/ICD9+)(categories not mutually exclusive)

Treatment	CDTA+, n = 662 (%)	CDTA–/ICD9+, n= 239 (%)	Odds ratio	p value
Any treatment for CDAD	607 (92)	214 (90)	0.78	0.35
Metronidazole	591 (89)	187 (78)	0.43	<0.01
Oral vancomycin	130 (20)	75 (31)	1.87	<0.01
Oral vancomycin and metronidazole	114 (17)	48 (20)	1.21	0.32

was made by colonoscopy alone. Second, the detection of CDAD cases transferred from other institutions indicates that CDAD cases diagnosed by methods other than the toxin assays are being captured by ICD-9 codes.

Use of ICD-9 codes to study CDAD rates has advantages and disadvantages. ICD-9 codes are readily available from billing databases. In the absence of a national surveillance system for CDAD, ICD-9 codes provide a standard method for determining CDAD rates that can be used at all types of hospitals. However, because ICD-9 codes are assigned at discharge and not on the date of diagnosis, determining which cases are hospital acquired and which are community acquired is difficult. Also, ICD-9 codes are assigned by medical coders, who may not be able to accurately identify a patient's principal diagnoses as well as a physician or medical professional. Despite these limitations, ICD-9 codes can likely be used to identify CDAD cases and track CDAD rates when *C. difficile*–toxin assay results are not available.

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Appendix

Details on ICD-9 Codes

The International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9) code used in this study was 008.45, "intestinal infection due to *Clostridium difficile*," and is the only ICD-9 code related to CDAD. To apply this code, medical coders must have documentation in a patient's medical record by the treating medical providers that a patient's gastroenteritis or colitis is due to *C. difficile*. Positive laboratory tests alone are not sufficient to warrant application of the code. At our institution, ICD-9 coding occurs, on average, 5–7 days after a patient is discharged from the hospital.

The ICD-9 system of classifying hospital discharge diagnoses is used throughout the United States. The definition for the code 008.45 is consistent between hospitals, although individual coding practices may vary. Although ICD-9 codes have limitations, they are readily available from administrative databases and have been used frequently to identify diagnoses and classify comorbidities (*1*).

A move to the International Classification of Diseases, 10th Revision, Clinical Modification (ICD-10) system is anticipated for US hospitals but the exact time of this transition is not yet known. The ICD-10 system does include a code for CDAD (A04.7, Enterocolitis due to *C. difficile*), so the ICD-based system presented here could be modified to be used with the updated coding system.

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Chikungunya Outbreaks Caused by African Genotype, India

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Chikungunya fever is reported in India after 32 years. Immunoglobulin M antibodies and virus isolation confirmed the cause. Phylogenic analysis based on partial sequences of NS4 and E1 genes showed that all earlier isolates (1963–1973) were Asian genotype, whereas the current and Yawat (2000) isolates were African genotype.

Chikungunya virus (CHIKV) outbreaks have been documented in Africa and Southeast Asia. In India, the first CHIKV outbreak was recorded in 1963 in Calcutta and was followed by epidemics in Chennai, Pondicherry, and Vellore in 1964; Visakhapatnam, Rajmundry, and Kakinada in1965; Nagpur in1965; and Barsi in 1973 (*1*). Recently, CHIKV has emerged in Southeast Asia and the Pacific region (*2–4*). Massive outbreaks have been reported from many islands in the Indian Ocean (*5*). *Aedes albopictus* is considered the vector in Reunion and other islands in the Indian Ocean (*5*), but *Ae. aegypti* is the main vector in Asia, including India (*1*). We investigated a large number of patients with fever with arthralgia, reported from October 2005 through March 2006, in many districts from Andhra Pradesh, Karnataka, and Maharashtra states.

The Study

Blood samples were collected from 1,938 suspected case-patients from the 3 states; serum was separated and transported to the laboratory on wet ice. Adult mosquitoes were collected from houses and sheds. Larval mosquitoes were collected from the affected areas by single-larva survey method. Adult household indexes and Breteau indexes were calculated for each area (*6*).

The C6/36 cell line was used for virus isolation (*7*). Immunoglobulin M (IgM) antibodies to CHIKV (IgM anti-CHIK) and dengue virus (IgM anti-dengue) (*8*) were assayed by IgM capture ELISA. For CHIKV ELISA, brain suspensions from mice infected with CHIKV were the source of antigen, and monoclonal antibodies were the source of antibodies (*9*). Dengue/CHIKV IgM antibodies and negative control human sera were included for respective tests. Approval for use of mice for antigen preparation was obtained from the institutional ethical committee according to national guidelines.

Immunofluorescence assay (IFA) was used to detect the virus in cell culture and in crushed heads of adult mosquitoes (*10*). A patient with the following was confirmed as having CHIKV infection: acute onset of moderate-to-high fever with joint pain of varying severity; negative test results for malaria, typhoid, and tuberculosis; and positive results for IgM anti-CHIKV antibodies, seroconversion, or CHIKV isolation. We used χ^2 test to compare proportions of cases in different age groups.

We studied CHIKV isolates obtained during current investigations and viruses isolated during earlier epidemics in India (1963–2000) (Table 1). RNA was isolated by using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Superscript II (Invitrogen, Carlsbad, CA, USA) was used for reverse transcription (42°C for 1 h). Initially, *Alphavirus* genus–specific primers that produced a 472-bp fragment (NS4 gene) were F1 5′ GAY GCI TAY YTI GAY ATG GTI GAI GG 3′ and R1 5′ KYT CYT CIG TRT GYT TIG TIC CIGG 3′ (*11*). The second set of primers that amplified a 294-bp product of E1 gene were CHIK/E1-S 5′ TAC CCA TTC ATG TGG GGC 3′ and CHIK/E1-C 5′ GCC TTT GTA CAC CAC GATT 3′ (*12*). For amplification, Platinum *Pfx* enzyme (Invitrogen) was used. Cycling conditions were 1 cycle at 94°C for 5 min; then 35 cycles each of 94°C (1 min), 50°C (1 min), and 68°C (1.5 min); followed by final extension of 7 min at 68°C. The PCR products were purified by using QIAquick PCR Purification Kit (Qiagen) and sequenced by using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an automatic sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems).

Using ClustalX, version 1.83, multiple alignments of nucleotide sequences were performed. The phylogenic status of the CHIKV isolates was assessed with the software MEGA 3.1 (*13*), Kimura 2-parameter distance, and neighbor-joining algorithm. The reliability of different phylogenic groupings was evaluated with the bootstrap test (1,000 bootstrap replications) available in MEGA.

Acute onset of moderate-to-high fever in association with body ache, backache, and headache was recorded. Joint pain of varying severity occurred within 2 days of onset of fever and, in decreasing order of affliction, involved knees, ankles, wrists, hands, and feet. Joint pain was severe and incapacitating and lasted for weeks to months. Inflammation of joints and transient macular rash on earlobes, neck, trunk, and upper extremities were

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Table 1. Chikungunya isolates sequenced, India, October 2005–March 2006

Identification no.	Location, state	Host	Year	GenBank accession no.
IND06KA2	Hajnal village, Karnataka	Human	2006	DQ520739
IND06KA3	Hajnal village, Karnataka	Human	2006	DQ520738
IND06AP4	Kalkada village, Andhra Pradesh	Human	2006	DQ520743
IND06AP6	Kalkada village, Andhra Pradesh	Human	2006	DQ520745
IND06AP5	Mungilipattub, Andhra Pradesh	Human	2006	DQ520744
IND06AP3	Devalammanagaram, Andhra Pradesh	Human	2006	DQ520742
IND06MS2	Kalkada, Andhra Pradesh	Mosquito	2006	DQ520740
IND06MS1	Devalammanagaram, Andhra Pradesh	Mosquito	2006	DQ520741
IND06MH1	Parbhani, Maharashtra	Human	2006	DQ520734
IND06MH2	Parbhani, Maharashtra	Human	2006	DQ520735
IND06MH3	Parbhani, Maharashtra	Human	2006	DQ520736
IND05 KA1	Kotgyal village, Karnataka	Human	2005	DQ520737
IND00MH4	Yawat, Maharashtra	Mosquito	2000	DQ520753
IND73MH5	Barsi, Maharashtra	Human	1973	DQ520752
IND71CH1	Chennai, Tamil Nadu	Human	1971	DQ520751
IND65MH6	Nagpur, Maharashtra	Human	1965	DQ520750
IND65MH7	Nagpur, Maharashtra	Human	1965	DQ520749
IND65AP7	Vishakhapattanam, AP	Human	1965	DQ520754
IND64CH2	Chennai, Tamil Nadu	Human	1964	DQ520748
IND63WB2	Calcutta, West Bengal	Human	1963	DQ520747
IND63WB1	Calcutta, West Bengal	Human	1963	DQ520746

reported for a few patients. Hemorrhage did not occur. The cases were reported predominantly from rural areas; distribution was focal. Multiple cases were recorded in families. All ages and both sexes were affected; significantly more cases occurred in persons aged ≥15 years (299 [89.8%] of 333, p<0.001). Cases were reported from 11 of 23 districts in Andhra Pradesh, 15 of 27 in Karnataka, and 16 of 35 in Maharashtra (Figure 1). Results of serologic testing and virus isolation are shown in Table 2.

State governments of Andhra Pradesh, Karnataka, and Maharashtra have declared outbreaks of CHIKV. By mid-April, the declared numbers of fever cases associated with this outbreak were >25,000 in Andhra Pradesh, >65,000 in Maharashtra, and >36,000 in Karnataka. In absence of active surveillance for this disease, these numbers may be underestimates.

The predominant mosquito species in the affected areas was *Ae. aegypti*. *Ae. albopictus* was either absent or present in negligible numbers. The population of *Ae. aegypti* was reasonably high in most of the localities; adult household indexes and Breteau indexes, respectively, were 10–60 and 13–75 in Andhra Pradesh, 20–70 and 40–200 in Karnataka, and 10–30 and 30–50 in Maharashtra. High density of *Ae. aegypti* populations in affected areas and 23 isolations or detections of CHIKV from adult mosquitoes indicates that this species is the main vector in India. Earlier outbreaks in India were mainly restricted to large cities; in contrast, the current outbreak is predominantly rural.

Anti-CHIKV IgM was detected in 33.5% to 41.9% of patients tested. The finding of antibodies to dengue virus in 0.9% to 9.9% of patients and to CHIKV and dengue virus in 0.4% to 4.3% of patients indicates that these viruses cocirculate in the area. Nine patients whose acute-phase serum sample was negative had anti-CHIKV IgM in the early convalescent-phase sample, collected during the second week of illness.

NS4-based phylogenic analysis identified the Yawat isolate (2000) from Maharashtra as central/East African genotype, not Asian genotype as reported earlier (*14*). This

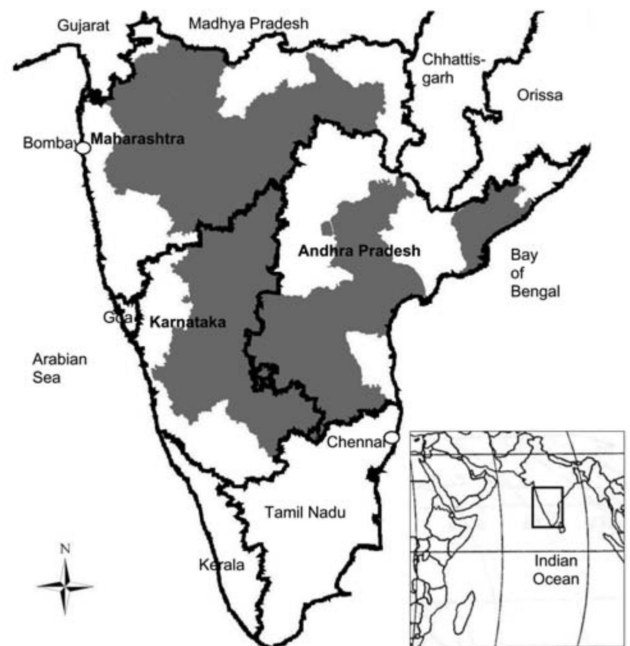

Figure 1. Southern India, 3 states affected by chikungunya virus (October 2005–March 2006). Gray shading indicates area affected in each state.

Table 2. Results of serologic testing and virus isolation, India, October 2005–March 2006*

	State		
	Karnataka	Maharashtra	Andhra Pradesh
No. blood samples	900	473	565
Anti-CHIKV IgM, n/N (%)	303/900 (33.7)	169/473 (35.7)	251/565 (44.4)
Anti-dengue IgM, n/N (%)	19/191 (9.9)	23/473 (4.9)	3/325 (0.9)
Anti-CHIKV and anti-dengue IgM, n/N (%)	1/191 (0.5)	2/473 (0.4)	14/325 (4.3)
CHIKV, human serum, n	83	9	20
CHIKV, *Aedes aegypti*, n	4	11	8

*CHIKV, chikungunya virus; IgM, immunoglobulin M.

finding led us to resequence all isolates in our repository. Phylogenic analyses based on NS4 (Figure 2A) and E1 regions (Figure 2B) yielded identical results. The Indian viruses isolated from 1963 through 1973 belonged to the Asian genotype, whereas the current isolates from the 3 Indian states and the Yawat isolate belonged to the central/East African genotype. Within the Asian genotype, all older isolates (India 1963–1973 and Thailand 1962–1978) clustered together, whereas later isolates from the Philippines (1985), Indonesia (1985), Thailand (1988, 1995, 1996), and Malaysia (1998) formed a distinct cluster. The sequence from Reunion Islands, which represents a recent outbreak of the disease (GenBank accession no. DQ443544), also grouped with the recent Indian isolates. Percentage nucleotide identity within earlier (1963–1973) and recent (2005–2006) Indian isolates was 99.71% ±

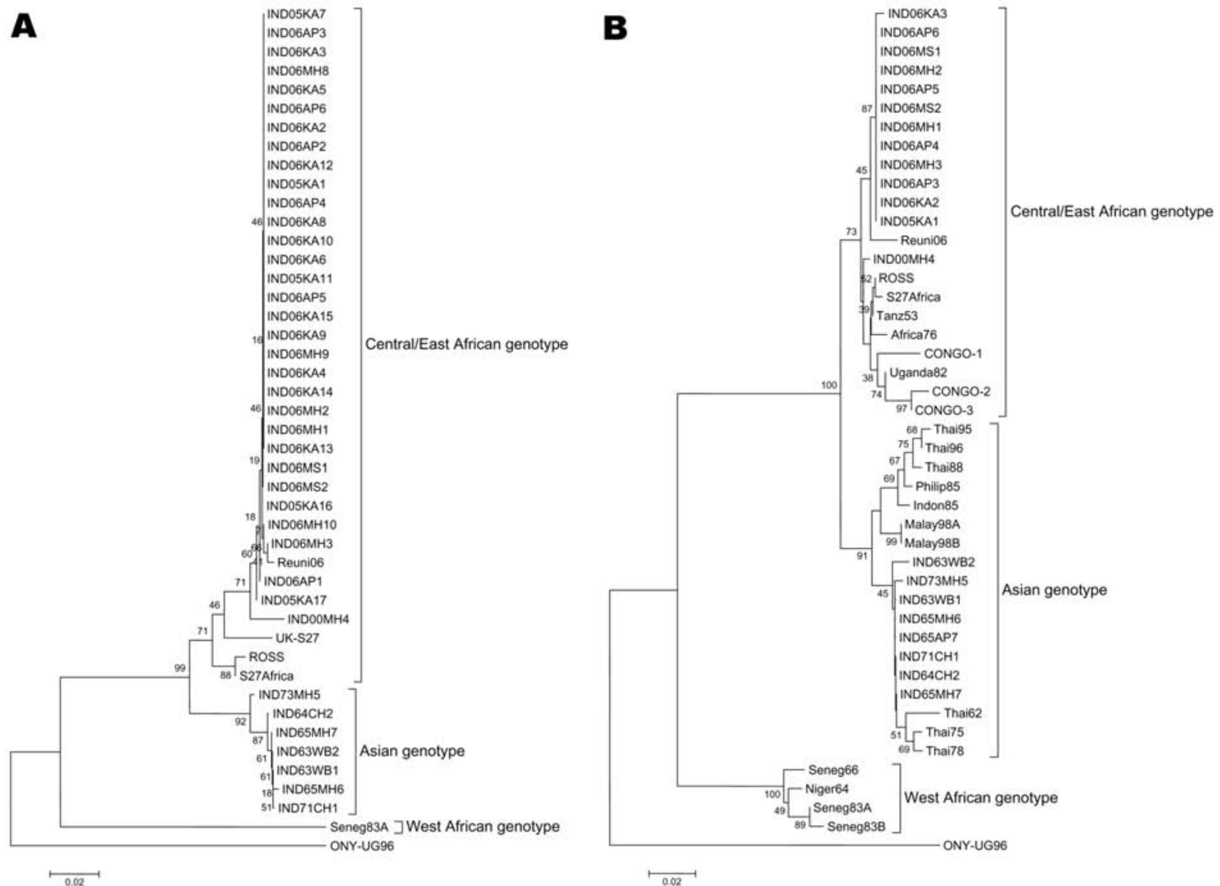

Figure 2. Phylogenic analyses of partial NS4 (456 nt, panel A) and E1 (294 nt, panel B). Refer to Table 1 for the details of the isolates sequenced during this study. Percentage bootstrap support is indicated by the values at each node. The following sequences were obtained from GenBank database: E1, ROSS (AF490259); S27Africa (NC-004162); Tanz53 (AF192905); Africa76 (AF192903); CONGO1 (AY549583); CONGO2 (AY549581); CONGO3 (AY549579); Uganda82 (AF192907); Thai95 (AF192897); Thai96 (AF192900); Thai88 (AF192896); Thai62 (AF192908); Thai75 (AF192898); Thai78 (AF192899); Philip85 (AF192895); Indon85 (AF192894); Malay98A (AF394210); Malay98B (AF394211); Seneg83A (AY726732); ONY-UG96 (AF079456); Reuni06 (DQ443544); Seneg83B (AF192892); Niger64 (AF192893); Seneg66 (AF192891); NS1, UK/S27 (AF345888); ROSS (AF490259); S27Africa (NC-004162); Seneg83A (AY726732); ONY-UG96 (AF079456); and Reuni06 (DQ443544). O'nyong-nyong virus (AF079456) was used as an outgroup.

0.16% and 99.94% ± 0.05%, respectively, whereas percentage nucleotide identity between these isolates was 96.11% ± 1.09%. The 2005–2006 Indian isolates were 98.61% ± 0.6% and 98.95% ± 0.57% identical with the Reunion and Yawat isolates, respectively.

Conclusions

This report confirms CHIKV as the causative agent for large outbreaks of fever with arthralgia and arthritis in 3 Indian states. Thus, chikungunya fever has emerged in outbreak form after 32 years.

The current epidemic is caused by central/East African genotype of CHIKV. That the Yawat isolate is grouped with central/East African genotype suggests that this genotype had been introduced ≥5 years before the current outbreaks. In this context, determining the genotype of currently circulating strains in Southeast Asia and understanding the modes of transportation of this strain in India and the conditions favoring such large outbreaks would be worthwhile.

Acknowledgments

We thank the staff of the health departments of Maharashtra, Karnataka, and Andhra Pradesh for help and support during the field investigations. We also thank M.V. Joshi and P.V.M. Mahadev for valuable guidance during field investigations, Kailash B. Gadekar and Ruben P. George for technical support during field investigations, and Santosh M. Jadhav and Atul M. Walimbe for data analysis and illustrations.

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Methicillin-resistant *Staphylococcus aureus* in Dutch Soccer Team

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An outbreak of community-acquired methicillin-resistant *Staphylococcus aureus* occurred among members and close contacts of a soccer team. Typing of the isolates showed the outbreak was caused by the well-known European ST80-IV strain. To our knowledge, this is the first report of an outbreak of this strain among members of a sports team.

Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is emerging as a cause of skin and soft tissue infections. CA-MRSA differs from hospital-associated MRSA in several ways (*1*). Most CA-MRSA isolates contain the virulence factor Panton-Valentine leukocidin (PVL) and carry staphylococcal cassette chromosome (SCC) *mec* type IV or V (*2*). In the Netherlands the prevalence of MRSA is still low (≈1%) (*3*), and ≈10 % of all MRSA isolates carry the genes for PVL (*4*).

Several reports have been published about CA-MRSA outbreaks among sports team members, communities, prisoners, men who have sex with men, military personnel, and drug users (*5*). Most of these cases occurred in the United States and were caused by USA300 MRSA sequence type (ST) 8 strains. In Europe the ST80-MRSA-IV strain is common (*4,6*) but has not been reported to have caused an outbreak in sports teams. A CA-MRSA outbreak in a British rugby team has been reported (*7*), but given the reported phage type, this strain was not ST80-MRSA-IV. So far, CA-MRSA outbreaks have usually occurred in intensive contact sports such as football (*8–10*), rugby (*7*), and wrestling (*11*).

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We report on the results of the screening that was conducted after MRSA was isolated from a boil on a Dutch soccer player and, subsequently, soft-tissue infections developed on other members of the soccer team.

The Study

Starting in June 2005, several players of a Dutch soccer team, consisting of ≈35 members, noted soft-tissue infections. In October 2005, the municipal health service received a report of a patient, a member of this soccer team, who had been hospitalized for an abscess resulting from MRSA. Other members of the team had skin infections as well, and screening was started.

A case was defined as a patient who had a culture-confirmed MRSA infection during the outbreak period October 2005 through January 2006. Healthcare staff obtained specimens by swabbing the patients' nose, throat, or wound. A total of 56 persons were screened: 42 members of the soccer club and 14 of their roommates. The 42 members consisted of soccer players, coaches, and people who used the same training facilities, locker room, and showers. The roommates screened were all those who lived with an MRSA-positive player and those who lived with an MRSA-negative player but had skin infections. Of the 56 persons screened, we identified an MRSA infection in 11 persons: 9 soccer players and 2 roommates (Table). Most infections lasted for several weeks. For all players who had soft tissue infections, MRSA was diagnosed. Among those in whom infections did not develop, no carriage of MRSA was found. One roommate had an abscess in the armpit, but the soccer player she lived with was MRSA negative. The mean age of the patients was 31 years (range 18–43). No histories of travel were reported.

To prevent further MRSA transmission, on October 28 the soccer club was instructed to increase hand hygiene, not share personal items, use liquid soap and disposable towels, put a towel on the bench before sitting, increase frequency of cleaning the facilities, and provide more ventilation to the locker room and showers. All patients were treated with cotrimoxazole for 1 week or, if needed, longer until wound infections were healed. Seven patients remained MRSA positive or had recurring wound infections. Perineum cultures from 3 patients showed carriage of MRSA in the gastrointestinal tract, and rifampicin was added to cotrimoxazole for 1 week or, if needed, longer until wounds were healed. Patients were advised to use clean bedding and clothing every day. Furthermore, patients used chlorohexidine, gluconate scrub, povidone iodine, and mupirocin ointment for 5 days. A patient was declared MRSA free after 3 cultures, taken at 1-week intervals, were MRSA negative. One patient, who also had eczema, remained MRSA positive.

Table. Characteristics of MRSA infections, soccer players and their contacts, the Netherlands, 2005*

No.	Patient	Age, y	Type of infection	Site of infection	MRSA carriage in nose	MRSA carriage in GI tract	Recurring infection
1	Player†	34	Abscesses	NA	NA	NT	NA
2	Player	30	Boil	Leg	Yes	NT	Yes
3	Player	43	NS	Arm	Yes	Yes	Yes
4	Player	33	NS	Forearm	Yes	Yes	Yes
5	Player	33	Boil	Leg	No	NT	No
6	Player	34	Boil	Knee	No	NT	No
7	Player	22	Boil	Leg	Yes	NT	Yes
8	Roommate of no.4	31	NS	Face	NA	No	NA
9	Player	18	NS	Buttock	Yes	Yes	No
10	Player	20	NS	Heel	Yes	NT	No
11	Roommate of an MRSA-negative player	39	Abscesses	Armpit	No	Yes	No
12	Opponent	33	Boil	Leg	NA	NT	No

*MRSA, methicillin-resistant *Staphylococcus aureus;* GI, gastrointestinal; NA, not available; NT, not tested; NS, not specified.
†This player was the index case-patient and was hospitalized.

In November 2005, MRSA was isolated from a 33-year-old soccer player with a boil, who was a member of a neighboring team. This soccer player had competed against the team with the MRSA-positive players on October 8, before MRSA-positive results were known and hygiene measures recommended. Immediately, this player's team was screened for MRSA; all other players were MRSA negative. The MRSA isolate from this player was included in the analysis.

To characterize the MRSA strains, the following typing methods were used: pulsed-field gel electrophoresis (PFGE), staphylococcal protein A (*spa*) typing, multilocus sequence typing, SCC*mec* typing, and PCR of PVL genes (*LukS-LukF*). All MRSA isolates were identical and identified as the European CA-MRSA ST80-MRSA-IV strain. All strains were PFGE type 28 (according to the Dutch PFGE classification system), *spa* type t044, ST80, SCC*mec* IV, and PVL positive. All MRSA isolates had identical susceptibility patterns; they were resistant to oxacillin (and thus to all β-lactam antimicrobial drugs), tetracycline, and fusidic acid. They were susceptible to rifampicin, ciprofloxacin, gentamicin, erythromycin, clindamycin, vancomycin, teicoplanin, and cotrimoxazole.

Conclusions

This study shows transmission of the CA-MRSA ST80-IV strain among members of a Dutch soccer team. Transmission apparently occurred not only between members of this team but also to a competing soccer team. Transmission of the USA300 strain between members of different teams was previously described for football teams (*8*). We cannot rule out the possibility of an independent colonization of the ST80-MRSA-IV strain in the competing team, but the 2 teams competed against each other during the period when the first symptoms were noted by members of the first team. Because soccer involves much less contact than football, rugby, or wrestling, MRSA transmission may not necessarily have been caused by skin-to-skin contact but could also have occurred by sharing equipment or personal items. This possibility has also been suggested in a report about a CA-MRSA outbreak among sports participants (*12*).

To our knowledge, this is the first report of an outbreak of the CA-MRSA ST80-IV strain in a sports team. As with the USA300 strain, more outbreaks of CA-MRSA ST80-IV are likely. Therefore, to identify and control an outbreak as early as possible, sports physicians and coaching staff should be alerted and informed about CA-MRSA.

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Helminth-related Eosinophilia in African Immigrants, Gran Canaria

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Of 788 recent African adult immigrants to Las Palmas de Gran Canaria, 213 (27.0%) had eosinophilia. The most frequent causes were filariasis (29.4%), schistosomiasis (17.2%), and hookworm infection (16.8%). Stool microscopy and filarial and schistosomal serologic tests gave the highest diagnostic yield. Country of origin and eosinophil count were associated with specific diagnoses.

We prospectively evaluated the prevalence and causes of eosinophilia in recent adult immigrants from Africa; the diagnostic usefulness of parasitologic and serologic tests; and the relationship between specific helminthic infections, country of origin, and degree of eosinophilia. After they gave written consent, 788 African immigrants were screened by examination of detailed medical records, physical examination, routine laboratory tests, serologic tests, the Mantoux test, and chest radiographs. Of the immigrants, 213 met the following inclusion criteria: 1) arrival within 6 months; 2) age $\geq$18 years, and 3) eosinophilia ($\geq 0.45\times10^9$ eosinophils/L). Direct parasitologic tests included the examination of 3 stool samples (both Kato-Katz and Ritchie techniques were used for each sample) and specific tests for *Strongyloides stercoralis* (Baermann test and agar culture) (*1*), optic microscopy of a terminal urine specimen, and Knotts test for microfilaremia. The immune chromatographic test for *Wuchereria bancrofti* (ICT Filariasis Binax, Portland, ME, USA), skin snips, and the Mazotti test were also used in selected cases.

ELISAs with crude extracts of adult *Dirofilaria immitis* adult worm antigens (AWA Di) (*2*), *Schistosoma bovis* worm antigens (*3*), *Fasciola hepatica* excretory/secretory antigens (*4*), and *Trichinella spiralis* L1 antigens (*5*) were used. Polystyrene microtiter plates were coated with 100 µL antigens per well in carbonate buffer (pH 9.6). Serum diluted 1:100 was added and incubated for 1 h at 37°C. Horseradish peroxidase goat anti-human immunoglobulin G (Sigma, Saint Louis, MO, USA) was added at different dilutions. Washes were performed 3 times with 200 µL phosphate-buffered saline–Tween 20 per well. After incubation for 1 h at 37°C, the substrate solution (ortho-phenylenediamine-H_2O_2) was added, and the reaction was stopped with 3N H_2SO_4.

Assay sensitivities were evaluated by using serum specimens from patients with a definite diagnosis of isolated helminthic disease (Table 1). In all patients, adequate parasitologic tests showed no other helminthic infection. To evaluate specificities, we used serum samples from Spanish blood donors; samples from healthy controls from sub-Saharan Africa; and samples from patients with isolated helminthic, protozoal, bacterial, or viral infections (Table 1). Healthy controls from sub-Saharan Africa were clinically evaluated; they did not have eosinophilia, and results of a systematic investigation for helminthic infections (using stool samples, urine samples, and Knotts test) were negative.

Moreover, an ELISA was used to test for strongyloidiasis with somatic larvae antigens from *Strongyloides venezuelensis*. Although the ELISA is 100% sensitive, its low specificity precluded its use as a diagnostic tool.

The SPSS 11.5 statistical package (available from http://www.spss.com) was used for analyses. The level of significance accepted was <0.05, and results were expressed as means plus standard deviation (SD). The receiver-operating-characteristic curve was used to establish ELISA cut-offs. The χ^2 and the Fisher exact tests were used to evaluate the association between demographic variables and final diagnoses, and the Student *t* test was used to compare the degree of eosinophilia among patients with single and multiple infections. Analysis of variance and post-hoc tests were used to compare the mean eosinophil counts in each final diagnosis.

We found that 213 (27.0%) of 788 immigrants whose conditions were analyzed had eosinophilia. Of these, 191 (89.7%) were male, with a mean age of 27.4 years (SD 8.3). Two hundred two (94.9%) patients were from sub-Saharan countries, mainly Nigeria (24.1%), Sierra Leone (17.3%), Ghana (15.0%), and Mali (8.9%); 165 (77.1%) patients had $0.450–0.999\times10^9$ eosinophils/µL, 47 (21.9%) had $1.000–2.999\times10^9$ eosinophils/µL, and 1 patient had $>3.000\times10^9$ eosinophils/µL.

One hundred fifty-four study participants (72.3%) were asymptomatic. In symptomatic patients (28.0%), the most frequent clinical features were lymphadenopathy (6.1%), pruritus (5.6%), and skin lesions (3.3%).

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[1]These authors contributed equally to this article.

Table 1. Characteristics of immunodiagnostic tests*

Test	Antigen	µg per well of antigens	Serum dilution	Anti-IgG peroxidase dilution	Sensitivity, %†	Specificity, %‡
Schistosoma spp.	AWA *S. bovis*	0.05	1:100	1:2,000	94	97
Filaria	AWA *Dirofilaria immitis*	0.08	1:100	1:5,000	90	97
Fasciola spp.	E/S *F. hepatica*	0.04	1:100	1:2,000	100	96
Trichinella spp.	L1 *T. spiralis*	0.03	1:100	1:2,500	100	91

*IgG, immunoglobulin G; AWA, adult worm antigens; E/S, excretory/secretory antigens; L1, larvae 1 antigens.
†Sensitivity: serum samples from patients infected with schistosomiasis (35), tropical filariasis (20), fascioliasis (12), and trichinellosis (3) were used.
‡Specificity: serum samples from healthy controls from sub-Saharan Africa (41), from healthy Spanish blood donors (52), from patients with other isolated helminthic infections (45), from patients with protozoa infections (25), and from patients with bacterial or viral infections (19) were used.

A final diagnosis was made in 161 cases (75.6%): 116 (54.5%) had 1 parasite, 30 (14.1%) had 2, and 15 (7.0%) had ≥3. The most frequent parasites were filariae (n = 63, 29.6%), schistosomes (n = 37, 17.4%), hookworms (n = 36, 16.8%), and *Trichuris* spp. (n = 18, 8.4%) (Figure 1). Direct methods were used in 60 (37.2%) patients, indirect methods were used in 80 (49.6%), and both methods in 21 (13.0%) patients. Stool microscopy and filarial and schistosomal serologic testing yielded the highest positive result rates (Table 2). The country of origin was statistically associated ($p<0.05$) with the final diagnosis: 77% of the patients with eosinophilia from Cameroon had filariasis, 63% of the patients from Mali had schistosomiasis, and 30.8% of the patients from Nigeria had hookworm infection.

The mean eosinophil count was significantly higher in patients with a final diagnosis than in those whose conditions were not diagnosed (871 ± 431 vs. 643 ± 179) ($p<0.05$), and the mean count was higher also in patients with 2 or more parasites than in patients with 1 (1,045 ± 641 vs. 827 ± 389) ($p<0.05$). Among patients with 1 helminthic disease, those with filariasis had higher eosinophil counts than those with schistosomiasis or geohelminthic infection ($p<0.05$) (Figure 2).

Eosinophilia is frequent in travelers and expatriates from tropical areas (*6–12*). However, its prevalence is variable (3.1%–50%), depending on the population studied (more frequent in immigrants than in travelers), the areas where infection occurs (mainly sub-Saharan Africa or Southeast Asia), and the design of the study (prospective or retrospective). In this prospective work, we studied a homogeneous population of immigrants who had recently arrived from Africa, and we detected eosinophilia in 27%.

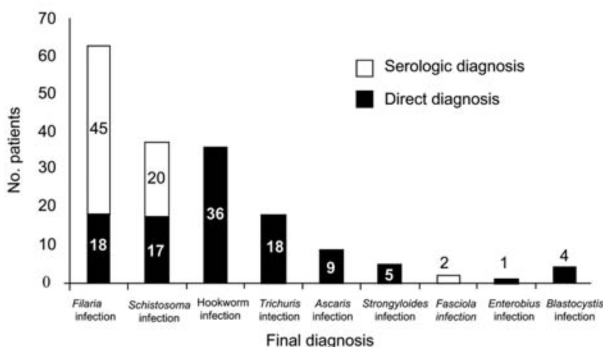

Figure 1. Final diagnosis of patients with eosinophilia. Filarial species detected by direct methods were *Mansonella perstans* (n = 13), *Loa loa* (n = 4), and *Onchocerca volvulus* (n = 1). Schistosomal species diagnosed by direct methods were *Schistosoma hematobium* (n = 10), *S. mansoni* (n = 6), and *S. intercalatum* (n = 1).

Studies of persons with imported eosinophilia have made a diagnosis that identified the etiologic agent in 15% to 64% of cases (depending on the population, the selected eosinophil count, and the methods) (*6–13*). Using direct and serologic methods (*10,13*), we detected helminthic infections in 75% of the patients. In all series, the main diagnoses are filarial, schistosomal, and geohelminthic infections. Only 27.7% of our patients had related signs or symptoms, which indicates that a proper investigation can detect many asymptomatic infections.

The sensitivities of our serologic tests were >90%, with specificities of 85%–97%. Using *D. immitis* antigens for the immunodiagnosis of tropical filariasis (*14*), we obtained a sensitivity of 90% for microfilaremia, with 97% specificity. The utility of adult worm antigens of *S. bovis* for serodiagnosis of schistosomiasis has been recently demonstrated (*3*).

Our high diagnostic yield with filarial (30%) and schistosomal (28%) serologic testing is similar to that obtained by Whetham et al. in travelers returning from West Africa (*10*). Among the direct methods, stool microscopy was the most sensitive (35%). However, serologic testing detected another parasitic infection (mainly filarial or schistosomal) when direct tests showed only a geohelminthic infection

Table 2. Diagnostic yield of etiologic tests*

Test	Test done, no. (%)	Yield of test, %
Stool (microscopy)	175 (81)	35
Filarial serology	189 (92)	30
Schistosoma spp. serology	213 (100)	28
Urine (microscopy)	66 (30)	16
Knotts test	123 (57)	13
Trichinella spp. serology	208 (97)	11
Fasciola spp. serology	209 (97)	7
ICT *Wuchereria bancrofti*	71 (33)	4

*ICT, immune chromatographic test.

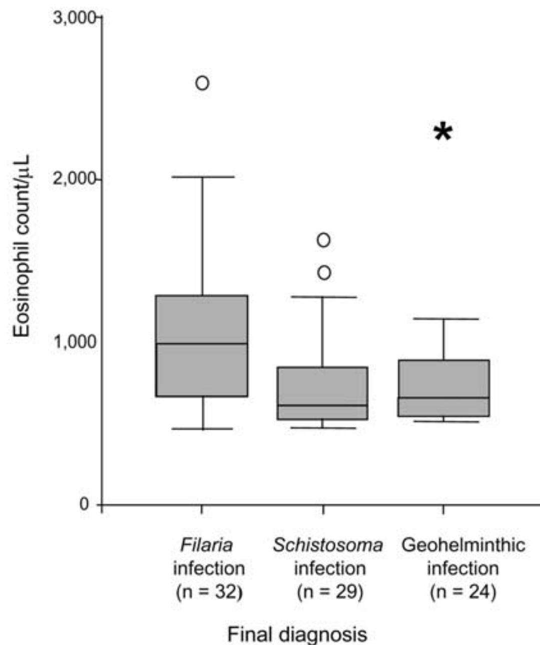

Figure 2. Relationship between eosinophil counts and the parasitologic diagnosis. Data are expressed as a box-and-whisker plot showing median, interquartile range (IQ), and extreme values. Circles indicate atypical outliers (values 1.5-3 × IQ), and asterisk represents extreme outliers (values >3 × IQ).

(13.2%), which suggests that direct and indirect tests are complementary in this population.

The proportion of *Strongyloides* spp. infection diagnosed was lower than in almost all other similar studies (*6–12*) because we could not ascertain it by stool positivity only, because of the low specificity of *Strongyloides* serologic testing available to us. Patients from Mali with eosinophilia had schistosomiasis more frequently, as reported in some European studies (*15*). However, we found a significant correlation between filarial or hookworm infection and immigration from Cameroon and Nigeria, respectively, an association not described previously. Finally, filariasis induces higher eosinophil counts than other parasitic infections, likely because the parasite inhabits blood and tissue and is not limited to the gut lumen. Our results show that 1) eosinophilia is frequent in recently arrived African immigrants, 2) helminthic infections can be diagnosed by using both parasitologic and serologic tests, 3) an immigrant's country of origin may suggest specific parasitic diseases, and 4) higher eosinophil counts usually indicate filariasis.

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Scrub Typhus in Himalayas

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Himachal Pradesh state of India is situated in the outer Himalayan ranges. During the rainy season, several cases of acute febrile illness of unknown origin occurred. *Orientia tsutsugamushi* was identified as the causative agent by microimmunofluorescence and PCR. Two new genotypes of *O. tsutsugamushi* were identified in the region.

Microimmunofluorescence (MIF) assay is the test of choice to diagnose rickettsial diseases (*1,2*), but more sensitive serologic tests and real-time quantitative PCR are expected to increase the number of cases diagnosed (*3*). Rickettsial diseases have been reported from various regions of India in the recent past (*4–6*); in the past few years, acute febrile illness with multiple organ involvement was diagnosed in several patients in our area. Results of tests for common causes of fever were negative, as were results of serologic tests for infectious mononucleosis, immunoglobulin M (IgM) for dengue fever, and IgM for leptospirosis. Sixteen of 31 serum samples from patients with suspected scrub typhus had titers 40–160 on Weil-Felix agglutination test with *Proteus* OXK antigen in 2003.

Himachal Pradesh is a mountainous state in northern India, situated in the outer Himalayas, with altitudes 350–7,000 m above sea level. It is the least urbanized state in India. During the rainy season, areas at lower altitudes have an average temperature of 20°C to 35°C, which is conducive to the spread of arthropod vectors. We recently reported an outbreak of scrub typhus in these areas (*7*). In an entomologic study in Himachal Pradesh, vector species *Leptotrombidium deliense* and *Gahrliepia* (*schoengastilla*) spp. were recorded (*8*). The aim of the present study was to retrospectively diagnose suspected rickettsial disease (scrub typhus) by using MIF assay and molecular methods in patients with acute febrile illness of unknown origin.

The Study

The study was conducted from July through October 2004. Scrub typhus was suspected by clinical manifestations such as febrile illness or fever with rash or eschar. After giving informed consent, patients filled out questionnaires about potential chigger exposure and symptoms or signs consistent with scrub typhus. Blood samples were taken from all patients for total blood cell count, biochemical analysis, serologic diagnosis, and molecular assays. This study was exempt from human subject review.

When they sought treatment, 5 patients had been symptomatic for 5 to 7 days, 14 had been symptomatic for 8 to 14 days, and the remaining 2 had been symptomatic for 18 to 25 days. Blood samples were collected at the time of admission to the hospital, and no serial assays were performed. All patients with clinical features that suggested scrub typhus received antirickettsial drugs (doxycycline/azithromycin) empirically.

Two serologic tests were used to confirm infections. The Weil-Felix *Proteus* agglutination assay with *Proteus vulgaris* OX-19 and OX-2 and *P. mirabilis* OX-K strains (Wellcome Diagnostics, Dartford, UK) was performed on each sample; a titer ≥80 was considered positive. Serum specimens were stored at –20°C. Serum specimens were tested by MIF assay with a panel of 11 rickettsial antigens, including spotted fever group (SFG) rickettsiae (*Rickettsia japonica*, *R. helvetica*, *R. slovaca*, *R. conorii* subsp. *indica*, *R. honeï*, *R. heilongjangensis*, and *R. felis*), *R. typhi*, and *Orientia tsutsugamushi* (Gilliam, Karp, Kato and Kawasaki strains) (*3*). The MIF assay was considered positive if antibody titers were >128 for IgG and >64 for IgM or if seroconversion was demonstrated (*9,10*).

DNA was extracted from the blood sample (buffy coat) by using QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Two amplification reactions were performed: 1) a real-time quantitative PCR with a TaqMan probe targeting the 47-kDa outer membrane protein with primers and probe previously described (*11*) and 2) a standard PCR targeting the 56-kDa protein with forward and reverse primers (OtsuF: 5′-AATTGCTAGTGCAATGTCTG-3′ and OtsuR: 5′-GGCATTATAGTAGGCTGAG-3′). The primers were purchased from Eurobio (Paris, France). The success of the amplification was confirmed by resolution of the products by electrophoresis on 1% agarose gel (Sigma Chemical Co., Saint Louis, MO, USA) in 1× Tris borate EDTA buffer for products of the 56-kDa gene. The sizes of the PCR-amplified products were determined by comparison with a molecular weight standard (Boehringer, Manheim, Germany) under UV light after ethidium bromide staining.

The PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Sequencing reactions were performed with a DNA sequencing kit, dRhodamine Terminator Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA). Sequencing was performed on an ABI PRISM 310 DNA Sequencer (Applied Biosystems). The sequences were identified by comparison with

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sequences available in GenBank by using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/).

In the MIF assay, samples from 21 patients showed positive antibody titers (both IgG and IgM) to *O. tsutsugamushi* (Gilliam, Karp, Kato, and Kawasaki strains). Twenty-eight of 51 serum samples had titers 80–320 to *Proteus* OXK antigen on Weil-Felix test, of which only 13 had titers on MIF. Moreover, samples from 15 patients with titers on Weil-Felix did not show titers on MIF, and samples from 8 patients without titers on Weil-Felix showed titers on MIF. None of the patients had a positive serologic test result for SFG rickettsioses. Real-time quantitative PCR to *O. tsutsugamushi* was positive in anticoagulated blood of 3 patients, and this result was confirmed by using primers for the 56-kDa antigen gene of *O. tsutsugamushi*. Patient 1 had a sequence (GenBank accession no. DQ530440) that matched the Karp type, close to strain LA-1 isolated in 1993 in Malaysia from mites (*12*). The sequence obtained from patient 2 (GenBank accession no. DQ530441) matched the sequence between the JG type and a recently described new subtype called Saitama, described in Japan (*13*). The results of MIF assays are shown in Table 1. The clinical features and laboratory abnormalities found in these 21 patients are shown in Table 2.

By MIF assay, 11 of 21 samples had positive titers of both IgG and IgM; 3 were positive for IgG (but not IgM) and were positive on PCR. These 14 patients had acute infection and are considered to have had scrub typhus, whereas the remaining 7 patients with only IgG titers are considered as probable scrub typhus case-patients. In cases of primary infection with *O. tsutsugamushi*, IgM appears at the end of the first week, whereas IgG appears at the end of the second week. However, in the case of reinfection with *O. tsutsugamushi*, IgG is detectable by day 6, and IgM titers are variable. The absence of IgM in 10 samples can be attributable to previous antigenic conditioning from reinfection (*9*). Moreover all 3 patients with positive PCR results had IgG titers but not IgM titers, which suggests that some of the remaining 7 patients with only IgG might also have been acutely infected.

Conclusions

We confirmed the diagnosis of scrub typhus in 21 patients from the Himalayas with several validated assays. PCR was performed in a few cases to further confirm *O. tsutsugamushi*, and we found that PCR was a good tool for molecular diagnosis, as recently reported (*6*). To the best of our knowledge, this is the first molecular detection of *O. tsutsugamushi* in southern Asia. The result was confirmed by using 2 different target genes in 2 different PCR assays. In our study, ≥2 different genotypes were identified; the phylogenetic position of 1 is between Karp and JP-1, and the other is between Saitama and JG type (Figure). Therefore, isolating these strains is recommended to increase understanding of the epidemiologic features of

Table 1. MIF assay results from 21 patients with suspected scrub typhus, Himalayas, 2004*

Patient no.	MIF titers (IgG/IgM)		Outcome
	Orientia Kato/Gilliam	*Orientia* Kawasaki	
1	128/0	512/0	Died†
2	128/0	256/0	Improved†
3	1,024/0	1,024/0	Improved
4	128/64	256/64	Improved
5	2,048/0	2,048/0	Improved
6	2,048/1024	2,048/1,024	Improved
7	1,024/128	512/128	Improved
8	2,048/64	512/0	Improved
9	512/0	512/0	Died†
10	2,048/0	2,048/0	Improved
11	1,024/256	1,024/256	Improved
12	256/128	512/256	Improved
13	2,048/0	2,048/0	Improved
14	0/128	0/128	Improved
15	512/256	256/256	Died
16	64/128	128/128	Improved
17	512/0	512/0	Improved
18	2,048/0	2,048/0	Improved
19	512/64	0/64	Improved
20	64/64	0/64	Improved
21	128/256	0/256	Improved

*MIF, microimmunofluorescence; Ig, immunoglobulin. IgM was considered positive at a titer of 64; IgG was considered positive at a titer of 128.
†Positive PCR result.

Table 2. Distribution of clinical features in 21 patients with suspected scrub typhus, Himalayas, 2004

Clinical feature*	Men (n = 13)	Women (n = 8)	Total (%)
Fever	13	8	21 (100)
Chills and rigor	10	5	15 (71.4)
Vomiting	6	3	9 (42.8)
Myalgia	4	4	8 (38.0)
Headache	4	4	8 (38.0)
Altered sensorium	2	3	5 (23.8)
Lymphadenopathy	9	2	11 (52.3)
Jaundice	5	6	11 (52.3)
Hepatomegaly	6	3	9 (42.8)
Congested eyes	5	2	7 (33.3)
Splenomegaly	4	3	7 (33.3)
Abdominal pain	2	4	6 (28.5)
Seizures	2	2	4 (19.0)
Cough	2	2	4 (19.0)
Abnormal bleeding	1	2	3 (14.2)
Eschar	2	0	2 (9.5)
Meningeal signs	1	1	2 (9.5)
Rash	2	0	2 (9.5)
Elevated transaminase levels	7	7	14 (66.7)
Renal dysfunction	8	6	14 (66.7)
Proteinuria	5	3	8 (38.1)
CSF abnormalities†	1	2	3 (14.3)
Acute RDS	1	1	2 (9.5)

*CSF, cerebrospinal fluid; RDS, respiratory distress syndrome.
†Elevated protein level and increased lymphocyte count.

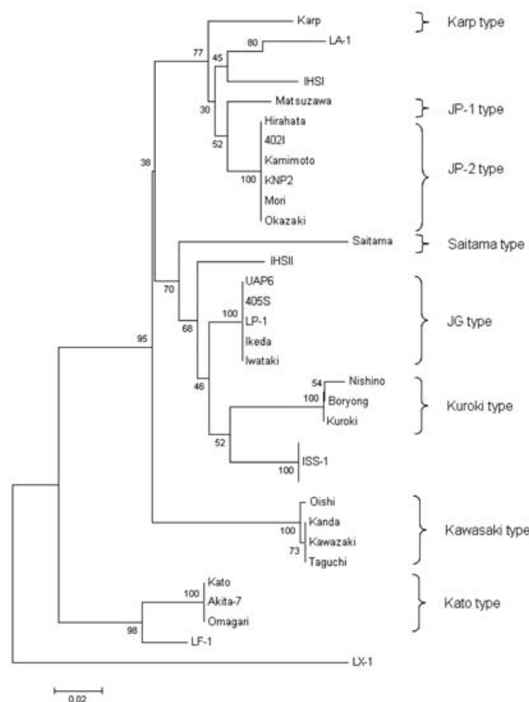

Figure. Phylogenetic tree produced by unweighted pair-group method with arithmetic means that shows the positions of IHS I and IHS II genotypes based on the partial 56-kDa sequence homologies. Numbers at nodes indicate bootstrap values, and the scale bar shows genetic distance of 0.02.

scrub typhus in India (*12*). In our study, an eschar, which is formed in few secondary infections (*2*), was noted in 9.5% of cases. Eschars are rare in Southeast Asian patients, and indigenous persons of typhus-endemic areas commonly have less severe illness, often without rash or eschar (*14*). The variation in cutaneous immunity has also been suggested as a possible explanation for the absence of an eschar in certain instances of scrub typhus (*15*).

The literature mentions this disease in hilly regions of the Himalayas and the Shimla region in Himachal Pradesh (*5,7,15*), but specific data are not available. The disease must have been present in the area but was not noticed because, in the past, most cases of fever were treated with drugs like tetracycline and chloramphenicol, which effectively treat scrub typhus also. The incidence of pyrexia of unknown origin with multiple organ involvement has increased for the past few years, which prompted us to conduct this study. Increasing prevalence of scrub typhus has been reported from some Asian countries, which coincides with the widespread use of β-lactam antimicrobial drugs and urbanization in rural areas (*14*).

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Dr Mahajan, registrar in the Department of Medicine, Indira Gandhi Medical College, Shimla, Himachal Pradesh, India, is working toward the control of scrub typhus in the state. His research interests include disease patterns at high altitudes.

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H5N1 Influenza Viruses in Lao People's Democratic Republic

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A prospective surveillance program for influenza viruses was established in Lao People's Democratic Republic (PDR) in July of 2005. We report isolation of H5N1 virus genetically distinct from H5N1 circulating in 2004, which indicates reintroduction of H5N1 into Lao PDR after its disappearance (i.e., no virologic or serologic evidence) for 2 years.

H5N1 influenza viruses have continuously circulated in southeastern Asia since 1996 (*1–5*). During late 2003 and early 2004, highly pathogenic H5N1 spread throughout China, Vietnam, Thailand, Cambodia, and Lao People's Democratic Republic (PDR) with a high lethality for domestic poultry; whereas in Vietnam, Thailand, and Cambodia, humans were infected but at a lower rate.

In Lao PDR, outbreaks were reported in the provinces of Vientiane Capital, Champasak, and Savannakhét. Approximately 155,000 poultry either died of disease or were culled, which contained the 2003–2004 outbreak. No human cases have been reported, and no scientific evidence has indicated avian influenza cases in poultry in Lao PDR since March 2004. This finding raises some questions. Had H5N1 viruses been eradicated from Lao PDR, or had they survived in domestic waterfowl to which they were nonpathogenic, as Chen et al. described in southern China (*6*,*7*)?

In response to outbreaks of H5N1, prospective surveillance programs have been established in affected southeastern Asian countries. In southern China, systematic surveillance of poultry markets has been ongoing since July 2000 (*7*). Thailand launched a comprehensive nationwide surveillance strategy (the x-ray survey) in October 2004 (*8*). Cambodia and Vietnam have also initiated surveillance programs, but little is known about the extent of the programs and their results. In Lao PDR since the outbreak in early 2004, surveillance programs have been limited to confirming the presence or absence of avian influenza. Given the frequency of H5N1 outbreaks in the region, a baseline for surveillance and determination of risk areas was essential. Therefore, in July 2005 we helped the Lao National Animal Health Centre establish a prospective surveillance program. We report the results of an 8-month surveillance program and the apparent reintroduction of H5N1 viruses into Lao PDR in early 2006.

The Study

Extensive virologic surveillance began in Lao PDR in July 2005. During 8 months, 8,592 samples were collected from healthy ducks, chickens, quail, and pigs at live markets in Vientiane, Champasak, and Savannakhét provinces, near the borders of Thailand, Vietnam, and Cambodia (Figure 1). Cloacal swabs were stored in 1.0 mL of virus isolation media. The samples were injected into chicken or duck eggs, and allantoic fluid was harvested after 72 h and analyzed as described (*9*). As of January 2006, no evidence of any influenza virus subtypes had been detected in any species.

In January 2006, serologic surveillance was initiated to clarify whether poultry in Lao PDR had been exposed to influenza viruses and recovered. Vaccination of poultry is

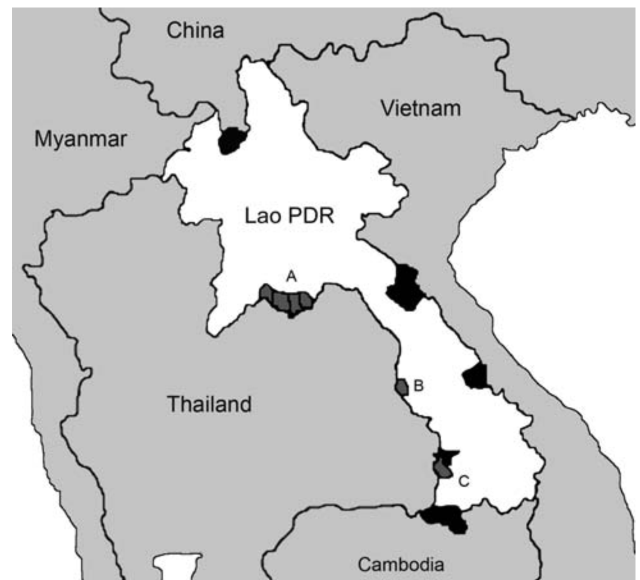

Figure 1. Map of Lao People's Democratic Republic indicates regions (black areas) of influenza virus surveillance. Outbreaks of H5N1 occurred in Vientiane (location of isolation of A/Duck/Laos/3295/2006) (A), Savannakhét (B), and Champasak (C) provinces during late 2003 and early 2004.

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not practiced in Lao PDR, which made serologic surveillance possible. Serologic analysis for antibodies to influenza viruses was performed with ELISA and hemagglutination inhibition (HI). The IDEXX FlockChek AIV (IDEXX Laboratories, Inc., Westbrook, ME, USA) was used to initially screen for antibodies to influenza. Sera were then tested by HI with antigens to H5, H6, H7, and H9 influenza subtypes as described (*10*). Initial results from serologic surveillance confirmed virus isolation results and absence of vaccine use; however, in early February 2006, in apparently healthy ducks in a live market in Vientiane, we detected antibodies to the viral hemagglutinins H5 (HI titers 40 to $\leq$640) and H9 (HI titer 80). Only 21 (3.5%) of 604 birds had positive test results at that time; 1 duck had antibodies to H5 and H9.

In late February 2006, H5N1 was isolated from healthy ducks at a layer farm in Vientiane Capital. The source of the isolated H5N1 is unknown, but our 8-month prospective surveillance program showed that it was recently introduced into this infected flock. Cloacal samples from 40 ducks at a duck farm in Vientiane tested negative for H5N1 virus in November 2005, but upon retesting in late February 2006, results for the same flock were positive for H5N1. To identify the source of the new isolate, we compared the sequence of its hemagglutinin gene with those of H5N1 viruses isolated since 2003 in China, Thailand, Vietnam, and Cambodia (Figure 2).

Sequence analysis indicated that the new H5N1 isolate (A/duck/Laos/3295/2006) (GenBank accession no. DQ845348) was not closely related to the other viruses circulating in Southeast Asia during 2003 and 2004. It clustered phylogenetically with H5N1 viruses isolated in China in 2005 and was most closely related to a 2005 human isolate from Anhui, China (A/Anhui/1/2005) (*11*). Furthermore, the 2004 Laotian H5N1 virus strains clustered with 2004 isolates from Malaysia, Thailand, and Vietnam. This evidence suggests that the H5N1 virus had recently been introduced into Lao PDR; however, its direct source is unknown. Despite its origin in China, it clustered with a 2005 Vietnamese virus isolate (A/duck/Vietnam/568/2005) and therefore may have crossed into Lao PDR from Vietnam. A Malaysian H5N1 isolate (A/chicken/Malaysia/935/2006) also clustered with this group rather than with Malaysian isolates from 2004, which indicates that it was likely a new introduction into Malaysia.

Surveillance for H5N1 continued in the area surrounding the infected farm. Two months after the isolation of H5N1, 40 cloacal swabs and 28 serum samples were collected from ducks at the infected and surrounding farms. All cloacal swabs had negative results for influenza viruses, and serum samples had negative results for antibodies to H5N1. As of June 2006, no reports of infected poultry have been made since the isolation, which indicates that H5N1 did not spread and its introduction may have been an isolated event; however, surveillance is ongoing.

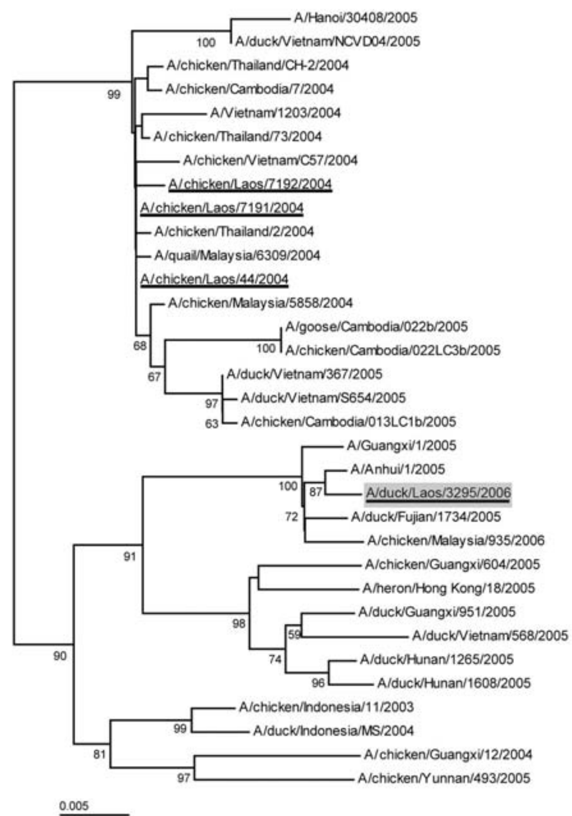

Figure 2. Phylogenetic relationship of the hemagglutinin (HA) gene of representative H5N1 influenza virus strains isolated in Southeast Asia from 2003 through 2006. Analysis was based on nucleotides 1–1012 (1,012 bp) of the HA gene. Viruses isolated in Lao People's Democratic Republic in 2004 and 2006 are underlined. The strain isolated from ducks in Vientiane in February 2006 is shaded.

Conclusions

As highly pathogenic H5N1 influenza viruses continue to spread into Eurasia, whether they will become endemic in Eurasian poultry or whether the highly pathogenic H5N1 will burn out and disappear with the possibility of reintroduction is unknown. We describe results from the first active surveillance study in Lao PDR since the 2004 outbreak. We extensively sampled live markets and local farms in Vientiane Capital and additionally sampled districts bordering Cambodia, China, Thailand, and Vietnam. A genetically distinct H5N1 was isolated from apparently healthy ducks, which indicates reintroduction of H5N1 in Lao PDR. Serologic data, antibodies to H5 in serum samples from apparently healthy ducks found in live markets, provide additional evidence of H5N1 in Lao PDR; however, we cannot rule out the illegal use of vaccines.

Evidence of an H9 subtype was also detected in a duck that was seropositive for H9 antibodies; however, no H9 influenza subtypes were isolated.

Whether H5N1 influenza viruses are endemic in wild migratory birds is unresolved; however, our study supports the notion that highly pathogenic H5N1 viruses are not endemic in Lao PDR. Our findings suggest that the H5N1 virus did not become established after the 2004 outbreak in Lao PDR, which is less densely populated than neighboring Thailand, Vietnam, and China (high summer temperatures in Lao PDR may have also been a factor). Instead, the virus disappeared and was later replaced by a newly introduced virus. Although no measures were taken to eradicate the virus, the newly introduced H5N1 has since vanished from the area. The area has been identified as high risk and has become the focus of ongoing surveillance.

This virus appears to have disappeared on its own in Lao PDR in the absence of vaccines and with limited surveillance, unlike what happened in the more densely populated Thailand, Vietnam, and China. In China, H5N1 continues to circulate in poultry despite ongoing surveillance and use of vaccines; heroic measures were required to eradicate H5N1 in Thailand, and since the use of vaccines in Vietnam in 2005, no more human cases have been reported.

These findings are encouraging for the less densely populated, developing countries of Africa and Asia because they suggest that H5N1 viruses are not being perpetuated through wild migratory birds. To date, wild birds have not been found to carry and spread highly pathogenic H5 and H7 influenza viruses; instead, each outbreak has been caused by a phylogenetically new virus, whose parent viruses were not pathogenic (*12*,*13*).

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Chimpanzee Adenovirus Antibodies in Humans, Sub-Saharan Africa

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Human sera from the United States, Thailand, and sub-Saharan Africa and chimpanzee sera were tested for neutralizing antibodies to 3 chimpanzee adenoviruses. Antibodies were more common in humans residing in sub-Saharan Africa than in humans living in the United States or Thailand. This finding suggests cross-species transmission of chimpanzee adenoviruses.

Vaccines to HIV-1 are needed to stem further spread of the HIV pandemic. One vaccine modality that has shown promise is based on adenovirus vectors of human serotype 5 (AdHu5) (*1–3*). However, immune responses induced by AdHu5 vectors are reduced by preexisting AdHu5-neutralizing antibodies found in humans in the United States (*4–7*).

The Study

To circumvent the negative effect of preexisting immunity to common human serotypes of adenoviruses on the efficacy of adenovirus vaccine carriers, we developed vectors based on chimpanzee-derived adenoviruses C68, C6, and C1 (*8*). We previously showed that neutralizing antibodies to chimpanzee adenoviruses are rarely found in US residents (*6*). Because vaccines to HIV-1 are most urgently needed in sub-Saharan Africa, we evaluated the prevalence of neutralizing antibodies to chimpanzee adenoviruses in sera from humans residing in 3 sub-Saharan countries with natural habitats for chimpanzees: Nigeria, Cameroon, and Côte d'Ivoire. Sera from captive chimpanzees in the United States and human sera from Thailand and the United States, including samples from persons with known exposures to chimpanzees, were tested for comparison.

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Neutralizing antibodies to AdHu5 were found in most serum samples from Africa and Thailand. Percentages of antibodies to AdHu5 were higher in serum samples from Africa and Thailand than in serum samples from the United States (Table 1). Titers to AdHu5 were comparable in serum samples from the United States and sub-Saharan Africa but were higher in the control group from Thailand (Table 2).

Neutralizing antibodies to AdC68, AdC6, and AdC1 were rare in sera from the United States and Thailand, with prevalence rates from 1.5% to 4% (Figure). Most positive samples had titers <80. Of 200 samples from Thailand, only 6 had high titers: 4 to AdC6 and 2 to AdC1. None of the samples from 50 US zoo workers, including those who reported regular contact with primates, had detectable antibodies to chimpanzee adenoviruses.

In contrast, serum samples from sub-Saharan African cohorts had higher prevalences of neutralizing antibodies to chimpanzee adenoviruses (Figure, Table 1). Samples from persons living in Cameroon and Nigeria showed a higher prevalence of neutralizing antibodies to AdC6 and AdC1. Titers of neutralizing antibodies to AdC6 and AdC1 were high, and samples were positive at dilutions ≥1:80. When compared with sera from Nigeria and Cameroon, sera from Côte d'Ivoire had a different pattern of antibodies reactive to the chimpanzee adenoviruses; prevalence rates were low to AdC1 but higher to AdC68 and AdC6. Although most samples had low to moderate titers, several samples had titers ≥80. No association was found between neutralizing antibodies to AdC68, AdC6, and AdC1 in any of the human samples tested, and only a few human serum samples had neutralizing antibodies to ≥1 chimpanzee adenovirus (data not shown). One serum sample from Cameroon neutralized all 3 chimpanzee adenoviruses and had titers of 20 to AdC68 and 160 to AdC6 and AdC1.

Circulating neutralizing antibodies to AdHu5 were found in 44% of captive US chimpanzees; titers in chimpanzee samples were comparable to those in human sera, suggesting that AdHu5 can readily cause an infection in captive chimpanzees. The prevalence of antibodies to AdC6 and AdC68 was high and exceeded that of antibodies to AdC1 (Table 1). Titers to AdC68 and AdC6 were higher in chimpanzees than in humans, but titers to AdC1 in both species were similar (Table 2).

Conclusions

Our data show that as expected, neutralizing antibodies to chimpanzee adenoviruses are rarely found in humans residing in the United States or Thailand. In contrast, their prevalence is higher in human sera from sub-Saharan Africa, where hunting and butchering of nonhuman primates for food are widespread and eating bush meat is com-

Table 1. Sera with neutralizing activity to different human and chimpanzee adenoviruses

Origin	% positive samples (p values)*†			
	AdHu5	AdC68	AdC6	AdC1
Human controls, United States (n = 50)	34.0	2.0	4.0	2.0
Human zoo workers, United States (n = 50)	28.0	0	0	0
Humans, Thailand (n = 200)	76.5	1.5	3.0	4.0
Humans, Cameroon (n = 405)	55.8	1.7 (0.6764)	7.9 (0.0045)	5.4 (0.1248)
Humans, Côte d'Ivoire (n = 169)	95.8	9.5 (0.0003)	10.7 (0.0008)	3.0 (0.9796)
Humans, Nigeria (n = 182)	89.0	4.9 (0.0267)	18.7 (<0.0001)	9.3 (0.0045)
Chimpanzees, United States (n = 50)	44.0	86.0 (<0.0001)	92.0 (<0.0001)	46.0 (<0.0001)

*p values show statistical difference between percentages of sera positive for neutralizing antibodies to human and chimpanzee adenoviruses. Reactivity of human sera from Cameroon, Côte d'Ivoire, and Nigeria and of chimpanzee sera to the 3 chimpanzee-derived adenoviruses were compared with human sera from the United States (n = 100) and Thailand (n = 200); the last 2 were combined because these countries do not offer natural chimpanzee habitats (similarity of USA and Thailand data for these adenoviruses was statistically confirmed). A logistic regression model was fitted to compare the percentages of samples positive for neutralizing antibodies between different groups. A p value <0.05 was considered statistically significant. All analyses were performed by using SAS version 9.1 logistic procedure (*9*).

†Virus tested for neutralization with a previously described neutralization assay (*10*). Samples that neutralized virus at dilutions ≥1:20 were scored as positive.

mon (*11*). Different prevalence rates of neutralizing antibodies to the 3 chimpanzee adenoviruses in human samples from the 3 African countries tested may reflect different infection rates in chimpanzees residing in these areas.

Cameroon, Gabon, and Republic of Congo are home to most Central African common chimpanzees (*Pan troglodytes troglodytes*). Most Western common chimpanzees (*P. t. verus*) inhabit Côte d'Ivoire and Guinea. The rare Nigeria chimpanzee (*P. t. vellerosus*) is found only in eastern Nigeria and western Cameroon, and Eastern African common chimpanzees (*P. t. schweinfurthii*) reside in a range from Central African Republic and the Democratic Republic of the Congo through western Uganda and Tanzania. No clear association was found between chimpanzee subspecies and neutralizing antibodies to different chimpanzee adenoviruses. Nevertheless, most samples were from *P. t. verus*, and only a limited number of samples were from *P. t. troglodytes* (n = 4) and *P. t. schweinfurthii* (n = 3). No samples from *P. t. vellerosus* were tested. In addition, the chimpanzee samples evaluated in the study were from animals kept in captivity and thus may not provide information on distribution of these viruses in free-ranging animals.

Increased prevalence of neutralizing antibodies to chimpanzee adenoviruses in sub-Saharan Africa may reflect cross-species transmission of these viruses from chimpanzees to humans. If transmission occurs, human-to-human spread of chimpanzee adenoviruses might further contribute to the comparatively high seroprevalence seen in equatorial Africa. Although we have no direct proof for viral cross-species transmission, this process has been previously described for other chimpanzee viruses. For example, the AIDS epidemic is believed to have originated from simian immunodeficiency virus–infected chimpanzees (*12*). Another chimpanzee retrovirus, simian foamy virus, has been reported to infect persons exposed to primates at zoos and research centers (*13*) and to infect Bantus in Cameroon (*14*). Chimpanzee adenoviruses do not appear to spread easily to humans through occupational contact with primates because none of the 23 persons who had routine exposure to primates, including chimpanzees, had serologic evidence of exposure despite the high prevalence of antibodies to chimpanzee adenoviruses in captive US chimpanzees.

Use of chimpanzee adenovirus vectors as vaccines for HIV-1 would require that most persons at high risk for HIV-1 infection lack neutralizing antibodies to these adenoviruses because such antibodies impair induction of transgene product-specific immune responses (*4*,*5*). Even in countries where chimpanzees are endemic, neutralizing

Table 2. Mean adenovirus neutralizing antibody titers for positive samples

Origin	Mean VNA* titer ± standard deviation†			
	AdHu5	AdC68	AdC6	AdC1
Humans, United States	**116 ± 111**	40 ± 0	20 ± 0	20 ± 0
Humans, Thailand	**303 ± 353‡**	20 ± 0	80 ± 44	**33 ± 25**
Humans, Cameroon	**125 ± 114**	109 ± 145	**82 ± 52‡**	**58 ± 41**
Humans, Côte d'Ivoire	**162 ± 212**	**60 ± 51‡**	**37 ± 34‡**	148 ± 275
Humans, Nigeria	**165 ± 206**	29 ± 20	**80 ± 115‡**	**42 ± 23**
Chimpanzees, United States	**164 ± 233**	**201 ± 204**	**137 ± 160**	**64 ± 72**

*VNA , virus neutralizing antibody.

†p values were determined by a Student *t* test to assess differences between titers in experimental samples and control samples. Human sera from the United States were used as a reference for antibodies to AdHu5, and chimpanzee sera were used as a reference for titers to AdC68, AdC6, and AdC1. Experimental samples in which ≥10 samples were positive for the given adenovirus are shown in **boldface**.

‡Samples that showed a statistically significant difference (p<0.05).

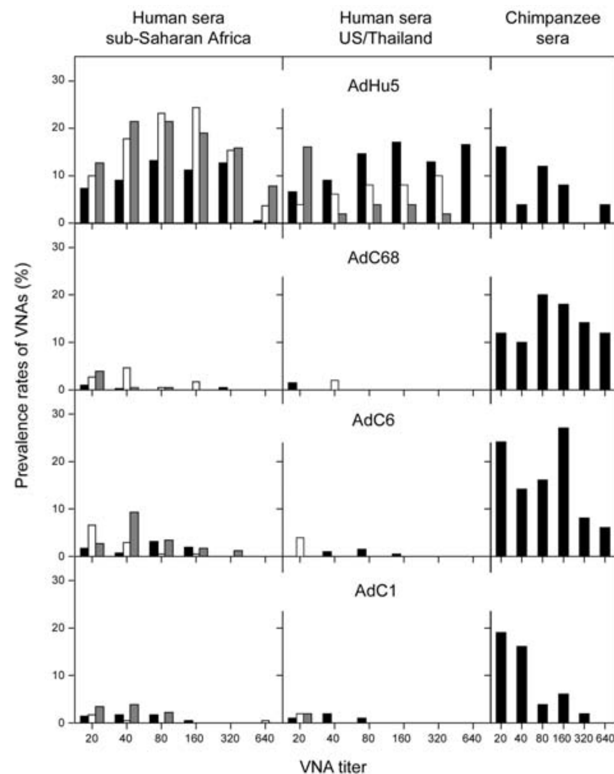

Figure. Prevalence of neutralizing antibody titers to chimpanzee adenoviruses. Percentages of negative samples are not shown. Left column: Cameroon, black bars; Côte d'Ivoire, white bars; Nigeria: gray bars. Middle column: Thailand, black bars; US controls, white bars; US zoo keepers or animal handlers, gray bars. VNAs, virus neutralizing antibodies. Coded human serum samples that had been collected for other studies were obtained under an institutional review board exemption.

antibodies to chimpanzee adenoviruses are less common in humans than those directed to AdHu5, which is currently in clinical trials as a vaccine for HIV-1 antigens in a vectored and replication-defective form. An alternative human serotype is AdHu35, which is being developed as a potential vaccine against HIV-1 (*15*). Prevalence rates to AdHu35 are low in the United States and Europe (<5%); however, rates are markedly higher (<20%) in equatorial Africa (*15*).

Although neutralizing antibodies to chimpanzee adenoviruses are also relatively more common in human sera from sub-Saharan Africa, they are found less frequently than antibodies to AdHu5 or AdHu35. Nonetheless, increased prevalence of neutralizing antibodies to adenoviruses in countries that are hardest hit by the AIDS pandemic needs to be taken into account in the design of vaccines based on chimpanzee adenovirus vectors. Vectors derived from other species are being developed and may provide additional or alternative vaccine carriers.

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Dr Xiang is a senior staff scientist at the Wistar Institute. His primary research interests are assessing immune responses to novel vaccine prototypes based on viral vectors and studying immune responses to vaccines expressing antigens of rabies virus or HIV-1.

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Spatial Epidemiology of *Plasmodium vivax*, Afghanistan

Simon Brooker,* Toby Leslie,*† Kate Kolaczinski,*† Engineer Mohsen,† Najeebullah Mehboob,‡ Sarah Saleheen,† Juma Khudonazarov,† Tim Freeman,† Archie Clements,* Mark Rowland,† and Jan Kolaczinski*†

Plasmodium vivax is endemic to many areas of Afghanistan. Geographic analysis helped highlight areas of malaria risk and clarified ecologic risk factors for transmission. Remote sensing enabled development of a risk map, thereby providing a valuable tool to help guide malaria control strategies.

An estimated 64 million persons are at risk for *Plasmodium vivax* malaria in the eastern Mediterranean region; as many as 25% of these people live in Afghanistan (*1*), where most (70%–90%) malaria cases are caused by *P. vivax* and the rest by *P. falciparum* (*2*). The main vectors in Afghanistan are *Anopheles stephensi* and *A. culicifacies* in the east, *A. pulcherrimus* in the north, and *A. superpictus* in hill areas north and south of the Hindu Kush mountain range. These vectors breed mainly in pools, rivers, and irrigated rice fields; their abundance is largely affected by the presence of water and variation in river flow, determined by spring snowmelt and summer rainfall. Because most of Afghanistan is a mountainous desert, the distribution of malaria is likely to be limited to areas where the climate suits the development of vector and parasite.

After 25 years of almost continuous war, no up-to-date nationwide cross-sectional surveillance data for exist for malaria; the last nationwide survey was conducted >50 years ago (*3*). Since the fall of the Taliban regime in Afghanistan in 2001, interest in the integration of malaria control into routine healthcare delivery has been renewed (*4*). To help guide this process and direct limited resources to the most vulnerable populations, accurate knowledge of national distribution of malaria is essential. We report the results of recent nationwide *P. vivax* surveys. We also investigated the geographic limits of transmission to develop a predictive spatial model of transmission to facilitate a malaria control strategy based on geographic risk stratification.

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The Study

Epidemiologic data were obtained from a nationwide survey of 269 villages conducted from August through September 2005. The country was divided into 4 ecologic zones on the basis of differences in elevation, temperature, and land cover type (Figure, panel A). The number of villages selected in each zone was proportional to the population in each zone. These data were combined with data from an additional 64 villages in known areas of malaria endemicity that were surveyed during 2000–2003, to give data from a total of 333 villages. Comparability was ensured by using the same sampling and parasitologic methods in each set of surveys. In each village, households were sampled along perpendicular transects. The survey team started from a central point and randomly selected the direction by spinning a bottle. Along the transect, every household was selected and every household member invited to participate. New transects were selected until 150 persons in each village were enrolled. Each village had ≥85% participation. A blood sample was collected

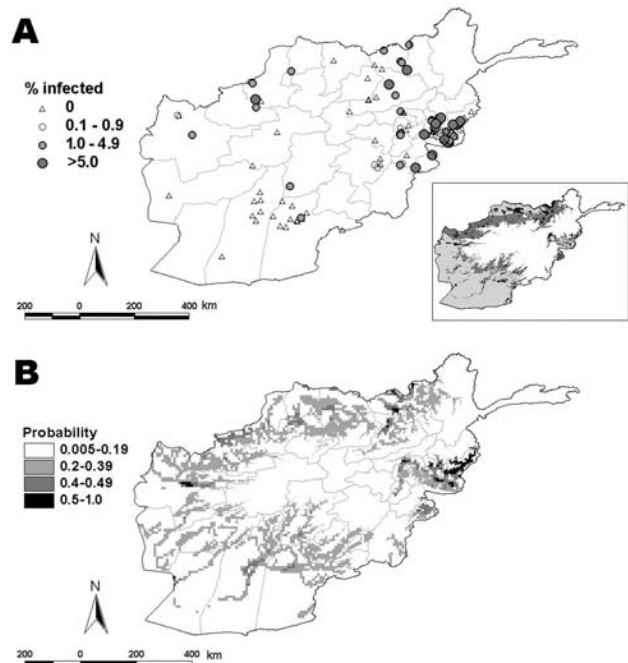

Figure. A) Prevalence of *Plasmodium vivax* in Afghanistan, according to a 2005 survey (n = 269) and previous prevalence surveys conducted by HealthNet-TPO, 2000–2003 (n = 64). Lower-right inset shows ecologic zones in Afghanistan according to differences in elevation, temperature, and land cover. White, high altitude rangeland; light gray, desert; dark gray, grassland; black, irrigated/marshland. B) Predicted probability of *P. vivax* transmission (prevalence >0%) in Afghanistan, according to logistic regression model.

from each person, and Giemsa-stained thick and thin blood films were prepared and stored for microscopic examination for the presence of malaria parasites. A case-patient was defined as a person for whom malaria blood stage parasites were seen after examination of 100 fields. All case-patients received antimalarial treatment according to national guidelines.

The geographic locations of villages were recorded in the field by using a nondifferential global positioning system (Garmin International Inc., Olathe, KS, USA). The village-level prevalence data were then included into the geographic information system (GIS) (ArcView, Version 3.2, ESRI Inc., Redlands, CA, USA). Global satellite sensor-derived data at 8×8 km spatial resolution were obtained from the United States Geological Survey, Distributed Active Archive Center (http://edcdaac.usgs.gov/1KM/comp10d.asp) and included the normalized difference vegetation index (NDVI) and land surface temperature. NDVI is an indicator of photosynthetic activity and is associated with saturation deficit and rainfall. The locations of rivers were downloaded from Afghanistan Information Management System project's website (http://www.aims.org.af), and minimum distance between each village and rivers was calculated by using ArcView. Elevation data were obtained from a global digital elevation model (http://edcwww.cr.usgs.gov/landdaac/gtopo30/). These environmental data were imported into ArcView and linked by location to the parasitologic data.

We used logistic regression analysis to investigate the relationship between environmental variables and the probability of transmission (*P. vivax* prevalence >0%). Initial variables were selected by developing univariate models; variables with Wald p>0.2 were excluded from further analysis. Colinearity was investigated between all possible pairs of potential predictor variables; if any pair had a correlation coefficient >0.9, the member of the pair that was less likely to be biologically important was excluded. With the remaining variables, backward-stepwise logistic regression analysis was conducted by using Wald p>0.1 as the exit criterion and p≤0.05 as the entry criterion. Nonlinear relationships were examined by using scatter plots. Entry of categorized predictor variables into the models was explored, but preliminary analysis indicated that linear forms were most significant. The final model was then cross-validated by using a jackknife procedure (*5*). Predicted occurrence was compared with observed occurrence by using receiver operating characteristic analysis. The statistic used for the comparison was the area under the curve, a plot of sensitivity versus 1 minus specificity (*6*). The coefficients from the best-fit model were then applied to the predictor variables to generate a map of predicted probability of transmission.

A total of 40,350 persons in 269 villages, ranging in age from 1 through 98 years, were examined. The overall prevalence of *P. vivax* was 0.49%, but infection levels varied considerably among areas of the country (Figure, panel A). Prevalence of *P. vivax* was highest in Faryab province in the north and in Nangarhar and Kunar provinces in the southeast part of the country. Small foci of *P. vivax* were found in Baghlan and Badakhshan in the northeast and Kandahar and Hilmand in the south. No transmission occurred in villages at elevations >2,000 m, likely because of variation in temperature. Prevalence was highest in river valleys, and no transmission occurred in villages >10 km from rivers.

The Table presents the logistic regression model for the probability of *P. vivax* transmission. The odds ratios indicate that transmission probability was much higher in locations adjacent to perennial rivers. *P. vivax* transmission and NDVI also showed a positive association. Validation of the model using an observed prevalence threshold of >0% gave an area under the curve of 0.67 (95% confidence interval 0.61–0.74), which indicates a moderately good predictive performance of the model. The map of predicted probability of transmission is presented in the Figure, panel B.

Conclusions

Spatial epidemiology aims to investigate spatial distributions of disease to identify geographic risk factors and populations at risk, which facilitates the rational implementation of control. Although *P. vivax* malaria is a serious problem in Afghanistan, only certain areas of the country are affected. Our analysis shows that this distribution is determined by climatic and other geographic factors, which affect mosquito and plasmodium reproduction. The use of GIS and remote sensing has enabled the first detailed description of the spatial variation of *P. vivax* malaria in Afghanistan and will facilitate implementation of a rational strategy by allowing differential, stratified control mechanisms to be used and resource allocation to be managed more efficiently. Afghanistan's malaria control strategy consists mainly of social marketing of insecticide-treated nets, coupled with support for healthcare

Table. Logistic regression model for the probability of *Plasmodium vivax* transmission, 333 villages in Afghanistan, 2005*

Variable	Odds ratio	Standard error	95% confidence interval	p value
Average normalized difference vegetation index	1.004	0.002	1.001–1.007	0.013
Distance to river <5 km	1.075	0.077	1.010–1.567	0.012

*Wald χ^2, 14.26; probability>χ^2, 0.0008; log pseudo-likelihood, –184.38873; pseudo R^2, 0.062.

providers in the delivery of effective diagnosis and treatment. The National Malaria Strategic Plan (2005), which adopted the Millennium Development Goals for malaria, calls for targeted interventions aimed at reducing the prevalence and effects of disease in those areas most at risk. Our results demonstrate that GIS and remote sensing are important tools for rapid mapping of disease patterns and for targeting limited control resources. Further work is ongoing to determine areas at risk for *P. falciparum* transmission, the less prevalent but more dangerous parasite, and to devise a combined risk map.

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ICD-9 Codes for Identifying Influenza Hospitalizations in Children

To the Editor: The effect of influenza on young children is substantial, but most infections are clinically unrecognized (*1*). As a result, without routine laboratory confirmation of influenza infection in patients admitted to the hospital with influenzalike illness, accurate estimates of influenza-related hospitalization rates are difficult to obtain. Several statistical models have been developed to generate estimates of excess or influenza attributable hospitalizations, all of which calculate the rate of hospitalization above baseline during periods in which influenza is circulating (*2–8*). However, their accuracy is limited when viruses such as respiratory syncytial virus (RSV) and parainfluenza are cocirculating with influenza.

International Classification of Diseases, 9th revision (ICD-9) diagnostic codes specific to influenza (487.0, 487.1, and 487.8) are easily retrieved from hospital discharge records. However, researchers and public health officials have rarely used them for influenza hospitalization surveillance, presumably because they lack sensitivity for identifying true influenza infections, although this assumption has never been tested.

To determine the sensitivity and positive predictive value of influenza-specific ICD-9 admission or discharge codes (487.0, 487.1, and 487.8), we conducted a retrospective cohort study of all patients <21 years of age hospitalized at the Children's Hospital of Philadelphia with laboratory-confirmed influenza during 3 consecutive influenza seasons (July 2001 through June 2004) (*9*). We compared admission and discharge ICD-9 codes with influenza laboratory results. All specimens were initially tested by rapid solid-phase immunoassay for RSV (Binax; Portland, ME, USA) and influenza (Binax). Direct fluorescent antibody testing for adenovirus, influenza A and B, parainfluenza virus types 1, 2, and 3, and RSV was performed on specimens negative by solid-phase immunoassay for RSV or influenza. Comprehensive viral culture was established for all specimens negative for respiratory viruses by direct fluorescent antibody test.

Of 715 cases of laboratory-confirmed influenza identified (Table), 617 (86%) were identified by rapid testing and 98 (14%) by viral culture after rapid test results were negative. A total of 529 patients had influenza-specific admission or discharge ICD-9 codes. The sensitivity of influenza-specific ICD-9 codes was 65% (95% confidence interval [CI] 61%–68%), and the positive predictive value was 88% (95% CI 84%–90%) (Table). Of 66 patients who had influenza-specific admission or discharge ICD-9 codes but negative influenza laboratory results, laboratory tests confirmed parainfluenza (n = 42), *Haemophilus influenzae* (n = 6; 1 with a positive blood culture and 5 with positive respiratory cultures), *H. parainfluenzae* (n = 1 wound infection), adenovirus (n = 1), and RSV (n = 2) infections. For 5 patients, influenza infection was documented in their charts, but they had either negative influenza test results or no influenza test performed. Seven patients had the expression "follow-up" written as "f/u" in the assessment section of their admission note, which may have been interpreted by medical coders as flu. We could not determine the reason for miscoding in 2 patients.

The sensitivity of influenza-specific diagnosis codes was related to the method of laboratory confirmation. Seventy-three percent (452/617) of patients (95% CI 70%–77%) who had positive rapid test results had influenza-specific admission or discharge diagnosis codes, whereas only 11% (11/98) (95% CI 6%–19%) who had positive influenza viral cultures (and negative rapid test results) had influenza-specific diagnosis codes.

Our results have a few policy implications. First, they suggest that in hospitals where routine influenza viral testing is performed, use of admission and discharge ICD-9 codes from hospital billing data for surveillance purposes will systematically underestimate actual influenza-related hospitalizations by 35%. The higher sensitivity of influenza-specific ICD-9 codes in patients with positive rapid test results compared with positive culture results suggests that unlike viral culture results, which generally are not available before discharge, rapid test results are often used to assign influenza-specific ICD-9 codes. Thus, rapid diagnostic tests that are more sensitive (e.g., PCR-based assays) may increase the sensitivity of influenza-specific ICD-9 codes in hospitals that routinely evaluate children admitted with respiratory symptoms of unclear cause. However, the imperfect specificity (94%–98%) of rapid influenza tests will produce a small but not negligible number of false-positive results. In hospitals where influenza testing is not commonly performed, the sensitivity of influenza-specific ICD-9 codes is likely to be lower.

Second, the high positive predictive value of influenza-specific ICD-9

Table. Influenza-specific admission or discharge ICD-9 codes (487.0, 487.1, and 487.8.) compared with influenza laboratory test results*

Parameter	LCI	No LCI	Total
Influenza-specific diagnosis codes	463	66	529
No influenza-specific diagnosis codes	252	–	–
Total	715	–	–

*ICD-9, International Classification of Diseases-9; LCI, laboratory-confirmed influenza. The sensitivity and positive predictive value of influenza-specific diagnosis codes were 65% and 88%, respectively.

codes observed in this study suggests that in hospitals where influenza testing is routinely performed, most patients whose hospitalization summary includes an influenza-specific ICD-9 code actually have influenza. However, misclassification of patients with parainfluenza and *H. influenzae* infections as patients with influenza demonstrates the potential for systematic coding errors even when influenza testing is routine.

Epidemiologists and public health officials should be aware that influenza-specific ICD-9 codes assigned in a setting of routine rapid diagnostic testing may be useful for following trends. However, these codes will substantially underestimate the actual number of influenza-related hospitalizations.

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Chikungunya Virus Strains, Reunion Island Outbreak

To the Editor: Chikungunya virus (CHIKV) is endemic in rural tropical Africa and is penetrating urban areas in Asia. CHIKV is maintained in a sylvatic cycle that involves mosquitoes of the genus *Aedes*, primates, and rodents. CHIKV infection induces fever, arthralgia, and maculopapular rash. Hemorrhagic complications have been reported in some outbreaks, but a more specific symptom is severe arthralgia, often persistent, which results in long-lasting disability.

After numerous cases of CHIKV infection had been reported in Comoros and Mauritius (*1*), an outbreak of febrile illness was reported on Reunion Island in March 2005 (*2*). The incidence of the disease remained relatively low until December 2005, when it increased dramatically. The outbreak resulted in >3,500 confirmed cases and an estimated 250,000 suspected cases (*2*), affecting >25% of the island's inhabitants. Encephalitic forms were reported on many occasions during the active phase of the outbreak, and >200 persons died while they were infected with CHIKV. Previously unreported complications, such as mother-to-child transmission, myocarditis, hepatitis, and extensive dermal lesions were also encountered.

Many samples, collected from patients during the outbreak, were sent to our laboratory (Virology Unit, Tropical Institute of the French Armed Forces Medical Service, Marseille, France) to identify the etiologic agent. Serum samples incubated with C6/36 cells according to previously published methods (*3*) yielded CHIKV. This virus was also isolated from cerebrospinal fluid collected from a patient with encephalitis, from corneas collected from asymptomatic human organ donors, and from pools of mosquitoes (*Aedes albopictus* and *Culex quinquefasciatus*) collected on the island.

Five isolates were partially sequenced. The CHIKV genome was partly amplified by using the specific primer pair OP16/OP17 (*4*), and reverse transcription (RT)-PCR products (1,200 nucleotides long) were cloned and sequenced (GenBank accession nos. DQ462746–DQ462750). Comparison of partial

sequences showed a high degree of identity between the strains isolated in Reunion, including the strain LR2006_OPY1 (*5*): paired identity was 99.3%–100% at the nucleotide level and 98.2%–100% at the amino acid level. The nucleotide and amino acid substitutions were homogeneously distributed across the sequence and were different for each isolate. Our strain IMT/6470, isolated from human serum, and the strain LR2006_OPY1 displayed the same nucleotide sequence in the sequenced region. The sequence identity among these isolates highlights the common origin of human and mosquito isolates.

The sequences of our isolates did not feature any codon deletions or insertions when compared with other CHIKV isolates from Africa and Asia available in GenBank (*4*,*6*). Strains from Reunion were also compared with the candidate vaccine strain TSI-GSD-218 (*7*). This strain showed 93%–94% and 96%–97% identity at the nucleotide and amino acid level, respectively, which suggests a sufficient antigenic community. Nevertheless, cross-neutralization experiments are necessary to confirm the protective effect of this candidate vaccine against Reunion strains.

In the phylogenetic tree based on the partial E1 sequences (Figure), all CHIKV strains isolated in Reunion clustered together. These strains were closely related to strains from the Central African Republic and the Democratic Republic of Congo (*4*,*6*). This finding suggests that the boundaries of the Central African CHIKV strains now extend to the Indian Ocean. The phylogenetic tree also illustrates the difference of lineage between the Reunion Island isolates and the Asian isolates.

CHIKV has been isolated from *Culex* spp. collected during outbreaks (*8*), but laboratory experiments have shown that *Cx. quinquefasciatus* failed to transmit CHIKV to monkeys

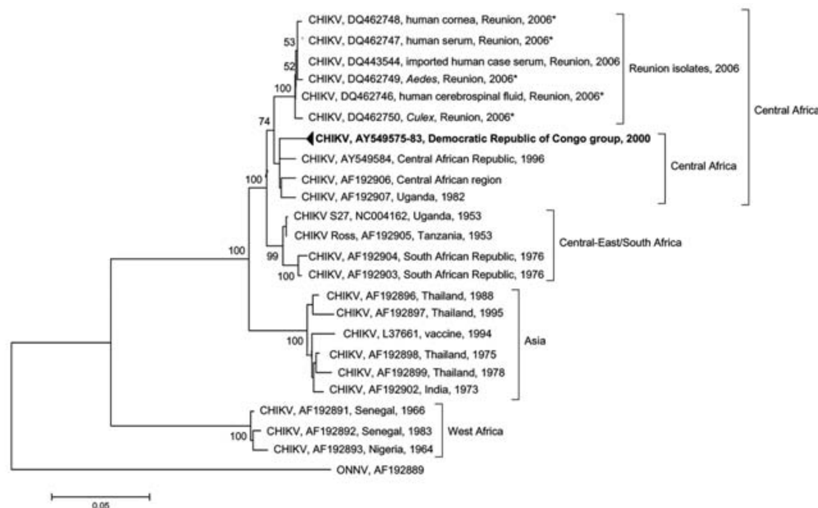

Figure. Phylogenetic tree of chikungunya virus (CHIKV) based on partial nucleotide sequences (3′ extremity of E1/3′ untranslated, position 10238–11367). Phylogram was constructed with MEGA 2 (http://megasoftware.net/mega2.html), and the tree was drawn with the Jukes-Cantor algorithm for genetic distance determination and the neighbor-joining method. The percentage of successful bootstrap replicates (1,000 bootstrap replications, confidence probability >90%) is indicated at nodes. The length of branches is proportional to the number of nucleotide changes (percentage of divergence). Asterisks (*) indicate strains isolated in this study. The dark triangle corresponds to viruses of the Democratic Republic of Congo clustering together (GenBank accession nos. AY549575–AY549583). O'nyong-nyong virus (ONNV) sequence has been introduced for correct rooting of the tree.

(*9*). Inside the Reunion cluster, the strain from *Culex* spp. was localized in a separate branch (bootstrap value 100%); this finding could be relevant to the different role of these mosquito species in virus epidemiology.

To our knowledge, CHIKV has never been isolated from human corneas. In our study, the cornea sample was obtained from an asymptomatic donor whose serum contained immunoglobulin M (IgM) but not IgG to CHIKV; this finding suggests the patient was recently infected with CHIKV. The presence of CHIKV in corneal cells will have to be confirmed because the samples we studied also included sclera, vascular tissue that could contain circulating virions; however, no virus was detected in the patient's blood sample by CHIKV-specific RT-PCR assay (*10*). Infected corneal or scleral cells may constitute a sanctuary that allows virus to persist after virus is no longer present in blood. Because viral persistence, which could explain long-lasting clinical complications of CHIKV infection, has never been demonstrated, this question deserves more investigation.

Our results indicate that CHIKV strains responsible for the outbreak in Reunion have a common origin and do not differ from strains circulating in East and Central Africa. More complete characterization of the 5 strains we report here, sequencing of the full-length genome, and phenotypic characterization of other CHIKV isolated in the area during the same period is currently underway in our laboratory.

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Legionnaires' Disease and Travel in Europe

To the Editor: The European Working Group for *Legionella* Infections (EWGLINET) conducts epidemiologic surveillance of Legionnaires' disease cases associated with travel (*1*) and provides epidemiologic typing markers of *Legionella pneumophila* among reference laboratories in collaborating countries. The procedures and criteria of notification are found in the Guidelines for Control and Prevention of Travel Associated Legionnaires' Disease (*2*). However, establishing the association of ≥1 case of this disease and a specific tourist accommodation site is difficult because of low attack rates and dispersal of people from the source of infection during the incubation period.

Collaboration promoted by this working group encourages the exchange of data instead of cultures. This distinction is critical when research is conducted on travel-associated Legionnaires' disease, in which strains from patients and environmental sources of infection studied are in different laboratories.

The value of such information is shown in a complex case study that was recently investigated. During July and August 2005, two patients with Legionnaire's disease living in 2 countries in Europe were reported to EWGLINET. Patient 1 was a 45-year-old woman who traveled in France and Spain July 1–6, 2005. Her symptoms started on July 6, 2005, when she was in Girona, Spain, where she was hospitalized. Patient 2 was a 56-year-old woman who traveled in Spain and France August 16–21, 2005. Her symptoms started on August 8, 2005, when she was in France, where she was hospitalized. Both patients tested positive for *L. pneumophila* serogroup 1 by specific urinary antigen test and culture, but they recovered and were discharged.

After routine notification to EWGLINET, it was established from the list of accommodation sites provided by the 2 patients that they each had stayed for 1 night at the same hotel in a French city within a 45-day interval. This finding led us to identify a cluster according to the definition in use (2 cases associated with the same accommodation within 2 years) (*2*). However, patient 2 spent 1 day in August in Zaragoza, Spain, during which an outbreak of Legionnaires' disease in the city affected 30 persons. Thus, illness in patient 2 could have been associated with the Zaragoza outbreak. Alternatively, both patients could have contracted the illness independently at different sites. Before onset, patient 1 stayed 5 days in her private residence in Girona and patient 2 visited 3 other hotels.

As soon as cultures from the 2 patients were available, the National Reference Laboratories of France and Spain shared their respective micro-

biologic results. Since both strains were identified as *L. pneumophila* serogroup 1, we performed sequence-based typing (SBT) (*3*) of 6 genes (*fla*A, *pil*E, *asd*, *mip*, *momp*S, and *pro*A) by using the protocol and database of EWGLINET. Both isolates showed identical SBT patterns (2,3,18,15,2,1).

Isolates from 4 patients in the Zaragoza outbreak were identified at the Spanish Reference Laboratory as *L. pneumophila* serogroup 1 (Philadelphia monoclonal antibody type) and had identical SBT patterns (3,4,1,1,14,9). Collaboration between public health authorities in France and Spain enabled us to eliminate the association of patient 2 with the Zaragoza outbreak and establish an association of both patients with the same site in France. Control measures were taken at the hotel, but we could not obtain environmental cultures for comparison with those of the patients. Lack of environmental data prevented investigation of the relationship with the other accommodation sites visited.

The SBT method provides robust genotyping with high discriminatory power (index of discrimination >0.94) (*3*). This method is less effective at discriminating between strains than pulsed-field gel electrophoresis (*4*), but it shows excellent reproducibility and may be useful in epidemiologic investigation of outbreaks caused by *L. pneumophila*. The availability of an online database with accessible information is key for sharing results and determining the geographic distribution of isolates that cause Legionnaires' disease (*4*,*5*).

This study demonstrates the critical role of sharing results between countries that participate in a network. Agreement is essential on a standardized questionnaire that includes more information on the patient's exposure to a disease. Moreover, despite the performance of the urine antigen test, cultures of clinical samples should be encouraged by clinicians and microbiologists. This step would permit use of techniques, such as SBT, in reference laboratories and sharing of results. Our investigation would have been more difficult without this technique in identifying the site where the infection potentially originated.

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Influenza A Virus PB1-F2 Gene

To the Editor: Recently, Chen and co-workers described the expression of an 11th influenza A virus protein, designated PB1-F2 because this protein is encoded in the +1 open reading frame of the segment-2 RNA (*1*). Later, Chen et al. presented a preliminary analysis of 336 PB1 sequences from GenBank (*2*). We have extended the work on PB1-F2 and analyzed 1,864 partial and complete segment-2 sequences deposited in GenBank; these sequences belong to 79 influenza A virus subtypes. In summary, the following 8 observations should receive attention:

First, the size of PB1-F2 polypeptides ranges from 79 to 101 amino acids (aa); most isolates encode versions of either 87 or 90 aa. Because polypeptides of 79 aa are located within mitochondria, their truncation has no effect on the protein function. The frequency of the 79-aa PB1-F2 is ≈5%.

Second, a functional PB1-F2 is expressed by 92% of all segment-2 sequences, i.e., a polypeptide >78 aa. The proportion of intact PB1-F2 varies according to host (humans 90%, swine 76%, other mammals 100%, birds 95%).

Third, the H1N1 subtype comprises 3 genetic lineages. One clade has 2 branches: 1 branch includes the human viruses, with the pandemic 1918 virus at its root; the other branch includes the classic swine viruses. The third clade represents the European porcine isolates. Although all classic swine sequences have a truncated PB1-F2 (in-frame stop codons after 11, 24, and 35 codons), the early human isolates (H1N1 sequences from 1918 through 1947) have an intact PB1-F2. After 1956, however, a mutation became prevalent such that the recent sequences starting from A/Beijing/1/56 terminate after 57 codons. An exception to

this rule is A/Taiwan/3355/97. Two human H1N1 isolates with an intact PB1-F2 coding sequence cluster in the H3N2 clade (A/Kiev/59/79, A/Wisconsin/10/98). The PB1 sequences of European porcine influenza A virus isolates cluster with European porcine H3N2 and H1N2.

Fourth, all H2N2 sequences are monophyletic and encode an intact PB1-F2. Fifth, the main sequence cluster of the H3N2 subtype comprises 3 branches: 1) porcine H3N2 and porcine H1N2 sequences from the United States, 2) porcine H3N2 isolates from Hong Kong and human H1N2, and 3) recent human H3N2 and some Japanese H3N2 isolates. Most of these sequences encode an intact PB1-F2.

Sixth, the cluster of European porcine influenza A virus isolates comprises the subtypes H1N1, H1N2, and H3N2. The lack of distinct clades for each subtype indicates frequent reassortment in the evolution of these viruses. Of the segment-2 sequences, 56% encode an intact PB1-F2.

Seventh, other porcine isolates of various subtypes represent trans-species infections or single reassortment events. And eighth, the segment-2 sequences of many avian influenza A virus isolates encode intact PB1-F2. Considerable proportions of truncated PB1-F2 genes were found in the H5N2, H6N6, H9N2, and H13N2 subtypes. However, because of the small number of sequences available, this observation may not be important.

In conclusion, PB1-F2 is expressed in most avian and many porcine influenza A virus isolates. This finding contrasts with those in the initial publication, which stated that PB1-F2 is not expressed in many animal isolates, particularly those of porcine origin (*1*). Because PB1-F2 was described as a proapoptotic protein probably counteracting the host immune response, why numerous human and porcine isolates lack this protein without selective disadvantage remains unclear.

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In response: Zell et al. (*1*) performed an extensive genetic investigation of PB1-F2, based on up-to-date GenBank sequences. Their sample size (1,864) greatly outnumbered ours (336) in a previous study (*2*) and thus definitely better portrays the genetic characteristics of PB1-F2. We appreciate their analyzing these samples by subdividing nonhuman strains into different species, which we did not do (*2*). Their analysis is especially meaningful for the global pandemic threat from avian influenza viruses, which increases the need to study interspecies adaptation and transmission.

Zell et al. found that 92% of PB1 RNA encodes a functional PB1-F2, compared with our 79% (264/334), which supports the increasingly crucial role of PB1-F2 in influenza virology. They found the proportion of intact human PB1-F2 to be 90%, a substantial boost from our 68% (67/99), which was based on data from late 2003 (*2*). This increase is apparently caused by the increasing number of human H3N2 sequences (mostly encoding an intact PB1-F2 compared with H1N1) deposited in the past 2 years.

Human H1N1 from 1918 through 1947 contains full-length PB1-F2, whereas human H1N1 beginning in 1956 has a truncated PB1-F2 after codon 57. As reported by Zell et al., only 3 human H1N1 strains contain full-length PB1-F2: A/Kiev/59/79, A/Taiwan/3355/97, and A/Wisconsin/10/98. The PB1 genes of A/Kiev/59/79 and A/Wisconson/10/98 were found clustered with human H3N2 as a result of natural reassortment between human H1N1 and H3N2 strains. On the other hand, the asynonymous mutation found on A/Taiwan/3355/97 enabled the translation to get past the usual stop codon at position 58, which other H1N1 strains exhibit. A/Taiwan/3355/97 (H1N1) was isolated from a patient with severe pneumonia. Animal study has demonstrated that the existence of full-length PB1-F2 contributed to pathogenesis in mice (*3*). We speculate that the expression of a full-length PB1-F2 may contribute to disease severity in humans.

The C-terminal domain of PB1-F2 contains the mitochondrial signal and can trigger apoptosis in specific immune-related cells. Our recent work (*4*) comparing avian and human influenza A viruses also found that many species-associated amino acid signatures are located on the C terminal of PB1-F2. This finding highlights the importance of further investigating the role of PB1-F2 on interspecies infection.

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Enterovirus 75 and Aseptic Meningitis, Spain, 2005

To the Editor: Although most human enterovirus (EV) (genus *Enterovirus*, family *Picornaviridae*) infections are asymptomatic, they can cause upper respiratory illness, febrile rash, aseptic meningitis, pleurodynia, encephalitis, acute flaccid paralysis, and neonatal sepsislike disease (*1*). Most EVs have been implicated in aseptic meningitis, most notably echovirus (E) 30, 9, 6, and 11 and coxsackie B virus (CBV) type 5 (*2*); other serotypes are less frequently associated with neurologic disease.

New EV serotypes have come to light, chiefly as a result of molecular typing methods (*3–6*). EV75 was proposed as a new serotype of the EV genus in 2004 (*5*). Retrospective analysis showed that it had circulated sporadically in Asia, the United States, and Africa since at least 1974. Only 8 isolates of this serotype have been reported worldwide, in 1974, 1985, 1986, 1987 (n = 2), 1998, and 2000 (n = 2). Infection in those cases was associated with respiratory disease, acute flaccid paralysis, neonatal jaundice, failure to thrive, or unspecified neurologic disease or was asymptomatic. At the time of writing this manuscript, EV75 had not been linked to aseptic meningitis.

From May 2005 through January 2006, 106 EVs were received for typing from Spanish hospital laboratories; 46 of them were from patients with aseptic meningitis, 10 from patients or contacts of patients with acute flaccid paralysis, 27 from patients with fever, 7 from patients with respiratory diseases, and 16 from other patients. Twenty EVs could not be typed by serum neutralization (*7*); however, 3′ terminus VP1 gene sequence analysis (*8*) showed that they were E18 (n = 7), CBV3 (n = 1), and E16 (n = 2); 2 could not be typed with serologic or molecular methods because the 3′ terminus of VP1 gene amplification was negative. The analysis of the 3′ terminus of VP1 gene of the remaining 5 cerebrospinal fluid (CSF) and 3 nasopharyngeal isolates showed that they were similar to the recently proposed EV75 serotype (*5*). These 8 isolates were obtained from samples from children in Bilbao (n = 3), Granada (n = 3), Barcelona (n = 1), and the Canary Islands (n = 1). In 4 patients with aseptic meningitis, EV75 was isolated from CSF. EV75 was isolated from CSF of a fifth patient who had symptoms of fever and irritability. The remaining 3 EV75 isolates were from nasopharyngeal swabs of children who had fever, respiratory disease, or gastroenteritis. All isolates were grown in cell lines (rhabdomyosarcoma, lung adenocarcinoma, and human fetal lung fibroblast) and identified as EV by immunofluorescence with pan-EV antibody assays (Pan Entero Blend Chemicon, Temecula, CA, USA, and Monoclonal Mouse Anti-Enterovirus, Dako, Glostrup, Denmark).

Phylogenetic analysis of the isolates from 2005 was performed on the basis of complete VP1 gene sequence (GenBank accession nos. DQ468137–DQ468142). The 5′ terminal domain was obtained by reverse transcriptase–PCR with specific primers EV75_sense: 5′-GAAAGCTTYTTC-CAAGGGGA-3′ and EV75_anti: 5′-GAGAAGTGKGACCAWCCATC-3′. Phylogenetic analysis of the Spanish isolates and representatives of all other species B EVs showed that the Spanish isolates clustered (bootstrap value 100, Figure) with strains USA/OK85-10362, ETH74-1341, USA/VA86-10363, USA/CT87-10364-5, OMA98-10366, and BAN00-10367-8 (accession nos. AY556063–AY556070), corresponding to the proposed EV75. The Spanish isolates constitute a subgroup (bootstrap value 100, Figure). The similarity between the Spanish cluster and other EV75 isolates was 82.8%–85.4% at the nucleic acid level. Although the entire VP1 sequence was not available for the isolates from 2006, the VP1 3′ terminal analysis showed the strains belonged to the same cluster.

To our knowledge, this is the first isolation of EV75 in Spain. Indeed, isolation of EV75 has not been reported in Europe. Given that the European EV75 isolate grows easily in a variety of cell lines, is detected by common EV genus-specific antibodies, and that EV surveillance and typing were performed in Spain since 1988 (*2*), EV75 might have begun to circulate in Spain recently. However, because isolates are not obtained from all aseptic meningitis patients and many EVs are detected by PCR but never typed, we cannot rule out the possibility of previous asymptomatic circulation.

The European strains of EV75 appear to represent a different evolutionary lineage than those previously

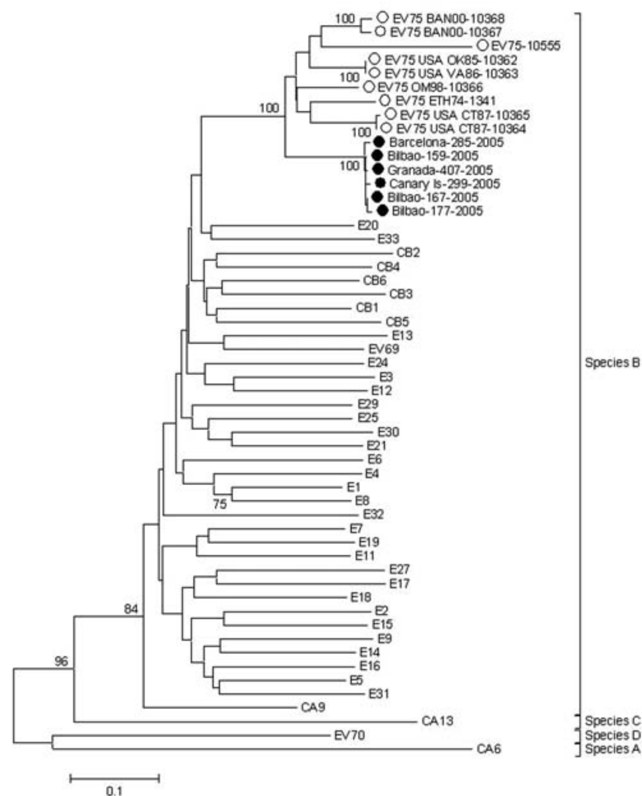

Figure. Phylogenetic analysis of complete VP1 sequences of Spanish enterovirus (EV) isolates (GenBank accession nos. DQ468137–DQ468142), the new proposed EV75 sequences (AY556063–AY556070 and AY919545), and prototype EV sequences (echovirus [E] 5, AJ241425; E31, AJ241435; E2, AF081315; E15, AJ241429; E14, AJ241428; E17, AF081330; coxsackie B virus [CBV] 2, AF081312; E26, AJ241433; E27, AF081338; E1, AJ241422; E8, AF081325; E4, AF081319; E21, AF081334; E30, AF081340; E25, AF081336; E29, AJ241434; CBV5, AF114383; CBV6, AF081313; E13, AF081327; EV69, AF081349; E24, AJ241432; E33, AF081346; E3, AF081316; E12, X77708; CBV3, M16572; CBV1, M16560; E6, AF081322; coxsackie A virus [CAV] 9, D00627; E16, AY302542; E9, AF524866; E7, AJ241426; E32, AF081345; E19, AJ241430; E11, AF081326; CBV4, X05690; E18, AF081331; E20, AJ241431; EV70, D17602; CAV6, AF081297; CAV13, AF081303; EV74, AY208118). Phylogenetic trees were constructed with the neighbor-joining method (MEGA version 3.0, available from http://www.megasoftware.net) with Kimura 2-parameter substitution model. Significance of phylogenies was estimated by bootstrap analysis with 1,000 pseudoreplicate datasets. Closed and open circles show Spanish and previously reported EV75 isolates, respectively.

described in the United States, Asia, and Africa (*9*). Only 1 of those EV75s was obtained from CSF (a nonspecific neurologic syndrome). Thus, EV75 has not been associated with aseptic meningitis, despite the fact that EV infections are a common cause of aseptic meningitis. Most of the Spanish isolates (5 of 8) were associated with aseptic meningitis in children. Although the number of EV75-associated cases was not high (as a percentage of the number of EVs isolated from aseptic meningitis patients, 10.8%), the wide distribution of the cases may indicate wide circulation. To avoid outbreaks of aseptic meningitis caused by previously noncirculating EVs (EV13, 2001 [*10*]) and to help define the extent of circulation of newly identified EV types, careful surveillance of aseptic meningitis should be undertaken.

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Ciprofloxacin-resistant *Salmonella* Kentucky in Travelers

To the Editor: Ciprofloxacin is the treatment of choice of severe nontyphoidal *Salmonella* infections in adults. Resistance to ciprofloxacin has been found exceptionally in nontyphoidal *Salmonella enterica* isolates and only in serotypes Typhimurium, Choleraesuis, and Schwarzengrund (*1–8*). Such isolates have been collected from humans and animals in Europe, Asia, and North America.

We report the emergence of ciprofloxacin-resistant isolates of *S.* Kentucky since 2002 in French travelers returning from northeast and eastern Africa. From 2000 through 2005, 197 *S.* Kentucky isolates from humans (1 per patient) were serotyped, from 69,759 total *S. enterica* isolates serotyped at the French National Reference Centre for *Salmonella*. Antimicrobial drug susceptibility was determined for 186 isolates by the disk-diffusion method with 32 antimicrobial drugs, as previously described (*9*). Resistance to several drugs, amoxicillin (18%), gentamicin (16%), nalidixic acid (21%), sulfonamides (24%), and tetracycline (24%), has been observed from 2000 through 2005.

A total of 17 (9%) ciprofloxacin-resistant *S.* Kentucky strains were isolated. A resistant isolate that was untypable by conventional serotyping (rough) but that had a pulsed-field gel electrophoresis (PFGE) profile associated with serotype Kentucky, was included in this study. Ciprofloxacin MIC levels in these isolates, determined by standard agar doubling dilution as previously described (*2*), were 4–16 mg/L. The first ciprofloxacin-resistant strain was isolated in December 2002 from a French tourist who had gastroenteritis during a Nile cruise in Egypt. In 2004 and 2005, 17 ciprofloxacin-resistant isolates were identified in unrelated adults who lived in different cities of France at different times of the year. The 16 patients we contacted acquired the infection during or immediately after travel to Egypt (10 patients), Kenya and Tanzania (3), or Sudan (1). In 2 cases, gastroenteritis occurred 2 months after travel to Egypt. None of the investigated cases were fatal or life-threatening.

The 18 ciprofloxacin-resistant isolates (17 serotype Kentucky and 1 rough) displayed various susceptibility patterns, from single resistance to quinolones to multiple resistance (up to 9 antimicrobial agents). To identify mutations responsible for ciprofloxacin resistance, the quinolone resistance–determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* were amplified by PCR and sequenced as described previously (*3*,*9*), except that different forward primers for *gyrB* (5′-TTATCGACGC-CGCGCGTGCGC-3′) and *parE* (5′-CGCGTAACTGCATCG-GGTTC-3′) were used. The 18 ciprofloxacin-resistant isolates had different double mutations in *gyrA* leading to amino acid substitutions, Ser83Phe and Asp87Gly (8 isolates), Ser83Phe and Asp87Asn (5), and Ser83Phe and Asp87Tyr (5), but had identical mutations in *parC* (resulting in Ser80Ile). An additional substitution was observed in ParC, Thr57Ser. This substitution, however, did not appear to be associated with quinolone resistance because it was also identified in nalidixic acid–susceptible isolates. No isolates had substitutions in the QRDRs of GyrB and ParE. All isolates tested by PCR for the plasmid-mediated quinolone resistance–conferring gene *qnrA* (*9*) were negative. In the presence of the efflux pump inhibitor Phe-Arg-β-naphthylamide, the MICs of ciprofloxacin were reduced from 4-fold to 16-fold, which suggests that an active efflux mechanism was present (*2*). The involvement of the AcrAB-TolC efflux system was determined by measuring AcrA expression with a method previously described (*5*). A moderate production of AcrA (3- to 4-fold increase when compared with the baseline production of AcrA in reference strain 98K) was observed in all but 1 ciprofloxacin-resistant isolate. This isolate overproduced (6-fold) AcrA, which correlated with a higher ciprofloxacin MIC (16 mg/L).

The 18 ciprofloxacin-resistant isolates and 14 ciprofloxacin-susceptible *S.* Kentucky isolates used for comparison were genotyped by PFGE with *Xba*I restriction and PulseNet's running conditions, as described previously (*9*). Each profile that differed by ≥1 clear band >50 kb was considered a distinct profile. The 18 resistant isolates displayed 9 profiles that differed by 1 to 3 bands (Dice correlation coefficient 55%) (Figure). Profile X1c was predominant (7 [39%] of 18). The 6 pansusceptible isolates tested displayed 5 different patterns unrelated to those of resistant isolates. Use of a

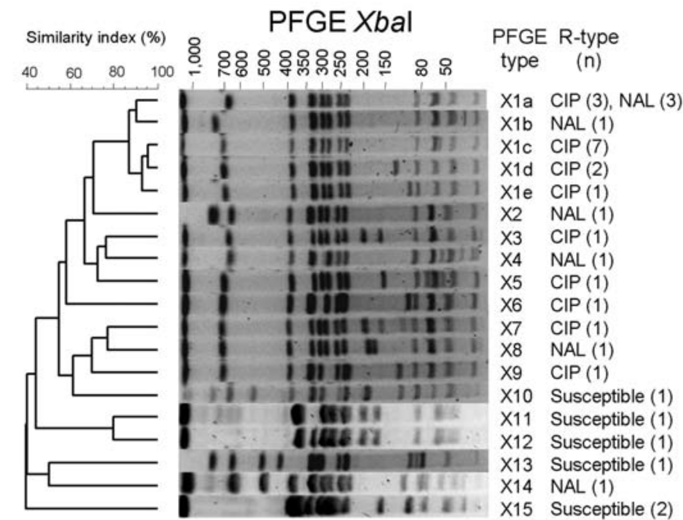

Figure. Dendrogram generated by BioNumerics version 4.1 (Applied Maths, Sint-Martens-Latem, Belgium) showing the results of cluster analysis on the basis of *Xba*I pulsed-field gel electrophoresis (PFGE) of *Salmonella enterica* serotype Kentucky isolates. Similarity analysis was performed by using the Dice coefficient, and clustering was performed by the unweighted pair-group method with arithmetic means with an optimization parameter of 0.5% and a 0.5% band position tolerance. The different PFGE profiles, the phenotypes of resistance to quinolones (R-type), and corresponding number of isolates are indicated. CIP, ciprofloxacin; NAL, nalidixic acid.

second restriction enzyme, *Spe*I, for the resistant isolates of X1 cluster enhanced discrimination. No clear correlations between combined PFGE patterns, *gyrA* mutations, and probable country of infection were observed.

Since *S.* Kentucky is infrequently isolated from human, animal, or environmental sources in France, these isolates must have been acquired abroad. Unfortunately, no investigations have been thus far conducted to identify the source of the contamination in probable countries of infection. Poultry products may be of particular interest because poultry is the main animal reservoir of *S.* Kentucky. Another possible source in East Africa is pork because a recent report identified quinolone-resistant (ciprofloxacin MIC >0.125 mg/L) *S.* Kentucky isolates in slaughtered pigs in Ethiopia (*10*). After identifying the source of the contamination, appropriate control measures should be implemented in the affected countries to control the spread of these isolates.

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KPC Type β-Lactamase, Rural Pennsylvania

To the Editor: Rural counties have been defined as those lacking a metropolitan center that has a population >50,000 persons (*1*). Little is known about antimicrobial drug resistance in such communities in the United States. Stevenson and colleagues (*2*) recently evaluated antimicrobial drug–resistant gram-positive infections in rural hospitals in Idaho and Utah. These researchers found that both methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci occurred in such settings, although some of the MRSA strains were probably community associated. Comparable studies on multidrug-resistant gram-negative infections have not been performed, to our knowledge.

Klebsiella pneumoniae producing a broad-spectrum β-lactamase, KPC, has been described in tertiary care centers and other metropolitan hospitals in New York City. Examples have also been found in similar settings in Boston, New Jersey, Maryland, and North Carolina (*3–5*). The carbapenems (such as imipenem and meropenem) are typically the most active antimicrobial agents against the *Enterobacteriaceae*. The KPC β-lactamases inactivate carbapenems and all other β-lactam antimicrobial drugs. Unfortunately, bacteria producing the KPC type β-lactamases are typically also resistant to trimethoprim/sulfamethoxazole, quinolones, and aminoglycosides, thereby making these pathogens truly multidrug resistant.

We describe a patient with KPC-producing *K. pneumoniae* in a rural setting in central-west Pennsylvania. The case highlights the potential for multidrug-resistant gram-negative organisms to occur outside their previously recognized settings in large metropolitan centers.

The patient was a 76-year-old woman who lived alone, closely attended by her daughter, in a small, central Pennsylvania community, 95 miles from a metropolitan center with a population of >50,000. Her medical history included a seizure disorder, hypertension, osteoarthritis of the knees, obesity, osteoporosis, and total hysterectomy. A month before isolation of the KPC-producing *K. pneumoniae*, the patient had a 3-day hospital admission to a 200-bed hospital in the nearest metropolitan center (population 7,000) after a fall. She was discharged to a local nursing home for rehabilitation. She is not known to have visited or been hospitalized in New York, Philadelphia, or New Jersey, nor did she share a room with a patient known to have been hospitalized in these areas. She had no known animal contact. She had received trimethoprim/sulfamethoxazole and levofloxacin for treatment for urinary tract infections in the month before the KPC-producing strain was isolated. She was readmitted to the 200-bed hospital with pyelonephritis in August 2005. Cultures of urine grew *K. pneumoniae*; the organism was resistant to all β-lactam antimicrobial drugs tested, including cefepime, ceftriaxone, piperacillin/tazobactam, imipenem, fluoroquinolones, trimethoprim/sulfamethoxazole, gentamicin, and tobramycin. The patient received therapy with amikacin in combination with cefepime, ertapenem, or tigecycline at different times over the following 4 weeks. Her symptoms improved, although her urine remained colonized with the multidrug-resistant *K. pneumoniae*. In October 2005, *Clostridium difficile* infection developed, accompanied by deep venous thrombosis and gastrointestinal bleeding, and the patient died. Multiple blood cultures collected before her death were negative, although the urine was persistently colonized with the multidrug-resistant *K. pneumoniae*.

The organism was referred to a research laboratory in a metropolitan center ≈100 miles away. Molecular analysis of the mechanisms of resistance was performed by using previously described methods (*6*). This analysis showed that the *K. pneumoniae* isolate produced the extended-spectrum β-lactamase (ESBL) SHV-11 and the carbapenemase KPC-2.

Since community-associated ESBL-producing organisms have been described in Canada and Europe (*7*,*8*), acquisition or in vivo development of ESBL and KPC-producing strains could have occurred outside of the healthcare setting. More likely, the patient acquired her almost completely resistant gram-negative organism in the rural hospital or her local nursing home. To our knowledge, no other clinical isolates with the same antimicrobial phenotype have been seen in patients in either setting before or after the patient's admission. An unsuspected reservoir of patients colonized with antimicrobial drug–resistant gram-negative organisms may exist (*9*). Ideally, an epidemiologic investigation at both the hospital and nursing home would have been performed, but facilities for an investigation involving use of selective microbiologic media and assessment of gastrointestinal carriage of resistant organisms are not typically available in a rural setting. Indeed, most rural hospitals do not even use routine diagnostic tests for detecting resistant gram-negative organisms such as ESBL producers (*10*).

Although much attention has been focused on the progression of antimicrobial drug resistance in gram-positive organisms, the development of alternative antimicrobial agents such as linezolid and daptomycin may mitigate the disastrous scenario of complete resistance to all commercially available antimicrobial agents. However, few drugs are active against

multidrug-resistant gram-negative pathogens, and enhanced measures are needed to prevent spread of these organisms. A greater understanding of the modes of spread and acquisition of these organisms is essential for effective control of this problem. We have reported just 1 case of infection with an almost completely resistant gram-negative organism. This case expands the known geographic spread of organisms with this resistance problem. This case also underscores the importance of studying the epidemiology of antimicrobial drug resistance in gram-negative organisms in the rural setting as well as in large metropolitan centers. Dissemination of knowledge regarding appropriate antimicrobial drug susceptibility testing for resistant organisms is also needed.

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Severe Pneumonia and Human Bocavirus in Adult

To the Editor: The newly identified human bocavirus (hBoV), a member of the *Parvovirus* family, is suspected to infect the cells of the respiratory tract and thus may be an etiologic agent of respiratory disease in humans (*1*). Although Koch postulates have not been fulfilled for HboV, it appears likely to cause a substantial number of respiratory tract infections, at least in children (*2*,*3*). We describe a case of severe atypical pneumonia associated with hBoV DNA in a bronchoalveolar lavage (BAL) sample from an adult.

The patient was a 28-year-old Caucasian woman with an angioimmunoblastic T–non-Hodgkin lymphoma (NHL) that changed into a highly malignant blastic B-cell lymphoma (T-cell–rich B-NHL state I with 70% CD20+ cells, initial stage IIIB). The patient was previously treated with vincristine and prednisone, followed by chemotherapy according to the R-CHOEP-14 protocol (3 cycles) (November 2003 through January 2004). From January through February 2004, chemotherapy was combined with antimicrobial drug therapy according to the R-DHAP protocol (which includes dexamethasone, the chemotherapy drugs cytarabine and cisplatin, and the monoclonal antibody drug rituximab) for persisting symptoms from the B-cell lymphoma. This regimen was followed by a therapy switch to alemtuzumab with ifosfamid, carboplatin, and etoposide (March 2004), which led to a therapy-induced leukopenia, thrombocytopenia, and high fever >40°C by the end of March and the beginning of April 2004. In May 2004, a second round of alemtuzumab with ifosfamid, carboplatin, and etoposide chemotherapy was initiated. In June 2004, a therapy-induced

hemorrhagic cystitis occurred. During July 2004, the patient had ongoing high fever and aplasia of bone marrow with unclear etiology. On July 22, hospital treatment was initiated; it consisted of antimicrobial drug treatment with ceftriaxone (1,000 mg once daily) and gentamicin (320 mg once daily), and antimycotic therapy was started with caspofungin (50 mg once daily).

Since cytomegalovirus (CMV) infection was suspected, ganciclovir (250 mg twice daily) was administered IV for 2 weeks. Although the patient reported an ongoing cough and pneumonialike symptoms, a severe atypical pneumonia that was refractive to antibacterial and antimycotic treatment was diagnosed for the first time during this hospital treatment. Computed tomography scan showed bilateral atypical reticulonodular infiltrations predominant in the lower zones of the lungs (Figure).

The BAL obtained during exacerbation of the pulmonary symptoms was tested for *Mycobacterium tuberculosis*, *Chlamydia pneumoniae*, *Pneumocystis jirovecii*, *Aspergillus* sp., *Candida* sp., *Cryptococcus neoformans*, CMV, Epstein-Barr virus, hepatitis B virus, hepatitis C virus, HIV, herpes simplex virus, and varicella-zoster virus by PCR and culture cultivation. Results were negative, except for a temporarily weak reactivity for *Aspergillus* antigen in serum and for CMV DNA in peripheral blood lymphocytes, which was positive before and became negative after ganciclovir therapy. An archived portion of the BAL was assayed retrospectively by PCR/reverse transcriptase–PCR for human bocavirus, respiratory syncytial virus, human coronaviruses including severe acute respiratory syndrome–associated coronavirus, influenza virus, and human metapneumovirus (hMPV). The only positive result was obtained for human bocavirus, which was confirmed by sequence analysis of the PCR product.

Within a few days, the patient's symptoms decreased, and she was discharged from hospital on day 41, despite ongoing bone marrow aplasia with antimicrobial and antimycotic prophylaxis, including trimethoprim/sulfamethoxazole (160 mg/800 mg once daily) and (voriconazole 200 mg twice daily). Clinical observations led to the primary assumption that the fever, cough, and pulmonary symptoms were likely caused by the postchemotherapeutic extended bone marrow aplasia and CMV infection accompanied by an unclear bacterial but fungus-typical infection. Retrospectively, however, human bocavirus DNA in the archived BAL strongly suggests that pulmonary symptoms were caused by this agent rather than by a yet unknown bacterial or fungal infection. Thus, in the clinical episode described here, the likely causative agent responsible for the severe pneumonia was the recently described bocavirus.

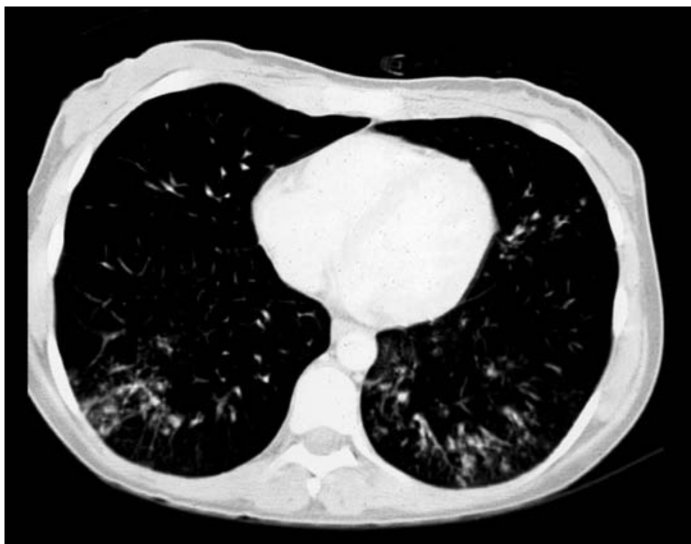

Figure. Computed tomography scan showing reticulonodular infiltrations of both lungs in the lower zones.

Respiratory viruses such as respiratory syncytial virus, hMPV, and hBoV seem to be the most prevalent etiologic agents of acute lower respiratory tract infection in children. Recently, evidence of human bocavirus infection was reported for 3.1% to 5.7% of children <3 years of age (*1–3*). Previously, only limited data on adults, including immunocompromised patients, were available, but the case we describe supports the hypothesis proposed for other newly identified respiratory viruses, namely, that these pathogens also contribute to severe infections in adult patients at high risk. For example, hMPV was found in 3% of stem-cell transplant recipients who underwent BAL because of lower respiratory tract infection (*4*). In those high-risk patients, hMPV also induced fatal infections (*4*). This finding led to the conclusion that a "new" virus that induces the identical clinical symptoms, like the human bocavirus, may also contribute to severe respiratory infections. In summary, this first report of a respiratory tract infection with hBoV in an adult immunocompromised patient strongly supports the assumption that hBoV is an emerging pathogen that requires our attention, even for adult patients (*1–3*).

Acknowledgments

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Leishmaniasis in Ancient Egypt and Upper Nubia

To the Editor: Leishmaniasis is a disease caused by parasites of the genus *Leishmania*. The infection is transmitted to humans through the bites of female sandflies and manifests mainly in 3 forms: visceral, cutaneous, and mucocutaneous. Visceral leishmaniasis or kala-azar, the often fatal form of the disease, is caused by species of the *Leishmania donovani* complex. These parasites were responsible for severe recent outbreaks in Sudan and other countries and are thought to originate in East Africa (*1–4*).

In this report, we describe the successful amplification of *L. donovani* DNA in ancient Egyptian and Christian Nubian mummies dating back 4,000 years. Besides the first proof for visceral leishmaniasis in paleopathology, we provide evidence that leishmaniasis was present in Nubia in the early Christian period and that the organism also infected ancient Egyptians, probably because of close trading contacts to Nubia, during the Middle Kingdom. We analyzed 91 bone tissue samples from ancient Egyptian mummies and skeletons and 70 bone marrow samples from naturally mummified human remains from Upper Nubia. The Egyptian material derived from the Pre- to Early Dynastic site of Abydos (n = 7; 3500–2800 BC), a Middle Kingdom tomb in Thebes West (42; 2050–1650 BC), and different tomb complexes in Thebes West, which were built and used between the Middle and New Kingdom until the Late Period (42; c. 2050–500 BC). The Nubian samples were taken before the flooding caused by the Aswan Dam from 2 early Christian burial sites at Kulubnarti, between the second and third cataracts of the Nile River in northern Sudan. One site was on an island in the Nile and dated from 550 to 750 AD. The other was on the western bank of the Nile and was in use from c.750 to 1500 AD. All samples were tested for *Leishmania* spp. DNA and further characterized by direct sequencing.

In 4 of the 91 Egyptian and 9 of the 70 Nubian samples, a 120-bp fragment of a conserved region of the minicircle molecule of kinetoplastid mitochondrial DNA of the parasite (*5,6*) could be successfully amplified and, with the first primer pair, unambiguously related to *L. donovani* species after sequencing (Figure). The positive samples from ancient Egypt exclusively originated from the Middle Kingdom tomb, while no molecular evidence for ancient *Leishmania* DNA was found in the Pre- to Early Dynastic and the New Kingdom to Late Period specimens.

In the Middle Kingdom, the Egyptians extended trade relationships and military expeditions to Nubia, the modern Sudan, with particular interest in the gold resources of the country and in obtaining slaves to serve as servants or soldiers in the pharaoh's army. Today, the Sudan is one of the highly endemic countries for visceral leishmaniasis or kala-azar, which is thought to have originated in East Africa and later spread to the Indian subcontinent and the New World (*4*). Therefore, the high incidence of *Leishmania* DNA in the Middle Kingdom samples (4 [9.5%] of 42) and the lack of findings in earlier or later time periods, may indicate that leishmaniasis was introduced into Egypt at this time. Leishmaniasis did not likely become endemic in the Egyptian Nile Valley because the disease is closely linked to its vector, the phlebotomine sandfly, and the distribution of Acacia-Balanites woodland (*7*). That ancient Egyptians became infected because of close trade contacts and associated travel with Nubia during the Middle Kingdom seems more plausible. The high frequency of

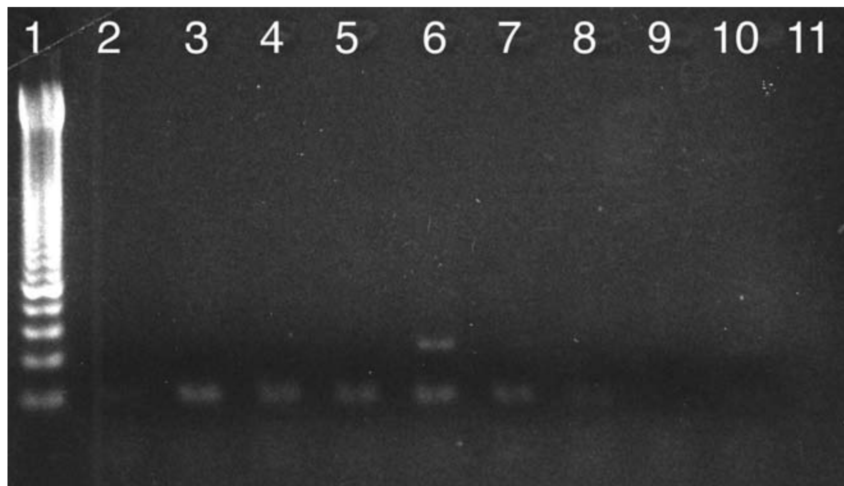

Figure. PCR amplification of a 120-bp fragment of kinetoplastid mitochondrial DNA of *Leishmania* spp. in Egyptian and Nubian mummies. Lane 1, 50-bp ladder; lanes 2–8, mummy samples; lanes 9,10, extraction controls; lane 11, PCR controls. Lane 6 provides a positive amplification product of the expected size.

Leishmania DNA–positive samples in the Nubian mummies (12.9%) suggests that leishmaniasis was endemic in Nubia during the Early Christian period and, in light of the data on the ancient Egyptian mummies, probably already several thousand years before. Taken together, our results support the theory that Sudan could have been indeed the original focus of visceral leishmaniasis (*4*).

Our study shows a completely new aspect of molecular paleopathology. The detection of ancient pathogen DNA is not only used to identify a certain disease and gain information on its frequency and evolutionary origin but also to trace back cultural contacts and their role in the transmission and spread of infectious diseases.

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Tickborne Encephalitis Virus, Northeastern Italy

To the Editor: Approximately 3,000 cases of tickborne encephalitis virus (TBEV) disease are registered annually in Europe (*1*). In Italy, indigenous TBEV infection cases have been only sporadically recorded from 1975 through 2001; in addition, serologic investigations in populations at risk in northern Italy have shown only a low prevalence of specific antibodies (0.6%–5%) (*2*,*3*). A surveillance system for TBEV infections was started after autochthonous TBEV was recognized in late summer and fall 2003 in Friuli-Venezia Giulia (FVG), a small region of northeastern Italy with nearly 1 million inhabitants (*4*). Surveillance is based on systematic microbiologic screening of all patients referred to the emergency departments of regional hospitals for suspected community-acquired central nervous system infections or fever and headache with a history of tick bite in the past 6 weeks. Screening for TBEV was performed on sera or cerebrospinal fluid (CSF) by enzyme immunoassay (Enzygnost Anti-TBE virus Ig, Dade Behring Marburg GmbH, Marburg, Germany) and repeated on convalescent-phase sera. Demonstration of specific immunoglobulin M (IgM) in serum or CSF in the acute phase or ≥4-fold rise in serum antibody titer in the convalescent phase was interpreted as an indicator of recent TBEV infection. For surveillance purposes, TBEV infection was defined when hemagglutination inhibition antibody test and neutralization assay by a reference laboratory confirmed ELISA results (*5*). Data were collected at a regional reference center, where cases were classified as possible, probable, and confirmed, according to the new TBEV case definition (*6*).

From July 2003 through November 2005, 20 cases of TBEV infection were detected; their demographic, epidemiologic, and clinical characteristics are given in the Table. Cases occurred throughout the year, with a biphasic peak in June and September–November. A biphasic clinical course was reported in 10 patients. The median period between tick bite and date of referral to hospital was 22 days (range 15–46 days). Seventeen cases were classified as confirmed, 2 as probable, and 1 case could not be classified because symptoms started after tick season (December) (*6*). Two patients were coinfected with *Borrelia burgdorferi*.

The most common symptoms were fever, headache, nausea, vomiting, and myalgia; the most common central nervous system signs were stiff neck, irritability, and limb paresis. Five patients only reported headache and fever without neurologic signs. Lumbar puncture, performed in 15 patients, showed mild pleocytosis with neutrophil predominance in 13 patients, elevated protein level in 14 patients, and normal glucose level in all.

The clinical syndrome was classified, in accordance with Kaiser et al., into febrile form (4 cases), aseptic meningitis (3 cases), encephalitis (2 cases), meningoencephalitis (8 cases), and meningoencephalomyelitis (3 cases) (*7*). None of the patients died, but 3 required respiratory support in the intensive care unit. Outcome was favorable for 9 patients; major neurologic sequelae were observed in 6 and minor sequelae in 5.

During the past 20 years, TBEV has reemerged in several European areas that had been disease free (*1*,*8*). In FVG, which borders disease-endemic areas such as Slovenia and Austria, the first cases of TBEV infection were documented recently (*4*). Several explanations, in addition to the well-established role of climate change, can be proposed (*1*). First, in Slovenia, after the end of the Communist regime, recreational activities increased considerably, with the creation of natural parks and hunting grounds, densely populated with deer, chamois, rodents, foxes, and other wild animals that can easily cross national borders (*9*). Second, after the 1976 earthquake that destroyed a large number of mountain villages in FVG, economic activities were progressively concentrated in the plains of the region, which rapidly increased urbanization of the plains towns. As a consequence, the mountains in the northern part of the region were progressively abandoned by humans and returned to wilderness. A final possible explanation is that TBEV cases were undiagnosed because awareness among local physicians was low; however, this variable likely played a minor role, since a recent serologic survey of persons at high risk (forest rangers) yielded a low positivity ratio (*3*). If even workers at risk had a low seroprevalence, TBEV cases were likely uncommon in the region.

The implementation of a regional active surveillance system allows the highest sensitivity in assessing the epidemiologic features of TBEV infections, which are characterized by highly disease-endemic microfoci in areas free of the problem (*10*). Precisely defining areas where risk is particularly will lead to optimal use of

Table. Demographic, epidemiologic, and clinical data for 20 patients with TBEV infection in Friuli-Venezia Giulia*

Patient	Sex	Age (y)	Tick bite	Hospitalization date (length of hospitalization [d])	Definitive diagnosis	Sequelae
1	F	36	Yes	2003 Jul 28 (31)	MEM	UL paresis
2	M	58	Yes	2003 Oct 13 (15)	E	Absent
3	F	42	Yes	2003 Oct 17 (19)	ME	Absent
4	F	27	No	2003 Dec 30 (25)	ME	UL paresis, paresis of VII cranial nerve
5	M	16	Yes	2004 Apr 28 (21)	ME	UL tremors
6	F	53	Yes	2004 Jun 21 (18)	ME	Diplopia
7	M	43	Yes	2004 Jul 17 (0)	FF	Absent
8	M	62	Yes	2004 Oct 10 (10)	ME	UL paresthesia
9	M	35	Yes	2004 Nov 8 (15)	ME	Absent
10	F	77	Yes	2004 Nov 22 (0)	FF	Absent
11	F	36	Yes	2005 May 8 (19)	MEM	UL paresis
12	M	12	Yes	2005 May 13 (27)	ME	Absent
13	M	64	Yes	2005 Jun 10 (11)	FF	UL paresthesia, hearing impairment
14	M	59	Yes	2005 Jun 20 (12)	M	Absent
15	M	15	Yes	2005 Sep 1 (10)	ME	Absent
16	F	39	Yes	2005 Sep 8 (8)	M	Absent
17	M	70	Yes	2005 Sep 16 (53)	MEM	UL paresis, RI, VAP
18	M	75	No	2005 Oct 18 (10)	FF	UL tremors
19	M	20	No	2005 Nov 2 (7)	M	UL tremors
20	M	61	Yes	2005 Nov 26 (13)	E	UL tremors, ataxia, opsoclonus

*TBEV, tickborne encephalitis virus; MEM, meningoencephalomyelitis; UL, upper limbs; E, encephalitis; ME, meningoencephalitis; FF, febrile form; M, meningitis; RI, respiratory insufficiency; VAP, ventilator-associated pneumonia.

prevention programs and design of educational programs for residents, tourists, and healthcare workers.

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Alex Langmuir and CDC

To the Editor: We were surprised and disappointed by the brevity of your article commemorating the 60th anniversary of the establishment of the Communicable Disease Center (CDC) (*1*). We realize that the accomplishments of the center and its derivative agencies are vast and that to give them full recognition would require far more space in Emerging Infectious Diseases than might be feasible. Nevertheless, your article that appropriately identified Joe Mountin as the administrative "father" of the center omitted any mention of Alex Langmuir, arguably the most influential of the infectious disease leaders over the years. Langmuir's creation and direction of the Epidemic Intelligence Service epitomized CDC's role in infectious diseases. His legacy deserves recognition in any chronicle of CDC, no matter how short.

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In response: We thank Drs. Winkelstein and Reingold for their comment regarding our article on the 60 years of CDC's progress in the area of infectious diseases. We certainly agree that Dr. Alexander Langmuir has made enormous contributions to this area, which we fully respect, We further agree that a more comprehensive approach would have required far more space than allotted for these types of commentaries. Allow us to emphasize that the omission of Dr. Langmuir and many other outstanding colleagues was not an oversight but an effort on our part to abbreviate an exceptionally long list of these deserving persons. We are looking forward to opportunities to provide a more comprehensive overview in which many of them will be appropriately recognized.

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Public Understanding of Pandemic Influenza, United Kingdom

To the Editor: Widespread outbreaks of influenza A (H5N1) in poultry and severe infections in humans have raised the possibility of an influenza pandemic. The 3 influenza A pandemics of the 20th century (*1*) were associated with considerable socioeconomic disruption as well as many deaths and pressure on health services. Experiences in the United States during the 1918–1920 pandemic (*2*) suggest that government advice that conflicts with personal or societal beliefs may not be followed, thus jeopardizing public health measures. Experience from the outbreak of severe acute respiratory syndrome has highlighted some pitfalls in achieving public understanding (*3*) and compliance (*4*) in the era of mass communication. Even if initial compliance is achieved, previous behavior patterns may reemerge during a pandemic as people begin to perceive that they have little control over the threat (*5*) or reduce their estimation of the risk (*6*).

Building robust public understanding has been made a priority in preparedness and response plans (*7*). However, despite widespread media coverage, little attention has been paid to assessment of public knowledge about the threat for pandemic influenza and surrounding issues. Such information may be essential to optimize public education strategies.

A questionnaire-based population survey was administered in March 2005 by 2 of the authors (MT and GB) to identify public knowledge about pandemic influenza, awareness of its potential effects, key information needs, and willingness to follow advice about public health measures. A structured interview consisting of 20 questions was used. Participants were approached at random and interviewed (in English) in public places including parks, shopping malls, libraries, and train stations in northern London. This area has considerable ethnic diversity (55% of the population is nonwhite) and a socioeconomic status similar to the rest of London. Recruited participants were >18 years of age and resided in the United Kingdom. They were excluded if another family member had previously completed the survey. Age and sex ratios were selected to reflect population centiles calculated from the 2001 UK population census. Statistical analyses were conducted with Fisher exact tests and epidemiologic tabulations in Stata version 8.2 (Stata Corp., College Station, TX, USA).

Of 273 persons approached for interview, 225 accepted and were eligible. Nine questionnaires were incomplete and therefore excluded, leaving 216 (79%) for analysis. Demographic characteristics of participants are summarized in the Appendix Table, available online at http://www.cdc.gov/ncidod/EID/06-0208-appT.htm). Half the respondents chose the correct definition of a pandemic from 5 options. Statistical analysis demonstrated that those 32–44 years of age were more likely than those of other age groups to choose correctly ($p = 0.001$). Persons who left school at ages ≥17 years were more likely than those who left school earlier to select the correct answer ($p = 0.007$).

Sex of the respondent did not influence correct response; 56% of those 18–31 years of age versus 86% of those ≥60 years of age were aware of the threat of pandemic influenza ($p = 0.006$). When asked the likelihood of a pandemic during the next 10 years, 71% responded that it was likely or very likely, whereas 16% considered it unlikely or very unlikely. When offered a list of 4 possible negative affects identified by experts (health-care service, food distribution, fuel distribution, and disruption to tourism), only one fourth thought that all 4 would occur. Details about symptoms of pandemic influenza were most frequently cited as the main public information need in the event of a pandemic. Television was rated by 68% of respondents as their preferred means of receiving information during a pandemic. Almost all respondents (97%) would wash their hands ≥5 times each day if requested, and 86% would definitely or probably be willing to stay away from public gatherings (unspecified) if asked. However, only 61% would stay away from work (unspecified period) as a means of avoiding pandemic influenza.

As far as we know, this is the first population-based study of knowledge and understanding of pandemic influenza. Public understanding of this threat and its potential effect in the United Kingdom appears to be limited. Our findings that older adults are more aware than younger persons has also been found in other settings (*8*) as has the increased public health awareness in more educated groups (*9*,*10*). Economic considerations retain high importance even with a potentially fatal threat, a phenomenon that has been previously noted with regard to self-quarantine (*4*). Our study did not address whether reluctance to take time off from work was more likely to be associated with public or private sector employment or self-employment. Further study in this area would help preparedness strategy.

This study was limited by a relatively small sample size, and its setting in 1 region of London may have implications regarding the extent to which the findings are applicable elsewhere. Further, larger assessments are needed both before and after specific pandemic influenza awareness programs as part of the ongoing process of pandemic preparedness.

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Influenza C Virus Infection in Children, Spain

To the Editor: Influenza viruses cause serious respiratory illness, particularly in infants <24 months of age (*1*). Despite serologic studies of French adults that showed an influenza virus seroprevalence of 60%–70%, influenza C infections have rarely been described (*2*). Given the technical difficulties involved in isolating influenza C virus in cell cultures, diagnosis is made only in certain laboratories. Detection of viral genome by reverse transcription (RT)–PCR in nasopharyngeal aspirates allows etiologic diagnosis of these infections (*3*). Mild upper respiratory infections in adults and adolescents are attributed to this virus (*4,5*). Some cases of lower respiratory infections have also been described in children (*6*).

A prospective study was conducted from September 1999 through July 2003. We determined the incidence and clinical manifestations associated with influenza C infection in all children <24 months of age admitted to Severo Ochoa Hospital in Madrid, Spain, with respiratory tract infections both with and without fever. All patients were evaluated by an attending physician. The study was approved by the Fondo de Investigaciones Sanitarias Committee of Spain.

Specimens of nasopharyngeal aspirates were obtained from each patient on admission (Monday to Friday) and sent to the Respiratory Virus Laboratory at the National Microbiology Center in Madrid for virologic studies. Specimens were processed within 24 hours of collection.

A multiplex RT-PCR was used for direct detection of respiratory syncytial virus A (RSV-A), RSV-B, adenoviruses, and influenza A, B, and C viruses in all nasopharyngeal samples, as previously described (*7*). Primers were specific for the nucleoprotein gene segment of influenza virus, the fusion gene of RSV, and the hexon gene of adenoviruses.

An internal amplification control was included in the reaction mixture to exclude false-negative results caused by specimen inhibitors or extraction failure. Given the high sensitivity of nested PCR, precautions were taken to prevent reactions from being contaminated with previously amplified product, as well as to protect target RNA or DNA from other specimens and controls. All procedures were performed in laboratory safety cabinets at locations different from those where amplified products were analyzed. Detection levels of 0.1 and 0.01 50% tissue culture infectious doses of influenza A and B viruses and 1–10 molecules of cloned amplified products of influenza C virus, RSV-A, RSV-B, and adenovirus serotype 1 were achieved.

A total of 706 hospitalized infants were enrolled in the study; 496 specimens were positive for virus (76.1% were RSV). Thirty children were infected with influenza virus (4.3% of all respiratory infections and 6% of all confirmed viral infections). Six patients had confirmed influenza C virus infections. Three of them had co-infections, 2 with RSV and 1 with adenovirus. Clinical characteristics of these 6 patients are shown in the Table. Although clinical characteristics for 24 influenza A virus infections were similar to those for influenza C virus infections (no influenza B virus was identified), statistical analysis was not conducted because of small sample size.

Influenza virus infections are a major cause of hospitalization and illness in young children, particularly those <2 years of age (*1*). Influenza A virus infections are more common than influenza B virus infections (75% vs 25%) (*8*). Our results indicate that influenza C virus is present

Table. Characteristics of 6 children with influenza C virus infections, Spain 1999–2003*

Characteristic	Patient 1	2	3	4	5	6
Age, mo.	12	15	24	3.5	11	7
Admission date	Jan 2000	Feb 2000	Mar 2000	Jan 2002	Nov 2002	Feb 2003
Sex	F	M	M	F	M	F
Temperature >38.5°C	Yes	Yes	Yes	Yes	Yes	Yes
Fever duration, d	15	5	3	10	2	2
Chest radiograph	Infiltrate	Normal	Infiltrate	Normal	Normal	Infiltrate
Diagnosis	Wheeze	Wheeze	Pneumonia	Bronchiolitis	Wheeze	Wheeze
O_2 saturation <95%	No	Yes	No	No	Yes	Yes
Days in hospital	9	8	2	2	2	13
Coinfection	No	No	Adenovirus	RSV-B	RSV-B	No
Diarrhea	Yes†	No	Yes‡	No	No	Yes†

*RSV-B, respiratory syncytial virus B.
†Rotavirus in feces.
‡Adenovirus in feces.

in infants hospitalized with respiratory infections.

In contrast to data for adults, in whom influenza C virus infection is associated with mild upper respiratory infections (*5*), our study showed that this infection in infants may be associated with illness severe enough to require hospitalization. Clinical symptoms of influenza C virus infection in our patients, such as high fever and respiratory symptoms, were similar to those described for infections with influenza A and B viruses (*1,8,9*). Nonrespiratory symptoms such as fever or diarrhea have often been associated with influenza A and B virus infections (*9*). Three patients with influenza C virus infections had diarrhea; 2 had rotavirus and adenovirus detected in feces, and 1 had rotavirus detected in feces. However, the high incidence of co-infections makes clarifying the role of influenza C virus as a causative agent in these conditions difficult.

Few studies have investigated influenza C virus infection in children. Katagiri et al. (*10*) described an outbreak characterized by fever and symptoms of upper respiratory infections in workers and children at a children's home. The largest pediatric study reported was that of Moriuchi et al. (*6*) of 20 cases of influenza C virus infection in children <15 years of age compiled in 2 years in a hospital setting among both inpatients and outpatients. They found clinical results similar to ours (lower respiratory infections). However, we could not make any conclusions about influenza C virus infections in nonhospitalized children.

Influenza C virus infection in hospitalized infants is responsible for a clinical condition with high fever and respiratory symptoms severe enough to require hospitalization. This virus should be studied in respiratory infections in hospitalized infants to further clarify its role.

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Zoonotic Cutaneous Leishmaniasis, Afghanistan

To the Editor: Recent reports from Afghanistan have focused on the current status of war- and refugee-related anthroponotic cutaneous leishmaniasis (ACL), caused by *Leishmania tropica*, in Kabul City, refugee camps, and Fayzabad City (Badakhshan Province) (*1–4*). In central Asia, ACL is transmitted mainly by the sandfly *Phlebotomus sergenti* in urban or periurban environments (*5*).

Zoonotic cutaneous leishmaniasis (ZCL), caused by *L. major*, occurs autochthonously in Afghanistan, but little is known about its regional and seasonal distribution or other disease characteristics (*6*,*7*). Recently, cases have been reported in troops deployed to the Mazar-e Sharif area: 19 cases among the British military contingent in 2004, 186 among Dutch troops in 2005 (overall attack rate 20.9%), and 14 among German troops in 2005 (*8*). This yet-undescribed *L. major* variant is highly aggressive, often disseminates and causes nodular lymphangitis, and is associated with delayed or poor response to treatment with sodium stibogluconate or miltefosine.

Regional epidemiologic reports, when available from Afghan or international health authorities, usually mention cutaneous leishmaniasis cases without further elaboration. To evaluate the current threat and specific epidemiology of cutaneous leishmaniasis in Mazar-e Sharif, medical entomologic and epidemiologic field investigations were conducted in June and October 2005. Results show that ZCL, ACL, and visceral leishmaniasis (VL) are endemic to Mazar-e Sharif. Data from patients seeking treatment from March 21, 2004, through March 20, 2005, at the Balkh Province Leishmaniasis Center, Mazar-e Sharif, showed that of 3,958 cases, 3,782 (95.5%) were ZCL, 174 (4.4%) were ACL, and 2 (0.05%) were VL; the number of unreported cases during this time is unknown. A sharp increase (4.4- to 5.4-fold) in ZCL cases was found when data from July 21 through August 20 and from August 21 through September 20, 2004, (30 and 169 cases, respectively) were compared with data for the same periods in 2005 (163 and 744 cases, respectively). ZCL chiefly affects farmers, nomads, and refugees. By sex, cases occurred in 1,991 (52.6%) male and 1,791 (47.4%) female patients. By age group, the rate for ZCL in young children was similar to that for other age groups: 1,167 (30.9%) cases in children $\leq$4 years of age, 1,218 (32.2%) cases in children 5–14 years of age, and 1,397 (36.9%) cases in persons $\geq$15 years of age. In the leishmaniasis center, cutaneous infections are confirmed by seeing *Leishmania* parasites in Giemsa-stained smears obtained directly from skin lesions. Although sporadically confirmed by culture and PCR, ZCL and ACL are usually differentiated by specific clinical aspects, especially of skin lesions: dry lesions characterize urban ACL; wet lesions, often superinfected and disseminating, characterize rural ZCL.

In terms of seasonal patterns in Mazar-e Sharif, ZCL peaks in mid-October and most ACL cases occur during mid-February (Figure). Observations at the leishmaniasis center in Mazar-e Sharif indicate that the incubation period for ACL is 4–6 weeks (associated with the cold, wet season) and that for ZCL is 8–12 weeks (associated with the hot, dry season). The minimum incubation period of ZCL in German and Dutch patients was 7 weeks, a figure derived from the known period of troop deployment rather than from hospital records.

The Mazar-e Sharif area, especially near the airfield, is semidesert, although extensive farming of seasonal crops is possible because of irrigation systems built in the 1980s. Irrigation canals are usually several kilometers long and 1.5–2 m deep and wide. Earth removed during canal construction is deposited as embankments that attract large numbers of the great gerbil, *Rhombomys opimus*, the

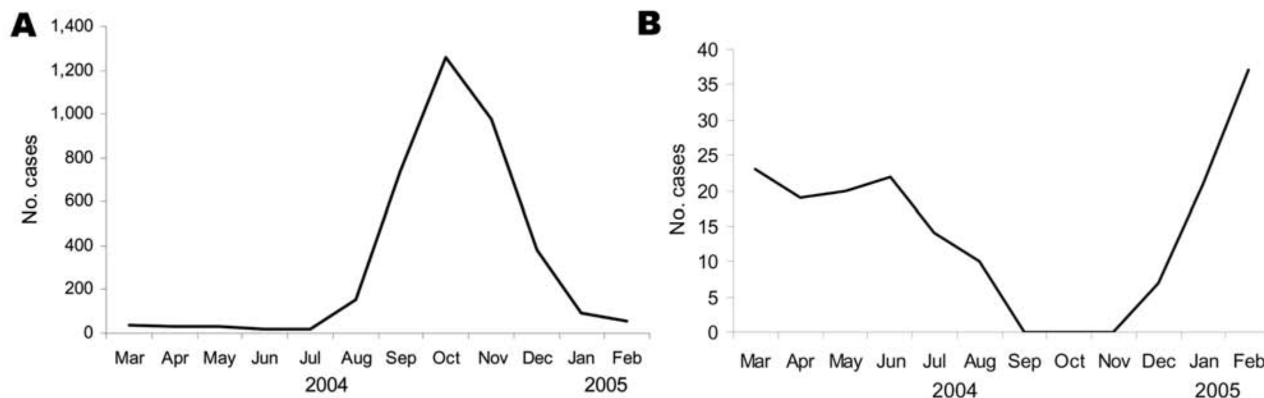

Figure. Seasonal distribution pattern of zoonotic cutaneous leishmaniasis (panel A) and anthroponotic cutaneous leishmaniasis (panel B) cases registered in the Balkh Province Leishmaniasis Center, Mazar-e Sharif, Afghanistan, March 21, 2004, through March 20, 2005.

main animal reservoir of *L. major* (*6*). Our studies showed an average of 3,380 *R. opimus* burrows per hectare, the highest density yet recorded, far exceeding the previous record of >1,000 rodent burrows per hectare in nearby Turkmenistan (*9*). Much lower *R. opimus* population densities were found at the Sakhi refugee camp, in the suburbs of Mazar-e Sharif, where Sherman box trapping for 18 nights was unsuccessful. Gerbil species were identified by photographs of live gerbils and by capture of 2. Both captured animals had positive results for *L. major* by microscopy and PCR. Because human enhancement of *R. opimus* habitat favors high infestations, the Mazar-e Sharif outbreak is an example of an anthropogenically induced emerging zoonosis. Sandfly surveillance was conducted in October 2005 by using unbaited CDC light traps (John Hock Co., Gainesville, FL, USA) and sticky traps placed in areas that were heavily infested with *R. opimus*. *Phlebotomus papatasi*, the principal vector of ZCL (*7*), was caught at low densities only, because of the cold night temperatures in October.

Our data suggest that foci of high-density enzootic ZCL occur in northern Afghanistan, especially in the Mazar-e Sharif area. ACL may also pose future problems because of its epidemic urban transmission potential (*10*). Effective disease surveillance and preventive measures should be promptly implemented to mitigate these health threats.

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Epstein-Barr Virus (Infectious Disease and Therapy)

Alex Tselis and
Hal B. Jenson, editors

Informa Healthcare,
New York, New York 2006
ISBN: 0824754255
Pages: 436; Price US $189.95

Epstein-Barr virus (EBV) was the first recognized human tumor virus, but it is not the causative agent for the tumor in which it was discovered, Burkitt lymphoma. Common to all Burkitt lymphomas, endemic or sporadic, are distinctive chromosomal translocations that reactivate expression of the *c-myc* protooncogene and comprise the primary oncogenic mechanism.

EBV is at least a contributory cofactor in endemic Burkitt lymphoma, but the virus is detected in <20% of sporadic cases in the United States. EBV does cause infectious mononucleosis, hairy leukoplakia, and B-lymphoproliferative neoplasms in immunocompromised persons. In addition, the early and utterly consistent presence of monoclonal EBV episomes in nasopharyngeal carcinoma worldwide suggests a crucial role for the virus in that neoplasm. While tantalizing, associations with other diseases, well reviewed in this volume, are inconsistent and suggest that the virus may have another role beyond the etiologic, namely, by affecting the phenotype of already existing tumor cells and possibly propelling tumor progression.

This book is assembled mostly from a clinical perspective, and useful chapters on several of the EBV diseases bring together information not easily found elsewhere. Well-informed chapters on the virology and epidemiology of EBV infection are also included. One of the editors (whose list of milestones displays the clinical emphasis of the book) has provided a nice historical summary.

As is usual with such compilations, the editors leave it to the contributors to speak for themselves, and the quality of the chapters is uneven. Some fall short in citation of primary sources or favor the author's view rather than one that weighs all the evidence. Withal it is a useful book, and having the less often discussed associations such as T-cell lymphomas and leiomyosarcomas assigned a place alongside authoritative chapters on the classic associations, nasopharyngeal carcinoma and Burkitt lymphoma, is convenient. The volume ends with a chapter on an EBV vaccine, which remains elusive after many years. In contrast, the penultimate chapter includes a brief summary of some successes with adoptive immunotherapy for posttransplant lymphoproliferative disease, which is generally refractory to conventional treatment. This volume is worth having for the cross-section of knowledge and developments in the EBV field it presents.

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The Power of Plagues

Irwin W. Sherman

ASM Press, Washington, DC, 2006
ISBN: 1555813569
Pages: 442; Price: US $39.95

The purpose of this book is to make the science of epidemic diseases accessible and understandable; to guide the general reader through the maze of contagious diseases, their past importance, the means by which we came to understand them, and how they may affect our future. This commentary on general and disease-specific concerns covers the nature of plagues; plagues, the price of being sedentary (an evolutionary view); 6 plagues of antiquity (urinary schistosomiasis, the plague of Athens, malaria in Rome, the Antonine plague, the plagues of Cyprian and Justinian); bubonic plague; AIDS (including a history of virology and an account of leukocyte function); typhus; malaria (plus an explanation of sickle cell disease and genetics); cholera; smallpox; preventing plagues (the immune system, with a coda on vaccine development); the plague protectors (antisepsis and antimicrobial drugs); syphilis; tuberculosis; leprosy; 6 plagues of Africa (sleeping sickness, river blindness, guinea worm, yellow fever, malaria, and hookworm) with the history of exploration and exploitation of this continent; plagues without germs (pellagra, beriberi, scurvy, and rickets); and emerging plagues (rodent-borne, West Nile virus, bovine spongiform encephalopathy and Creutzfeldt-Jakob disease, and influenza). The text covers not only the geography, history, microbiology, and physiology of these infections but also their influence on plastic art, movies, literature, and music (with a special fondness for nursery rhymes).

Surprisingly, the role of contemporary epidemiologic methods and governmental institutions is not examined. No explanation is included of how present-day public health officials go about detecting a problem, how they define an epidemic, how they use data such as incidence or attack rates to identify the cause, and how laws and regulations (e.g., vaccine requirements for school entry and rules for production of food and biological materials) are indispensable for disease prevention. The text would have profited from another round of editing to modify overly forceful generalizations, tighten the discussion, and check for historical and medical accuracy. For example, acyclovir is not AZT, and AZT was not available for first-line treatment of AIDS in the early 1980s; cholera is not slowly creeping into the Western Hemisphere, but it produced large epidemics in Central and South America in the 1990s; Figure 9.7B is not an antivaccination statement from Boston in 1902 but, as the engraving itself indicates, a provaccine statement from England in 1898; vaccination with *Mycobacterium bovis* BCG does not cause the tuberculin test result to be negative; and malaria control efforts in the United States were not interrupted by World War II but, on the contrary, were enhanced by the creation of an agency called Malaria Control in War Areas.

This is a concise and clear account of the biologic and historical determinants of epidemic diseases. It is marred by a small number of factual errors and a failure to include epidemiologic and public health methods as components of the equation that determines the power of plagues.

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Molecular Principles of Fungal Pathogenesis

Joseph Heitman, Scott G. Filler, John E. Edwards Jr, and Aaron P. Mitchell, editors

ASM Press, Washington DC, 2006
ISBN: 1555813682
Pages: 684; Price: US $149.95

This book offers advanced treatment of a broad selection of topics in molecular medical mycology authored by leading investigators. It assumes a foundation of knowledge of mycotic pathogens and is suitable for the reader who is well-grounded in molecular microbiology. It is highly recommended for investigators planning to conduct medical mycology research. The book is divided into 5 sections: General Principles, Model Systems, Specific Pathogens, The Host, and Future Directions. Only selected highlights are described here because of space limitations.

The book reviews the development of transforming and gene-silencing methods for identifying virulence determinants. An overview of *Candida albicans* virulence underlines that molecular subtyping has elucidated 3 major clades, which differ in their potential for producing superficial versus deep-seated infection. The first step in pathogenesis is adherence to host tissues. The endothelial and epithelial specificity of members of the *C. albicans* Als family of adherence molecules is defined by the adherence profile of null mutants. The discovery through the genome sequence of *C. albicans* mating type locus and the delineation of the unique pathway of a parasexual cycle are discussed. Although the population is largely clonal and seems locked in a diploid state, the species has a demonstrated ability to undergo recombination.

The phylogenetic species concept has led to a better understanding of the lineage of pathogenic fungi, especially for the mitosporic fungi, which have no known sexual stage. The evolution of fungal species, shown by multilocus sequence typing, enabled construction of a phylogenetic tree of all known fungal pathogens with assignments to well-described families and orders.

Mechanisms of resistance to antifungal agents are discussed, including insights from genome sequence analysis and recent clinical observations such as the role of transcription factors in upregulating efflux pumps in the presence of antifungal agents or steroids. How environmental fungi have acquired their pathogenic potential for humans, even those whose immune function is intact, is a puzzle, but clues come from the interaction of fungi with soil-dwelling amebae. Fungi escape endocytosis by converting from yeast to hyphal forms; this not only conditions them for intracellular survival but also suggests how dimorphism may have originated. Transcriptional profiling using microarrays is a powerful tool for identifying genes expressed during mold-to-yeast morphogenesis and host-fungus interactions in infected tissue cultures and biofilms. Interspersed in the book are examples of exploiting this technology to discover key regulatory pathways.

No subject attracts more interest, yet is strewn with more pitfalls, than

fungal vaccine development. Two major types of vaccine development are preventive vaccines and monoclonal antibody immunotherapy. Individual recombinant antigens have evoked mild to moderate protection. Interest in a potential attenuated live vaccine has been stimulated by the finding that targeted disruption of the *Blastomyces dermatitidis* gene, which encodes the surface adhesin BAD-1, renders the mutant avirulent.

Of the chapters on specific pathogens, the one covering virulence mechanisms in *Coccidioides immitis* is notable. It discusses application of molecular approaches to identify key proteins expressed during arthroconidial and spherule morphogenesis at each stage of the disease process and to dissect the corresponding interactions with the immune system.

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Pierre-Auguste Renoir (1841–1919). Luncheon of the Boating Party (1880–1881)
Oil on canvas (130.175 cm × 175.5775 cm). Acquired 1923. The Phillips Collection, Washington, DC, USA

Πάντα ῥεῖ καὶ οὐδὲν μένει[1]

– Herakleitos of Ephesus, c. 500 BC

Polyxeni Potter*

"I'm doing a painting of oarsmen which I've been itching to do for a long time. I'm not getting any younger, and I didn't want to defer this little festivity which later on I won't any longer be able to afford already it's very difficult…; one must from time to time attempt things that are beyond one's capacity," wrote Pierre-Auguste Renoir to Paul Bérard, his friend and supporter (*1*). The ambitious painting might have been Renoir's response to author and critic Émile Zola's challenge to impressionists in 1880 that, instead of "sketches that are hardly dry," they "create complex paintings of modern life" after "long and thoughtful preparation" (*2*).

Zola was not alone in challenging artists of his generation. Poet Charles Baudelaire back in 1863 had called for a "painter of modern life," inviting his contemporaries to create art of their own times (*3*). Heeding the call, French artists of the late 19th century broke with the past, forging perhaps the most popular movement in the history of art. Radical in their departure from tradition, the impressionists abandoned history as source of inspiration and moved the studio outdoors to capture a moment, an impression, under the changing light of the sun. No longer interested in telling a story, they replaced standard narrative techniques with feathery, interrupted brush strokes that best described an angle of interest or a fleeting scene of daily life. Their innovations forever changed how art was created and viewed, producing works of unprecedented spontaneity and lightness.

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The child of working-class parents, Renoir was born in Limoges, the city in France known for its porcelain and fine china. When he was still young, the family moved to Paris and settled in the Louvre area, where he grew up lighthearted and easygoing in the shadow of the great museum. Despite early affinity for scribbling and drawing, his fine singing voice was noticed first, and he studied under composer Charles Gounod, who encouraged a music career for him. Because of financial constraints, at age 13, he was apprenticed instead to a porcelain painter with the prospect of long-term work at a large factory outside Paris.

At the porcelain shop, his talent for painting was quickly acknowledged, but he continued to paint china, fans, café murals, and window shades, to study in the evenings, and to copy the masters at the Louvre until he could enroll in the École des Beaux-Arts. There, he met Claude Monet (*4*), Frédéric Bazille, and Alfred Sisley. The foursome, who shared an aversion to established rules, bonded quickly, painted together, influenced each other, and became founding members of impressionism, along with Paul Cézanne, Edgar Degas, Berthe Morisot, and Camille Pissarro.

"There are no poor people" Renoir believed, denying that lack of means could interfere with happiness, success, or the imagination (*5*), even though living expenses and painting supplies were hard to come by for much of his career: "I would several times have given up if Monet had not reassured me with a slap on the back" (*5*). And when near the end of his life he was invited to the Louvre to view

[1]Everything flows. Nothing stands still.

the hanging of one of his works, he mused, "If I had been presented at the Louvre 30 years ago in a wheelchair I would have been shown the door" (*5*).

Many innovations of this era, among them mixed paints in metal tubes and use of poppy seed oil as binding medium, facilitated the artists' task. And the advent of photography freed them from the need to paint in realistic detail, proposing new ways to focus on subjects. But turmoil brought on by the Franco-Prussian war in 1870 interfered with the full impact of these changes as with the progress of impressionism. Bazille was killed in action; Monet, Pissarro, and Sisley moved to England; and Renoir joined the cuirassiers, though he saw no action.

After the hostilities, Renoir returned to Paris, where he continued to struggle for acceptance. He worked with Monet, painting on the banks of the Seine and the coast of Normandy; traveled to Africa, Spain, and Italy; and by the end of the decade, he had found his own unique voice. His style became more refined and traditional, focusing closely on the human form. "For my part," he freely admitted, "I always defended myself against the charge of being a revolutionary. I always believed and I still believe that I only continued to do what others had done a great deal better before me" (*5*).

They are "lumpy and obnoxious creatures," wrote the New York Sun about Renoir's famed female nudes, exhibited in New York in 1886 (*5*). Nonetheless, popular success and creature comforts were finally within the artist's reach. He remained extremely prolific (more than 6,000 paintings); despite debilitating arthritis and other health problems, he continued to paint until his death.

No other painting captures Renoir's characteristic joie de vivre and conviviality better than Luncheon of the Boating Party on this month's cover. The social ease and camaraderie of his youth seem to have permeated this painting, while gaiety and charm emanate from it toward the viewer, who is tempted to join in. "The picture of rowers by Renoir looks very well," wrote Eugene Manet to his wife Berthe Morisot (*6*). Holiday-making was a favorite theme of the impressionists, who "…show their particular talent and attain the summit of their art when they paint our French Sundays…kisses in the sun, picnics, complete rest, not a thought about work, unashamed relaxation" (*6*).

Renoir worked on the complex composition for months, frustrated at times with the unavailability of models, the clustering of figures, the landscape: "…I no longer know where I am with it, except that it is annoying me more and more" (*1*). On this single canvas, he combined still life, genre, landscape, and portraiture to capture food, friends, and conversation near the waterfront. Carefully structured and meticulously finished, this moment at play was to become a cultural icon.

The gathering took place near Chatou, Renoir's favored retreat on the Seine. Once the domain of the affluent, the area now offered pastimes for all. A group of friends assembled on the balcony of the Maison Fournaise. Among them, a historian and art collector, a baron, a poet and critic, a bureaucrat, actresses, and artists Paul Lhote and Gustave Caillebotte, who sat backward in his chair in the right foreground and gazed across the table at Aline Charigot, the young seamstress who later would become Renoir's wife. The youths leaning against the rail are proprietors of the establishment (*7*).

"…[O]ne cannot imagine these women…having been painted by anybody else," wrote art critic Théodore Duret, "They have…that graciousness, that roguish charm, which Renoir alone could give to women" (*8*). Earthy, savvy, and engaging, they light up the scene. The luncheon is finished. The crowd "hangs out" against the clutter of leftover food and drink, gracing the intimate tableau all of us want to be in.

Just beyond the awning, the river flows discreetly in the background. Soon, it will turn dark, the crowd will disperse, the moment will end. The moment and its transient place in constant change, so well understood by the impressionists and masterfully captured by Renoir, have also long puzzled philosophers and scientists and are central to the study of emerging disease. In a world where "everything flows," organisms and their surroundings are constantly changing, and "nothing stands still," vigilance is order of the day. Disease control is as good as the next set of natural circumstances, for as Herakleitos of Ephesus put it 2,500 years ago, "You cannot step twice into the same river, for fresh waters are ever flowing upon you" (*9*).

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the November issue for the following topics:

Aerosol Transmission of Influenza A Virus

Pregnant Women and Emerging Infection and Bioterrorism Emergencies

Impact of Emerging Infections on the Pregnant Woman

Hepatitis E Virus Infection, Rural Southern China

Invasive Meningococcal Disease, Germany

Schistosomiasis among Travelers

Staphylococcus aureus–associated Skin and Soft Tissue Infections in Ambulatory Care

Clostridium difficile PCR Ribotypes in Calves

Susceptibility of North American Ducks and Gulls to H5N1 Highly Pathogenic Avian Influenza Viruses

Social Distancing Designs for Pandemic Influenza

Avian Influenza at Food Markets

Fatal Avian Influenza A H5N1 in Dog

Chikungunya Fever, Hong Kong

Reemergence of Chikungunya in Thailand

Complete list of articles in the November issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

March 7–9, 2007
6th International Symposium on Antimicrobial Agents and Resistance (ISAAR 2007)
Raffles City Convention Centre Singapore
http://www.isaar.org

March 20–23, 2007
ISOPOL XVI: 16th International Symposium on Problems of Listeriosis
Marriott Riverfront Hotel
Savannah, GA, USA
Abstract submission deadline: November 1, 2006
Contact: 240-485-2776
http://www.aphl.org/conferences/ISOPOL.cfm

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Editorial Policy and Call for Articles

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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.

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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.

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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 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. 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.