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On the Cover

Krishna Storms the Citadel of Naraka (detail) (from a Bhagavata Purana). India, Karnataka, Mysore (ca. 1840). Opaque watercolor and gold on paper (25.1 cm × 36.8 cm). San Diego Museum of Art (Edwin Binney 3rd Collection)

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Theme Issue on Poverty and Human Development. Emerging Infectious Diseases will be dedicating its October 2007 issue to the theme of poverty and human development as part of an effort by more than 175 scientific journals to simultaneously publish articles on this topic. Manuscripts should be submitted no later than **June 1, 2007**.

Response to Malaria Epidemics in Africa

Tarekegn A. Abeku*†

Malaria epidemics affect nonimmune populations in many highland and semi-arid areas of Africa. Effective prevention of these epidemics is challenging, particularly in the highlands where predictive accuracy of indicators is not sufficiently high to allow decisions involving expensive measures such as indoor residual spraying of insecticides. Advances in geographic information systems have proved useful in stratification of areas to guide selective targeting of interventions, including barrier application of insecticides in transmission foci to prevent spread of infection. Because rainfall is associated with epidemics in semi-arid areas, early warning methods based on seasonal climate predictions have been proposed. For most areas, response measures should focus on early recognition of anomalies and rapid mass drug administration. Vector control measures are useful if abnormal transmission is highly likely and if they can be selectively implemented at the early stages of an outbreak.

Malaria epidemics frequently affect highlands and semi-arid areas where populations lack immunity. Rapid response to these epidemics can be made where effective surveillance systems are in place for early recognition of disease incidence anomalies. Recent advances in research on malaria early warning systems are potentially useful to reduce the effects of epidemics, but they are associated with challenges that face most developing countries. Here, different intervention decisions will be discussed within the context of research findings relevant to epidemic malaria and implementation capacities of epidemic-prone countries.

Highlands and Semi-arid Areas

Most malaria epidemics follow abnormal weather conditions, often in combination with other causes, including increased resistance of the parasite to antimalarial drugs, population movement due to seasonal labor and civil unrest, and reduced malaria control operations, in particular, the cessation of regular vector control (*1*).

In highlands, transmission is unstable due to fluctuations in temperatures that are normally low (*2*). Temperature affects duration of the sporogonic cycle of the *Plasmodium* parasite within the *Anopheles* vector, survival and feeding frequency of the adult female, and duration of the aquatic stages. Most of the epidemics affecting highlands that support short, annual, transmission are superimposed over normal seasonal increases in malaria incidence, a phenomenon that makes early detection difficult. Other areas experience occasional transmission in specific years with more pronounced levels of illness and death, and substantial spatial and temporal variations (*3*). In addition to explosive epidemics, highland areas in Africa have shown a trend of increasing malaria transmission in recent years (*4*). This trend has important implications for choosing response mechanisms.

Semi-arid areas, on the other hand, have mostly warm climates, and epidemics are associated with anomalous rainfall, which causes increases in vector breeding and survival. In Botswana, more than two thirds of the variability observed between years in malaria incidence during January–May could be explained by variation in rainfall during December–February (*5*). A major epidemic that affected semi-arid regions of northeastern Kenya in January–May 1998 was reportedly caused by abnormal rainfall and floods during November–December 1997 (*6*). In these areas, monitoring rainfall can provide a fairly accurate forecast of transmission risk (*5,7,8*).

Preventive Interventions

The type and targets of interventions depend on the forecast probability, available resources, and the timing of the events or available lead time. In most cases, highly accurate forecasting is not possible as yet (e.g., in highlands); hence, emphasis should be placed on improving surveil-

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lance for early detection of abnormal incidence to minimize delays in responding (*9*).

Malaria control programs are usually faced with uncertainties regarding decisions on whether seasonal preventive measures should be used routinely in areas known to be at risk due to their geographic characteristics or whether developing mechanisms for rapid response is a better strategy. Although decisions have to be made rapidly in epidemic situations, they also have to be economically justifiable. Interventions that are appropriate in semi-arid areas can be difficult to implement or may be less useful in highland situations. In particular, measures aiming at prevention are more suited to semi-arid areas and sometimes to highlands with moderate malaria endemicity, rather than to highlands that are normally malaria free and only occasionally affected by epidemics.

In most highlands with substantial interannual variability of incidence, measures such as regular indoor residual spraying (IRS) of insecticides for preventive purposes may be an unnecessary waste of resources. However, IRS on an annual basis may be justified in some highland fringe areas. For example, in Madagascar, annual IRS was restored during 1993–1998 to reverse the spread of epidemic malaria that reappeared following recolonization of the central highlands by *A. funestus*, which had disappeared after effective control campaigns (*10*,*11*). After 5 years of spraying DDT mostly in areas between 1,000 m and 1,500 m in altitude, vector density and malaria prevalence rates were greatly reduced. Annual IRS campaigns in the 1950s in the epidemic-affected highlands of Kenya have also produced similar results (*12*,*13*). Selective application of insecticides for a limited number of years in areas most at risk for unstable transmission, followed by focal use as and when required, should be considered, especially in areas bordering malaria-endemic lowlands or in transmission foci in valleys in highland regions.

Highland valleys are often the source of malaria infection. A survey in an altitudinal transect in the Usambara mountains in Tanzania has shown the importance of local topography in explaining variations in splenomegaly among residents (*14*). Not surprisingly, altitude correctly predicted whether an occupant had an enlarged spleen in 73% of households. Moreover, when land where water was likely to accumulate within 400 m of each household was included in the model, prediction significantly improved in areas with an altitude from 1,000 to 1,200 m, where malaria transmission is unstable. In the western Kenyan highlands, the indoor density of *A. gambiae* s.s. vectors has been shown to be negatively associated with distance from swamps (*15*). Areas near man-made breeding sources also have high transmission risks. For example, human activities such as brick making have played a role in creating vector-breeding sites during the dry seasons (*16*). A spatial analysis of the distribution of *P. falciparum* in the highlands of Kenya has indicated that prevalence of infection and parasite densities decreased with distance from valley bottoms (*17*). These foci maintain low levels of transmission through the dry periods and are a potential source of infection when weather and other conditions favor spread of the disease to surrounding highlands. Selective annual insecticide spraying of houses in and around these valley bottoms and areas in the vicinity of man-made transmission sources may help protect the populations in the highlands during transmission seasons. An effective insecticide with a long residual life (such as DDT) should be used. The potential use of geographic information systems and remote sensing technologies to map transmission foci and risk factors and to guide targeting of interventions has been extensively reviewed (*18*). Spatial epidemic risk maps, based on the climate profiles of epidemic-affected localities, have been proposed for highland areas in the Horn of Africa and East Africa (*2*). Static spatial maps have been used for general stratification of areas and, in some cases, for regular interventions in countries such as Madagascar (*10*), South Africa (*19*), and India (*20*).

In the past few years, several international collaborative efforts have been initiated to develop and test temporal risk maps based on rainfall anomalies by using remote sensing technologies, with special applicability in semi-arid areas (*7*). Continually updated rainfall anomaly maps are available free of charge from the websites of the Famine Early Warning Systems Africa Data Dissemination Service (http://igskmncnwb015.cr.usgs.gov/adds) and the International Research Institute for Climate and Society (http://iridl.ldeo.columbia.edu/maproom/.Health/.Regional/.Africa/.Malaria). The practical use of these technologies for early warning of abnormal transmission and selective application of prevention or preparedness is yet to be fully evaluated, although encouraging results have been documented in southern Africa, especially in Botswana (*7*). Because rainfall variability in Botswana has high predictability of interannual variations in malaria incidence (*5*), an early warning system has been proposed to provide probabilistic forecasts of anomalously high or low incidence in semi-arid areas on the basis of seasonal precipitation forecasts (*8*). Although claims have been made that the system can add up to 4 months of warning over methods that use observed precipitation, the system's sustainable applicability for targeting preventive measures remains to be seen.

In the highlands, monitoring temperature anomalies can provide crude forecasts. Studies have shown that major epidemics in the 1980s and early 1990s in Ethiopia were significantly more often preceded by a month of abnormally high minimum temperature than would be expected by random chance (*3*). Similarly, higher than average tempera-

tures in Zimbabwe were associated with severity of epidemics and deaths in the following year (*21*). Studies involving concomitant longitudinal follow-up of weather patterns, indoor resting densities of vectors, and malaria incidence are ongoing in highland sites in Kenya and Uganda to clarify and quantify epidemic-triggering mechanisms (*22*).

Several studies have shown epidemic malaria to be associated with El Niño events in many parts of the world (*23*). For example, analysis of malaria data from Colombia for 1980–1997 appears to indicate that El Niño events intensify the annual seasonal transmission cycle (*24*). Prediction based on El Niño indicators may prove useful for ensuring availability of resources at national levels, in particular, drugs and insecticides, because of the relatively long lead times (*25*).

Rapid Assessment

Early detection capacity is essential for effective and rapid containment of epidemics. An important aspect of such capacity is the existence of efficient disease surveillance systems. In East Africa, research has shown that computer-assisted, weekly sentinel surveillance can be successfully implemented at district levels (*22*).

After detection of abnormal increases, mostly increased numbers of clinically diagnosed cases, the actual causes of the increases should be rapidly confirmed through laboratory diagnosis before intervention measures are recommended. Confirmation should be followed by assessing the magnitude and geographic extent of the outbreak, prioritizing areas, and deciding on the types of interventions required. These steps, however trivial they might seem, are nevertheless essential and must be taken within the shortest possible time. In most instances, simple rapid assessment methods are sufficient for sound decisions. As an example, in an epidemic that affected Uasin Gishu District in Kenya, school absenteeism was used as an indicator to determine priority areas for mass treatment of fever (*26*).

More advanced techniques that are economical but powerful and rapid can also be implemented, especially if sampling procedures are adopted in advance of an epidemic event. One such technique is lot quality assurance sampling (LQAS). The LQAS method was first used in industrial sampling to identify batches of products (or lots) with unacceptable number of defective items. In public health, this method has been used to select communities with disease prevalence rates beyond acceptable levels (*27*). Its advantage is that relatively smaller sample sizes are required than in other sampling methods. After an acceptable probability of error and the maximum sample size are determined, the population is sampled until a certain number of infections is exceeded; the sampling then stops and the area is classified as having high prevalence. In Madagascar, this procedure was compared with a conventional sampling plan to select areas where prevalence rates among schoolchildren were at least 15%; such areas were candidates for some specific action (*28*). A plan in which 2 persons were found positive among a random sample of 36, denoted as (36,2), classified communities correctly with 100% sensitivity and 94% specificity. After such assessments, the use of IRS for epidemic control should only be considered if continuation of transmission is anticipated over a long period and if rapid implementation of IRS is feasible at the early phase of an epidemic. The main challenge is that few countries have the capacity to rapidly organize and implement IRS as a meaningful epidemic-containment method.

Sufficient evidence that insecticide-treated nets (ITNs) are beneficial for short-term epidemic control is lacking, so their use should be limited to situations in which their availability and rapid implementation is possible, such as in refugee camps (*25*). Nevertheless, widespread use of ITNs in epidemic-prone areas with moderate malaria endemicity will, in the long run, contribute to transmission reduction and should be encouraged. Larval control may have a limited role in some situations, for example, in semi-arid areas, with well-defined mosquito breeding sites after rainy periods, or in man-made sites such as wells and water storage tanks. Larviciding is likely to be effective when bodies of water cannot be eliminated due to essential economic activities such as brick making (*16*).

Occasionally, widespread epidemics affect large geographic areas such as the highlands of Ethiopia, affected in 1988 (*3*). In such situations, carrying out large-scale preventive IRS operations would be difficult, if not impossible, even if early warning systems are in operation. Health services need to focus on more feasible measures such as strengthening preparedness by stocking drugs and diagnostic materials, closely monitoring changes in malaria incidence, educating communities to seek prompt treatment, classifying areas according to their risk levels, and making contingency plans to rapidly deploy mobile treatment teams.

Mass Drug Administration and Mass Fever Treatment

Mass drug administration (MDA) is the presumptive treatment of an entire affected population with a therapeutic dose of an antimalarial drug, whereas mass fever treatment (MFT) refers to treatment of febrile patients only. These approaches require that sufficient and appropriate antimalarial agents are available and that mobile treatment teams can be deployed in affected areas in the shortest possible time. In all epidemic situations, rapid distribution of effective treatment in affected communities is recommended. This requires local surveillance and logistics capacity for early detection of abnormal incidence and timely mobilization of resources.

In areas where reliable early warning systems are not in place due to technical, logistical, or other reasons, stocking contingency antimalarial drugs in health units across areas at risk before known transmission seasons provides an alternative approach. Epidemics tend to occur during those seasons of known transmission, which mostly follow the rainy period. Areas historically known to be most at risk should be identified to prioritize health units for drug distribution. If available, risk maps may be used for classification of areas. A practical alternative for inter-area comparisons is to use proxy measures such as adult-to-child ratios of patients attending health facilities. This approach has been used in Kenya to study stability of malaria in the highlands (*29*). Malaria patients admitted to the hospital were classified into 2 age groups: <15 years of age ("children") and ≥15 years ("adults"). Depending on the age structure of the developing country populations, the adult-to-child ratio of hospital admissions approached unity for an unstable malaria situation in which adults were as likely as children to be at risk for severe malaria.

MDA has been used alone or in combination with IRS to prevent and control malaria in various settings. The primary objective of this measure in the epidemic control context is to reduce the reservoirs of the parasite by reducing infectiousness to vectors, while providing curative and prophylactic benefits to treated persons. To have the desired effects on transmission, antimalarial drugs with schizontocidal and gametocytocidal effects should be used. In the past, primaquine was given in combination with 4-aminoquinolines for its effect on gametocytes. In a malaria-endemic area in Tanganyika (present day mainland Tanzania), the repeated use of amodiaquine and primaquine combination considerably lowered transmission by reducing the sporozoite rates (*30*). Repeated MDA with proguanil has been shown to substantially reduce transmission in the highlands of western Kenya in the late 1940s (*31*). Although many MDA trials did not interrupt transmission, most succeeded in considerably reducing parasite prevalence, and some showed marked reduction in incidence of cases and deaths (*32*).

Alternatively use of MFT as an important rapid measure, rather than using MDA for the entire population, has been proposed for epidemic control (*25*). Attaining high coverage is crucial; epidemiologically relevant questions include the practicality of diagnosing fever cases in emergency situations and whether a large enough proportion of the population can be treated in this way in order to have a considerable impact on transmission. In Ethiopia, the Ministry of Health guidelines recommend rapid sampling of households to determine the proportion of occupants with illness in the previous 7 days; a cut-off value of 50% would then be used to decide whether to use MFT or MDA (*33*).

The introduction of combination therapy with artemisinin derivatives, which have gametocytocidal effects, has recently led to the hypothesis that its use for large-scale MDA might be a potential malaria control measure (*32*). Artemisinin-based combination therapy (ACT) drugs have been shown to moderately reduce transmission by reducing the duration of gametocyte carriage and the proportion of mosquitoes that are infected by carriers (*34*). In The Gambia, children treated with the combination of chloroquine and artesunate were significantly less infectious to mosquitoes than children treated with chloroquine alone (*35*). Treatment with the combination containing artesunate also significantly reduced the prevalence and density of gametocytes, as well as the duration of gametocyte carriage, although the effect was transient as it did not prevent emergence of mature gametocytes at day 28 after treatment (*35*). Another study in an area with highly seasonal but intense transmission in The Gambia showed that MDA with a single dose of artesunate combined with sulfadoxine-pyrimethamine failed to interrupt transmission overall, but incidence in the first 2 months was significantly lower in treated villages than in control villages (*36*). The failure of MDA to interrupt transmission in the longer term was attributed to the high entomologic inoculation rate in the area. Nevertheless, MDA with a full therapeutic dose of ACT can likely play a major role in the control of epidemics and malaria in areas with a short transmission season (*32*).

Treatment of patients with fever, whether at health facilities or as part of epidemic control, presents the challenge of balancing costs in time and other resources with accuracy of clinical diagnosis. This diagnostic method is particularly less accurate in areas of low endemicity than in areas of high endemicity (*37*), although sensitivity and specificity tend to increase during transmission seasons (*38*). As a result, overdiagnosis of malaria in areas of low transmission remains a major problem, especially when expensive ACT drugs are to be used for treating patients with fever. Furthermore, surveillance systems that rely entirely on data generated from health facilities without laboratory confirmation can lead to false epidemic alerts (unpub. data).

The cost-effectiveness of rapid diagnostic tests in epidemic situations in relation to the use of ACT has been compared with presumptive treatment by using a model based on actual cost data (*39*). The threshold prevalence beyond which treatment based on rapid diagnostic tests becomes more expensive than presumptive treatment was shown to be 21% for artesunate-amodiaquine and 55% for artemether-lumefantrine. During epidemics, the percentage of highland populations infected or incubating infection is usually higher than the threshold for artesunate-amodiaquine. A recent study in western Kenyan highlands showed

that nearly 44% of the sampled population were infected over a 10-week period during an epidemic, with adults and children similarly affected (*40*). These observations indicate that relatively less expensive ACT drugs such as artesunate-amodiaquine can be cost-effective when used in MFT without laboratory confirmation. For large-scale epidemics when most of the population are either infectious or incubating the infection, MDA with relatively inexpensive ACT distributed once or repeated within 1–2 weeks can substantially reduce transmission.

The choice of treatment sites largely depends on the magnitude of the epidemic. Treatment at existing health facilities should be given special attention, and health services should ensure that essential drugs for the treatment of both uncomplicated and severe malaria are in stock. In many situations, mobile treatment centers will be required to cover remote rural areas. In Kenya, mobile treatment teams could be assembled in 1 week through a provincial health system to control an outbreak in Uasin Gishu District (*26*).

Conclusion

Many countries still need to improve their technical and logistics capacity to deal with high demands in resources to prevent or contain malaria epidemics. Nevertheless, better targeting of interventions by using recent technologic advances in spatial analysis and risk mapping, optimal use of computing facilities in disease surveillance and efficient use of information, and better preparedness especially in terms of antimalarial drug stocks will potentially provide a feasible means of effective epidemic control in many countries. In semi-arid areas, early warning methods using actual or probabilistic prediction of rainfall may be used for making decisions to implement prevention measures. In these areas, most vector control measures, including those targeting the aquatic stages of vectors, may be feasible and useful. In the highlands, anomalies in weather patterns, especially minimum temperatures, may be used as crude early warning indicators of malaria epidemics, mainly for preparedness purposes. However, the focus in these areas should be on early detection of abnormal incidence for rapid initiation of response. In normally malaria-free highlands, measures attempting to prevent transmission based on crude forecasts may not be cost-effective or feasible in most situations. At present, the use of IRS and other vector control measures in these areas should be limited to special situations where selective and timely application is feasible to contain proven, ongoing transmission. More research is required to increase our understanding of the genesis of epidemic malaria in the highlands and to develop better predictive models. Research should also focus on ways of developing local epidemic management capacities within the health service systems. The use of relatively inexpensive ACT drugs such as artesunate-amodiaquine for MDA or MFT should be a primary strategy for rapidly reducing transmission in all epidemic situations.

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Plague and the Human Flea, Tanzania

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Domestic fleas were collected in 12 villages in the western Usambara Mountains in Tanzania. Of these, 7 are considered villages with high plague frequency, where human plague was recorded during at least 6 of the 17 plague seasons between 1986 and 2004. In the remaining 5 villages with low plague frequency, plague was either rare or unrecorded. *Pulex irritans*, known as the human flea, was the predominant flea species (72.4%) in houses. The density of *P. irritans,* but not of other domestic fleas, was significantly higher in villages with a higher plague frequency or incidence. Moreover, the *P. irritans* index was strongly positively correlated with plague frequency and with the logarithmically transformed plague incidence. These observations suggest that in Lushoto District human fleas may play a role in plague epidemiology. These findings are of immediate public health relevance because they provide an indicator that can be surveyed to assess the risk for plague.

Plague, caused by infection with *Yersinia pestis*, persists in many parts of the world; several hundred cases are reported to the World Health Organization each year, mostly from Africa (*1*,*2*). In Tanzania, a persistent focus of human plague was discovered in 1980 in the Lushoto District, in the northeastern part of the country. By 2004, 7,603 cases had been reported from this region (*3*). The distribution of plague cases in Lushoto is limited to an area of ≈1,200 km^2, and a strong variation in plague frequency and incidence is seen among the villages in this region (*3*). Although evidence of infection with *Y. pestis* has been observed in several wild rodent and flea species, the actual reservoir in which the infection survives between epidemics has not yet been identified, and the ecology of the infection and the source from which humans acquire infection are poorly understood (*4*–*8*). In Lushoto District, frequent plague outbreaks occur in some villages, but the disease is uncommon in other villages in the same vicinity. A study is under way to compare the ecologic conditions in villages having frequent outbreaks with those in villages where plague is relatively rare, with the objectives of understanding, predicting, and ultimately controlling human plague. Comparing host and vector communities is an important part of such studies.

In Lushoto District, it has been suggested that the fleas *Xenopsylla cheopis*, *X. brasiliensis*, and *Dinopsyllus lypusus* are plague vectors among sylvatic rodents, but *Pulex irritans*, the human flea, has received little attention (*9*). *P. irritans* has been collected in several plague-affected and plague-free villages of the Lushoto area during epidemics and interepidemics (*10*), as well as on *Rattus rattus* (B.S. Kilonzo and S. Msingwa, unpub. data). Plastering a mud house is recommended in the area as a way of keeping the house free of fleas (*9*) and involves mixing soil (without manure) with water and rubbing the mixture over the floors with a piece of cloth. We report differences between plague-affected and plague-free villages in the numbers of free domestic fleas present in mud houses and consider whether this variation can be linked to house plastering as an antiflea measure.

Materials and Methods

Lushoto District is situated in Tanga region, in the West Usambara Mountains, a part of the Eastern Arc Mountains. With an elevation ranging from 900 to 2,250 m above sea level, Lushoto District (04°22′–05°08′S, 038°05′–038°38′E) covers a surface area of 3,500 km^2, of which 2,000 km^2 are arable land and 340 km^2 are forest reserve. Soils are mainly low-pH loams, rich in iron, manganese, and magnesium. Agriculture is the major economic activity, on which >90% of the population depends (*11*,*12*).

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The temperate climate is characterized by a short rainy season during November–December and a longer one during March–May. A minor and unreliable rain, the Mlwati, occasionally occurs in August and September. The region is the most densely populated area in Tanzania, with an annual growth rate of 2.8% and 102 inhabitants per square kilometer. Inhabitants belong to 1 of 3 major tribes: Wasambaa (80%), Wambugu (10%), and Wapare (5%); the remaining 5% are immigrants from diverse other regions (*13*) Most Lushoto residents (70%) keep livestock in their houses, but cats and dogs are usually kept outside (*9*).

We selected 12 villages from throughout the plague-endemic area, ensuring a variation in both the frequency and incidence of plague, based on the earlier study by Davis et al. (3). Plague frequency is expressed as the percentage of plague seasons from 1986 to 2004 with reported plague cases, while plague incidence is the mean annual number of plague cases per 1,000 inhabitants at village level for the same period. Table 1 lists the 12 villages we surveyed, ranked according to plague frequency, and Figure 1 shows a map of the study area in Lushoto District. The 7 villages where plague on average occurred in >3 years per decade were considered "high plague frequency" villages, and the 5 villages where it occurred on average in <2 years per decade were considered "low plague frequency" villages. In all 12 villages, the common housing is mud houses with dirt floors and iron sheet or thatch roofs. Cattle are kept outside the house attached to poles during the day and feed on grass, but they are kept inside overnight.

Collection of domestic fleas began in May 2005 because earlier literature reported that plague cases in Tanzania usually appear 2 times per year, in October/November and May/June (*14*). A more recent detailed investigation of Lushoto hospital data, however, showed a consistent seasonal pattern in which the highest number of plague cases occurs in January (*3*). Taking into account the practical limitations of extended fieldwork periods overseas, a second collection period was started in January 2006. Fleas were thus collected every month from May through August 2005 in 4 core study villages (Gologolo, Emao, Kiranga, and Magamba) and from January through March 2006 in all 12 villages.

Houses surveyed were randomly chosen after the chief of each village granted authorization. Fleas were trapped by using a kerosene hurricane lamp hung above a 3-cm–high tray with a 45-cm diameter, half full of water. The lamp was lit at dusk and switched off at dawn during 3 consecutive nights. All traps were checked every morning between 9 AM and 11 AM, and captured fleas were preserved in 70% ethanol. The head of each household was interviewed by questionnaire to assess the perception of flea nuisance in the house and the frequency of plastering.

Table 1. Data on villages in Lushoto District, Tanzania, surveyed for domestic fleas, ranked by plague frequency*

Village	Population†	Plague frequency	Plague incidence	Coordinates, South–East	Altitude, m asl	Year of most recent plague case	Year(s) flea trapping conducted	No. forms received‡
Dule	3,036	0.059	0.637	04.58370–038.31657	1,405	1986	2006	20
Mtae	3,407	0.059	0.121	04.48421–038.23758	1,632	2000	2006	20
Handei	5,745	0.118	0.137	04.59514–038.32390	1,376	1990	2006	20
Kiranga	868	0.176	1.056	04.57571–038.27021	1,821	1996	2005–06	33
Magamba	2,676	0.176	0.836	04.72895–038.30148	1,743	1998	2005–06	52
Goka	1,116	0.353	1.175	04.56680–038.25990	1,843	1997	2006	20
Mambo	5,669	0.353	0.722	04.51167–038.21976	1,828	1997	2006	20
Nkelei	1,305	0.647	3.434	04.56062–038.24209	1,904	2000	2006	20
Shume-Nywelo	3,757	0.647	10.460	04.70025–038.19687	1,890	2001	2006	20
Emao	2,054	0.706	5.180	04.56276–038.25304	1,827	2000	2005–06	41
Gologolo	2,202	0.765	18.544	04.69707–038.22692	1,950	2002	2005–06	26
Manolo	10,464	0.765	6.320	04.62058–038.22260	1,809	2003	2006	9

*Data about plague are extracted from (*3*). Villages with a plague frequency of >0.3 on average, ≥3 years in a decade, were considered high frequency villages. asl, above sea level.
†From 2002 census.
‡No. questionnaire responses received from village during survey.

Figure 1. Map of the Lushoto District of Tanzania showing locations of willages with high and low plague frequency. All other villages with known locations are also plotted. The solid lines represent altitude contours (200-m elevation lines). To the west, a steep escarpment demarcates the edge of the district.

We calculated the *P. irritans* index (*Pii*), per village per month, as the average number of *P. irritans* collected in a house. Frequency and incidence are village-specific characteristics based on long-term data, while for *Pii* we had to rely on relatively sparse and heterogeneous sampling during a short period. Simple averaging and testing for a correlation may be misleading if findings vary between days, months, or years. Therefore, we analyzed the relation with a mixed model that included these sources of temporal variation in *Pii* (first applying a log transformation to ensure normality). This model included log(*Pii*) as the dependent variable and frequency or log(incidence) as the (continuous) independent factor. Year and month (nested within year) were added as fixed effects, while village and the year–village interaction were treated as random effects to control for temporal variation in *Pii*, as well as for the fact that village is the independent unit of observation. Correlations in day-to-day estimates of *Pii* were modeled by using an autoregressive correlation coefficient. Model selection was based on backward elimination of nonsignificant fixed effects. All random effects were retained in the model to ensure appropriate weighting and approximation of degrees of freedom by using the Kenward-Roger method.

We also tested for lagged relationships between monthly values of *Pii* and monthly plague incidence; that is, monthly incidence was related to the mean *Pii* for the previous month. Per village, the average number of people infected with plague each month was further expressed as a proportion of total number of cases in the district and was termed the monthly incidence. Daily flea numbers per house per village were summed to obtain a mean monthly *Pii* ± SD. The natural logarithm of the monthly incidence +1 was the dependent variable, while the natural logarithm of the mean monthly *Pii* was the predictor variable. Possible temporal dependence of the monthly incidences within villages was modeled by using an exponential decay of the degree of temporal autocorrelation. We also tested for an association between plastering frequency (independent variable) and the total number of captured fleas (dependent variable, log transformed) in a mixed analysis of covariance model with village and village-by-frequency as random effects. In all the analyses above, standard error and denominator degrees of freedom were estimated by the Kenward-Roger method. We also tested for an association between altitude and the mean monthly *Pii* (log transformed to ensure normality) by using a linear regression model. Finally, the association between flea abundance and the time since the previous plastering (the number of days between the last time the householder said the floor was plastered and the date of our first visit) was analyzed with a Cox proportional hazards model. If no fleas were trapped, the observation was considered to be censored. All analyses were performed in SAS version 9 (SAS Institute Inc., Cary, NC, USA).

Results

P. irritans was the predominant species (72.4%) among domestic fleas. Other species collected were *Echidnophaga gallinacea* (15.1%), *Ctenocephalides felis* and *C. canis* (6.5%), *Xenopsylla brasiliensis* (3.4%), and *Tunga penetrans* (2.6%). *P. irritans* and *E. gallinacea* were the only species found in every village. *P. irritans* accounted for 61.5% and 75.2% of all fleas collected in low and high plague frequency villages, respectively (Table 2). Twice as many houses were infested by *P. irritans* in high plague frequency villages than in low plague frequency villages (Table 2).

For all the trapping sessions in 2005 and 2006, the *P. irritans* index was 2–9× greater in high plague frequency than in low plague frequency villages (Figure 2). The statistical analysis also showed that *Pii* was strongly positively correlated with plague frequency ($F_{1,11.3} = 14.08$, $p = 0.003$), with the logarithmically transformed plague incidence ($F_{1,11.5} = 12.62$, $p = 0.004$) and altitude ($F_{1,11H} = 8,641$, $p = 0.015$). The abundance of other species was much lower than that of *P. irritans*, and none of the other

Table 2. Distribution of flea species within villages and houses, Lushoto District, Tanzania

Domestic flea species	Common hosts in Tanzania	Flea species composition, %		Houses with given flea species, %	
		Low*	High†	Low*	High†
Pulex irritans	Humans	61.5	75.2	28.8	65.4
Ctenocephalides felis, *C. canis*	Cats, dogs, other animals	8.8	5.8	6.8	10.7
Echidnophaga gallinacea	Domestic fowl, *Rattus rattus*	19.6	13.7	12.2	15.7
Tunga penetrans	Humans, dogs, goats	2.0	2.8	2.0	6.9
Xenopsylla brasiliensis	*Rattus rattus, Mastomys natalensis*	6.8	2.1	4.1	5.0

*Villages designated as low plague frequency.
†Villages designated as high plague frequency.

flea species indexes were correlated with plague frequency or incidence.

The questionnaires (301 valid responses) showed that in 8 of the 12 studied villages, some persons plaster their houses daily (Table 3). The figures suggest a great variability in the frequency of plastering between and within villages and that frequency of plastering has no relation with the frequency of plague. For example, in Shume-Nywelo (high plague frequency) and Dule (low plague frequency), 55% and 50% of housekeepers, respectively, said they never plaster the house; in Gologolo (high plague frequency), 65.4% plaster their houses 7 times a week, but in Emao (another village with high plague frequency), only 19.5% do so.

The frequency of plastering did not correlate with the natural logarithm of the total number of fleas caught (t_{20} = 0.88, p = 0.39), and this lack of association did not vary across villages (no significant random village–frequency interaction, χ^2_1 = 0.4, p = 0.47). Frequent plastering did not appear to prolong the time between the last plastering and the occurrence of the first fleas in the trap (χ^2_1 = 0.36, p = 0.55).

Discussion

Our results show that the density of domestic fleas is higher in villages with a higher plague frequency or incidence. Moreover, the human flea *P. irritans* accounts for a larger percentage of the domestic fleas in these villages. The factors that contribute to the presence of plague in some villages in Lushoto while it is absent from others (*3*) are so far unknown. It is tempting therefore to attribute an epidemiologic role to *P. irritans*. This has been suggested recently for another focus of human plague; Arrieta et al. (*15*), working in the Peruvian Andes, observed that 69.9% of fleas collected in domestic environments (on domestic animals and inside houses) were *P. irritans* (or, perhaps, *P. simulans*, a sister species) and found the same positive relation between high plague risk areas and *P. irritans* densities.

The human flea was first mentioned in tropical Africa (Ethiopia) in 1868 (*16*). In Tanzania, plague was first reported in 1886 in the Iringa region, but no information is available about the flea species present at that time. The presence of *P. irritans* in Tanzania dates at least to 1915, when it was found in Dar-es-Salaam. In northeastern Tanzania, its presence was reported in 35% of the beds examined by Smith in 1959 (*17*); in 1977, 82.5% of the fleas collected in human dwellings belonged to this species (*18*). *P. irritans* is often found in high densities in habitations, especially those with a dirt floor and a thatched roof, and is considered a possible plague vector in Angola, Brazil, Burundi, Democratic Republic of Congo, Iran, Iraq, Nepal, People's Republic of China, and Tanzania (*19–21*).

Although a substantial body of literature describes the ecology of plague, the relation between the bacterium *Y. pestis* and the human flea *P. irritans* during epizootics and epidemics is poorly understood. The classic epidemiologic model for plague considers it an enzootic infection of mostly resistant wild rodents. An outbreak of human plague may begin with an epizootic in peridomestic rats, from which rodent fleas (in tropical regions typically *X. cheopis*) questing for a host may infect humans (*22*). In this scenario, human ectoparasites do not play an important role. However, epidemiologic investigations based on historical accounts of the Black Death in the 14th–16th centuries in Europe show that the epidemics do not conform to this classic model, even leading to suggestions that the Black Death may have had a cause other than *Y. pestis* plague, an issue that is still hotly debated among historians (*23*,*24*). Recently, Drancourt et al. (*25*) reviewed earlier biologic studies that have presented experimental evidence for or against the role of *P. irritans* in the transmission of plague.

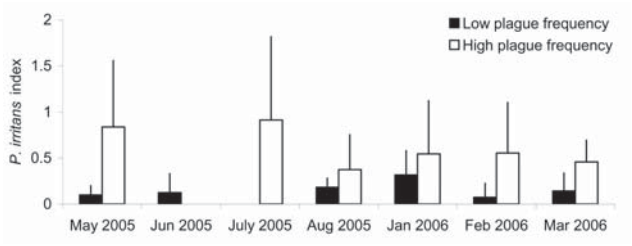

Figure 2. Monthly domestic *Pulex irritans* index, *Pii*, averaged for low plague frequency villages (black columns) and high plague frequency villages (white columns). The error bars indicate standard deviation from the mean. No data were available for high plague frequency villages in June 2005 or for low plague frequency villages in July 2005.

Table 3. Questionnaire responses about plastering frequency, Lushoto District, Tanzania

	How many times per week do you plaster the floor?							
Village	0	1	2	3	4	5	7	n*
Low frequency								
Dule	10	–	1	1	7	1	–	20
Mtae	9	–	–	–	2	1	8	20
Handei	–	1	–	–	–	–	19	20
Kiranga	13	1	8	5	4	–	2	33
Magamba	24	6	3	8	4	1	6	52
High frequency								
Goka	–	1	–	–	–	–	19	20
Mambo	5	–	–	2	3	–	10	20
Nkelei	6	–	–	–	1	–	13	20
Shume	11	–	3	3	3	–	–	20
Emao	26	–	–	4	1	2	8	41
Gologolo	9	–	–	–	–	–	17	26
Manolo	5	–	–	–	1	–	3	9
Total	118	9	15	23	26	5	105	301

*No. respondents per village.

P. irritans is frequently infected with *Y. pestis* (pestiferous) but is rarely infective (China, Ecuador, Kazakhstan, Democratic Republic of Congo, Brazil; [*21*]), mainly because it is not an easily blocked species (*21*). Blocking of the proventriculus by massive replication of the *Y. pestis* bacteria is known to enhance flea vectorial capacity and occurs in known plague vectors *X. cheopis* and *Nosopsyllus fasciatus* (*26*). Therefore, the role for *Pulex* spp. as plagues vector was classically believed to be no more than mechanical transmission by way of soiled mouthparts, which is only possible if a high level of bacteremia exists in the pestilent host, if new potential hosts are available within 3 days after the infective blood meal, and if multiple bites occur (*21*). Such levels of ectoparasitism are realistic in a rural habitat; for example, in 1 night in our study in Gologolo, a basic light trap caught 26 fleas in a single room.

The role of unblocked fleas may, however, be more than just mechanical. Eisen et al. (*27*), studying alternative fleaborne transmission mechanisms, recently showed that *Oropsylla montana*, which rarely becomes blocked, is immediately infectious, transmits efficiently for at least 4 days postinfection (early phase), and may remain infectious for up to 8 weeks postinfection because the fleas do not undergo block-induced death. This scenario of efficient early-phase transmission by unblocked fleas matches historical observations of rapidly spreading epizootics and epidemics and their highly focal nature. During the second plague pandemic, in Europe, *P. irritans* was a suitable vector because it was abundant on persons and in their homes, as it is today in some remote foci in Central Asia (*25*,*28*). In Ecuador, during a plague outbreak in the Chimborazo region in 1998, *P. irritans* was abundant in human bedding (*29*). The findings of the study by Eisen et al. (*27*) would also be consistent with a role for human fleas in the epidemiology of plague in Lushoto. In contrast, in the Ituri plague focus in the Democratic Republic of Congo, Devignat noticed the total absence of domestic *P. irritans* (*16*,*30*), just as in the epidemics in Saigon-Cholon in 1943 (*31*). *P. irritans* also appeared later in foci in the Democratic Republic of Congo, and the primary human fleas at that time (1946) were *X. cheopis* and *X. brasiliensis* (*32*).

Among the other domestic species collected, *C. felis strongylus* and *C. canis* are commonly found on cats and dogs in Lushoto (*5*). These species are poor plague vectors but can be pestiferous, as observed in Democratic Republic of Congo (*30*). *T. penetrans*' status as plague vector is unknown. The females of this species are embedded in the host epidermis (humans, dog, rat, cat), but males are free hematophagous ectoparasites (*33*). *E. gallinacea* is frequent in human homes where hens are kept, but it was never observed on humans in Lushoto. It has been found to be infected with *Y. pestis* in the field (*34*,*35*) but is considered a poor plague vector due to its "stick tight" behavior (*36*). Finally, *X. brasiliensis* is the African counterpart to Asian *X. cheopis* in the sense that it is considered an excellent plague vector (*7*,*30*). Notably, the abundance of *X. brasiliensis* could not explain the village-level variation in either incidence or frequency of human plague in the present study.

During our study, no human plague cases were recorded in the test region, and the small mammals we trapped in the 4 core villages tested negative for *Y. pestis* (n = 925, tested in a multiplex PCR; data not shown). Thus, the study period could be atypical in the sense that it is a period in which plague was absent. Whatever the explanation for the absence of plague cases, it is nevertheless clear that the abundance of *P. irritans* differs significantly between villages with different histories of human plague cases.

Because the vectorial status of *P. irritans* is still under discussion, and because of the correlative nature of our re-

sults, the observed relations must be interpreted with care. For example, *P. irritans* may not be a significant plague vector but a biologic indicator of the conditions that are conducive for the occurrence of plague in a village. Flea larvae are very sensitive to moisture excess and dehydration, 2 conditions that are caused by abiotic factors, mainly air/soil humidity and temperature, factors likely to vary locally and annually. Climatic conditions are further linked with altitude and orientation of slopes in mountainous areas, and those do not change from 1 year to another. Indeed, elevation cannot change the transmission of plague, but it can create conditions that are more conducive for plague, such as the distribution of particular flea species. Altitude effects on the distributions of sylvatic flea species are partly explained by host availability and population density but also by local climatic conditions (*37*). For example, in the Madagascar highlands, at an altitude <800 m, the sylvatic flea *Synopsyllus fronquerniei* is absent, even though its common host, *R. rattus*, is present (*38*). Soil texture can also affect both development time and survival of preimaginal stages of fleas through differences in soil moisture (*39*).

Our data suggest that human fleas may play an important role in spreading plague in Lushoto, or that human fleas at least are correlated with other factors that are important in this respect. These observations are of immediate public health relevance because they provide a clear indicator that can be surveyed to assess plague risk. Also, they suggest a clear target to be included in disease control efforts and indicate where to continue looking for factors that are responsible for the persistence of plague foci. Earlier studies have so far not been able to pinpoint such factors in the Lushoto plague focus, nor in the similar focus of Okoro County, Nebbi District, Uganda, which has been surveyed for 13 years (*4*,6,*14*,*40*). Plague has always been associated with poor home and environmental sanitation, and plague control in Africa has always focused on rodents and their fleas. Our results show the importance of including human ectoparasites in control programs and that plastering of houses, a locally accepted means of flea (and plague) control, does not have the expected effect on flea densities.

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Trends for Influenza-related Deaths during Pandemic and Epidemic Seasons, Italy, 1969–2001

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Age-specific patterns of death from influenza vary, depending on whether the influenza season is epidemic or pandemic. We assessed age patterns and geographic trends in monthly influenza-related deaths in Italy from 1969 through 2001, focusing on differences between epidemic and pandemic seasons. We evaluated age-standardized excess deaths from pneumonia and influenza and from all causes, using a modified version of a cyclical Serfling model. Excess deaths were highest for elderly persons in all seasons except the influenza A (H3N2) pandemic season (1969–70), when rates were greater for younger persons, confirming a shift toward death of younger persons during pandemic seasons. When comparing northern, central, and southern Italy, we found a high level of synchrony in the amplitude of peaks of influenza-related deaths.

In Europe and the United States, the geographic pattern of influenza epidemics has been studied extensively, yet mostly at the national level with few local studies (*1–6*). Description of local influenza patterns can contribute to understanding of transmission and seasonality, which are influenced by factors such as demographic differences, climatic variability, and virus virulence.

Age patterns and geographic trends for influenza are commonly assessed by using data on influenza-related deaths, which are indirectly quantified by using statistical methods to estimate seasonal increases in death from pneumonia and influenza (P&I) or all causes (AC) (*7–11*). This approach has shown that age-specific influenza death patterns vary according to whether the influenza season is epidemic or pandemic. During epidemic seasons, proportion of influenza-related deaths is greatest among persons ≥65 years of age, whereas during all 3 influenza A pandemics in the 20th century, persons in this age group accounted for a lower proportion of influenza-related deaths in the United States (*12*) and Europe (*8,13*).

Although patterns of influenza-related deaths have been investigated in many countries (*14–18*), few studies have focused on southern Europe. With regard to Italy, these methods have been applied only to death data for elderly persons during 1970–2001 and only at the national level (*19*). Our objective was to use the above-described approach to assess age patterns and geographic trends for influenza-related deaths in Italy; our focus was on differences between epidemic and pandemic seasons.

Methods

Death and Population Data

We obtained the monthly number of deaths caused by P&I and AC in Italy from 1969 (first available data year) through 2001 (most recent data year) from the Italian National Census Bureau, which records all causes of death reported on death certificates and classifies them according to the International Classification of Diseases (ICD). For our analysis, we considered only the underlying cause of death. To select P&I deaths, we used ICD-8 codes 480–486 and 470–474 for the years 1969–1979 and ICD-9 codes 480–486 and 487 for the years 1980–2001.

The geographic areas considered were the 3 administrative regions of Italy: northern Italy, central Italy, and southern Italy, as defined by the Italian National Census Bureau (*20*). Northern Italy comprises Piedmont, Lombardy, Autonomous Province of Trento, Autonomous Province of Bolzano, Val d'Aosta, Veneto, Friuli Venezia Giulia, Liguria, and Emilia Romagna. Central Italy comprises Tuscany, Um-

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bria, Marche, and Lazio. Southern Italy comprises Abruzzo, Molise, Campania, Puglia, Basilicata, Calabria, Sicily, and Sardinia. For each year, we generated summary datasets of the monthly number of deaths from P&I and AC, stratified by age group (0–14, 15–44, 45–64, and ≥65 years) (*9*). We calculated the annual number of persons in each age group and the monthly number of deaths per 100,000 population for each age group and standardized these to 30.4-day months.

Virologic Surveillance

To determine which influenza viruses were circulating each season, we reviewed publications listing viral subtypes identified in Italian laboratories (*21–23*). For the most recent years (1999–2001), we obtained these data from the Italian National Influenza Center, which has performed virologic surveillance since 1999.

Statistical Analyses

To estimate age-specific excess deaths (an indirect measure of death attributable to influenza) from P&I and AC for the 32 influenza seasons, we applied a Serfling-type regression model to monthly time series of deaths (*7*,*9*). As described in previous studies (*9*,*19*), we removed the seasonal trend from the time-series data (de-trended) by fitting a smooth spline function to the average death rates in summer (June–August). Then, we applied a seasonal regression model to the de-trended series, excluding values for December–April, to model the expected mortality rates in the absence of influenza activity. Monthly mortality rates were calculated as the observed minus the predicted mortality rates for all epidemic months. We identified epidemic months by applying the above-mentioned procedure to deaths coded specifically as influenza (ICD-8 code 470–474 and ICD-9 code 487). We defined epidemic months as those winter months for which influenza-specific mortality rates exceeded the upper 95% confidence limit of the seasonal model.

Seasonal excess deaths were estimated as the sum of monthly excess deaths, after back-adjusting for the true month length and removing the spline transformation. The model was applied to P&I and AC mortality rates separately for each age group. We achieved an excellent fit for all age groups. All model terms included were statistically significant ($p<0.0001$), but additional terms were not ($p>0.05$).

Age Patterns and Geographic Trends

To determine whether variations in age structure biased the geographic comparisons, we generated excess mortality rates for each area and age group and standardized them on the basis of the age distribution of the Italian population in 2001 (the year of the most recent Italian census). This permitted a comparison of age-adjusted P&I and AC excess mortality rate estimates across areas.

To compensate for nondemographic differences among areas (e.g., differences in access to healthcare and in coding for cause-specific deaths) (*24*), we also calculated the percentage increase in mortality rates as the excess deaths divided by the baseline deaths in winter (expected deaths), for P&I and AC, separately. This measure has been used successfully in past research (*7*,*8*). To estimate correlations of influenza-related death across the 3 geographic regions, we calculated the pairwise Spearman correlation coefficients of seasonal estimates for the 32 years considered.

Results

Geographic Trends, Synchrony, and Effect of Epidemic Seasons (All Ages)

For the 32 influenza seasons studied, excess deaths averaged 3 per 100,000 population (range 0–38) for P&I and 18 per 100,000 for AC (range 0–107). Influenza accounted for an estimated 57,243 deaths from P&I (average 1,789 per season) and 318,806 from AC (average 9,963 per season).

The highest number of excess deaths was found for the 1969–70 pandemic season; no measurable number of excess deaths was found for 5 seasons (1981–82, 1984–85, 1986–87, 1990–91, 2000–01) (Figure 1). The 27 seasons that had excess P&I and AC deaths had an average of 2.4 epidemic months per season (range 1–4). The influenza seasons with higher excess deaths tended to be characterized by a predominance of influenza A (H3N2) viruses (Figure 1). For these seasons, the average excess deaths from P&I and AC (4.5 and 23.4 per 100,000 population, respectively) was 4× higher than that for the 11 seasons in which influenza A (H1N1) or B viruses were predominant (0.8 for P&I and 7.4 for AC, per 100,000 population).

For the overall study period, the excess deaths per 100,000 population from AC was 15 for northern Italy, 14 for central Italy, and 22 for southern Italy; from P&I they were 4, 3, and 3, respectively (online Appendix Figure 1, available from www.cdc.gov/EID/content/13/5/694-appG1.htm). Also for these 32 years, no statistical differences among the 3 geographic areas were noted for excess deaths from P&I or AC (Kolmogorov-Smirnov test, $p = 0.8$ and $p = 0.9$, respectively). Patterns were similar with the percent increase in excess deaths from P&I and AC. The 95% confidence intervals for estimates for individual seasons were within 6% of given values (Table 1). When conducting this analysis for seasons in which influenza A (H3N2), A (H1N1), and B predominated, area-level differences were again not significant. The strong correlation of excess-death estimates in the 3 regions suggests a high level of synchrony in the amplitude of local influenza epidemics (number of excess deaths peaked in the same month in each region) across Italy (P&I, Spearman $\rho = 0.88–0.93$,

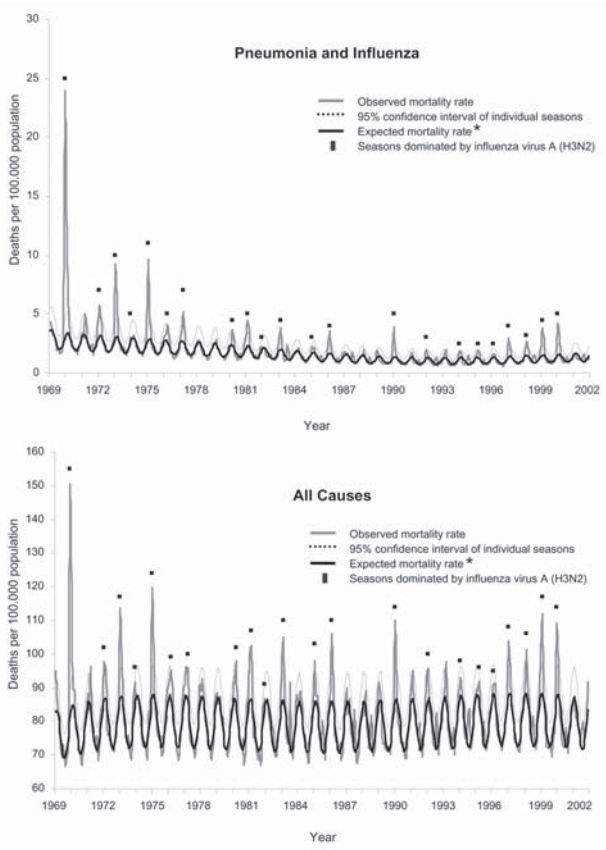

Figure 1. Monthly mortality rates from pneumonia and influenza and all causes for Italy, January 1969–December 2001. *Baseline mortality rates determined by Serfling model.

p<0.0001; AC, Spearman ρ = 0.80–0.93, p<0.0001) (Figure 2; online Appendix Figure 2, available from www.cdc.gov/EID/content/13/5/694-appG2.htm).

Magnitude and Trends of Influenza-related Deaths during Pandemic and Epidemic Seasons, by Age

During epidemic seasons, most influenza-related deaths at the national level (84%) occurred in persons ≥65 years of age, for P&I and AC; by contrast, during the 1969–70 influenza A (H3N2) pandemic season, deaths markedly affected all age groups, especially the 45–64 group.

In Italy the proportion of excess deaths from AC in persons <65 years of age was 3-fold higher during the pandemic season than during all other epidemic seasons. In particular, when the pandemic season was compared with the season with the second highest number of deaths (1974–75), the number of influenza-related deaths was 7× higher for persons 0–14, 4× higher for persons 15–44 and 45–64, and 2× higher for persons ≥65 years of age. Similar results were obtained for all 3 geographic areas (Table 2). The number of excess deaths from AC during the influenza A (H3N2) pandemic season was 1- to 9-fold higher in Italy than in other European countries (France, England, and Wales), in North American countries (United States, Canada), and in Asian countries (Japan, Australia) (Table 3).

Discussion

This study showed a high level of correlation in the amplitude of influenza epidemics (i.e., peaks in rates were similar) in the 3 Italian regions during a 32-year period spanning epidemic and pandemic seasons. The analysis of local influenza-related death patterns did not show differences in mean mortality rates among geographic areas These findings are consistent with the high level of synchrony found in other area-level studies in Europe and in the United States (*1*,*2*,*5*,*6*).

The first season analyzed was the 1969–70 pandemic season. In Italy, as in other European countries (*8*), the pandemic season was more destructive in the second season of circulation of influenza A (H3N2) virus (i.e., in 1969–70), 1 year after the pandemic strain was first introduced to Italy (*25*–*27*). The pandemic season seems to have had a greater effect in Italy; excess mortality rates were estimated to be 38 (20,000 deaths) for P&I and 107 (57,000 deaths) for AC. These unexpectedly large excess mortality rates were 3-fold higher than that in the United States and 1-fold higher than those in other European countries. The increase in percentage of deaths reduced but did not eliminate these differences. However, the percentage of deaths in persons <65 in Italy (29%) was lower than the percent-

Table 1. Mean age-standardized excess all-cause deaths per 100,000 population and the winter seasonal percent increase attributable to influenza, Italy, 1969–2001*

Deaths	Northern	Central	Southern
Pandemic season (1969–1970)			
Excess	103.6	85.3	105.2
Percent increase	21.7	21.0	25.9
Epidemic seasons (1970–2001)			
Excess	13.7	13.2	17.1
Percent increase	3.4	3.6	5.1
Entire study period (1969–2001)			
Excess	16.6	15.4	19.9
Percent increase	4.0	4.1	5.7

*95% confidence intervals for individual season estimates were within 6% of values listed.

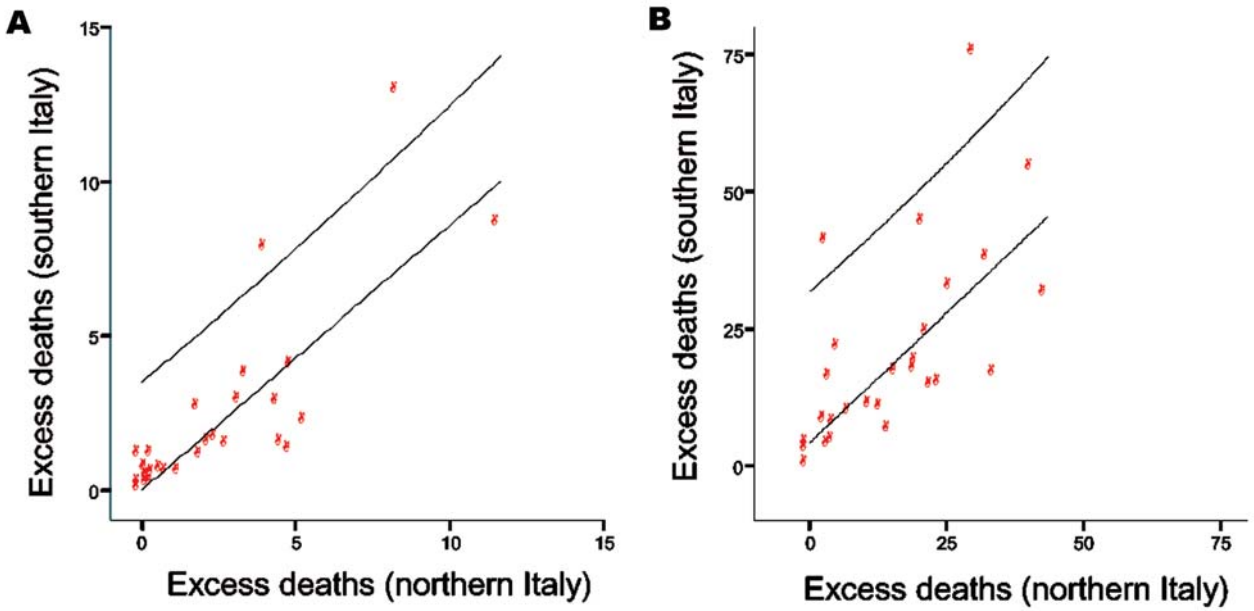

Figure 2. Correlation in the influenza epidemics for 31 influenza seasons (1970–2001), measured by excess deaths from pneumonia and influenza (A) and all causes (B). Excess mortality rates per 100,000 in southern and northern Italy.

age in that age group in the United States (34%) but similar to the percentages in other European countries, especially France (27%) (Table 3). Future studies could address these differences in numbers of deaths that may stem from underlying differences in baseline mortality rates or perhaps in influenza transmission.

During the pandemic seasons, compared with epidemic seasons, the relative increase in mortality rates was lower for elderly than for younger persons in Italy, confirming that during pandemics, children and young adults have a greater relative risk for death than the elderly (*12*). A possible explanation is the partial immune protection of elderly persons who may have been exposed before 1891 to H3 antigens (*28*), whereas persons born after 1891 would not have been exposed to these antigens.

Several limitations should be mentioned. First, deaths from P&I were not always confirmed by laboratory methods, which could have resulted in misclassification of deaths. However, patterns of death from P&I were very similar to those from AC, which are not subject to this bias. A second limitation was that demographic and nondemographic differences could have biased the geographic comparison. However, we performed age standardization and calculated the percentage increase in deaths over the winter baseline, which reduces baseline differences in deaths, unrelated to influenza.

A third, more critical, caveat stems from the surveillance of viral subtypes. The proportion of laboratory-confirmed cases has only been available since 1999 (i.e., for only 2 years of the study period). However, in the latest years the proportion of laboratory-confirmed cases was ≈15% (range 11%–28%), with >3,000 samples tested (*29*), which could have affected the accuracy of influenza diagnoses over time and perhaps across regions. For example, during the 1998–99 season, when influenza B viruses were predominant, the death rate was high compared with that found for the other influenza B seasons, which indicates that the characterization of viral subtypes is limited by the geographic distribution of the sites participating in virologic surveillance.

Our findings suggest that influenza epidemics are strongly correlated in amplitude across the 3 regions of Italy. Different factors have been reported to drive the spatial and temporal correlations of epidemics: population move-

Table 2. Mean age-standardized excess deaths per 100,000 population, Italy, 1969–2001

	Pneumonia and influenza						All causes					
	1969–70			1970–2001			1969–70			1970–2001		
Age, y	Northern	Central	Southern	Northern	Central	Southern	Northern	Central	Southern	Northern	Central	Southern
0–14	2.4	3.3	7.8	0.0	0.1	0.1	8.6	8.7	30.3	0.3	0.6	1.6
15–44	3.8	2.5	3.0	0.1	0.1	0.1	7.7	7.0	8.4	0.6	0.8	0.8
45–64	38.8	25.4	27.4	0.7	0.4	0.7	112.1	75.1	109.0	4.3	4.3	6.6
≥65	288.7	221.8	218.2	14.0	12.7	14.2	694.8	621.6	859.2	75.6	71.2	115.7
Total	43.0	31.5	29.3	2.2	1.9	1.9	103.6	85.3	105.2	12.0	11.6	18.6

Table 3. Multinational comparison of influenza A (H3N2) Hong Kong pandemic, based on all-cause excess deaths estimates*

Deaths	Italy†	England, Wales†	France†	Australia‡	Japan†	USA§	Canada§
Overall no./100,000 population	107	77	72	64	49	27	12
Increases over baseline, %	24	20	21	16	20	8	6
Persons <65 y,	29	23	27	20	N/A	34	24

*Data from this study and (*8*). Estimates are for the major pandemic seasons, for which timing varied by country. N/A, not applicable.
†Second season of virus circulation, 1969–70.
‡Second season of virus circulation, 1970.
§First season of virus circulation, 1968–69.

ments and environmental factors such as climate or weather conditions (*5*,*30*,*31*). Although population movements are assumed to play a key role in the global spread of influenza epidemics, they have been quantified only in the United States (*5*,*32*,*33*). The role of environmental factors and differences in circulating viruses among the geographic areas in Italy also remains to be clarified.

In conclusion, our results suggest that geographic synchrony of influenza in Italy is high and that for persons <65 years of age, death rates are likely to be substantially elevated in a future pandemic as compared with other epidemic seasons. Our study adds to others that have found strong spatiotemporal patterns in illness and death from influenza in the United States, France, Australia, and across Europe (*1*,*3*,*5*,*34*). Such results provide insight for the Italian pandemic preparedness and response efforts (*35*,*36*) and could be used in mathematical models for influenza spread at the national level.

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Respirator Donning in Post-Hurricane New Orleans

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We evaluated correctness of N95 filtering facepiece respirator donning by the public in post-hurricane New Orleans, where respirators were recommended for mold remediation. We randomly selected, interviewed, and observed 538 participants, using multiple logistic regression for analysis. Only 129 (24%) participants demonstrated proper donning. Errors included nose clip not tightened (71%) and straps incorrectly placed (52%); 22% put on the respirator upside down. Factors independently associated with proper donning were as follows: ever having used a mask or respirator (odds ratio [OR] 5.28; 95% confidence interval [CI], 1.79–22.64); ever having had a respirator fit test (OR 4.40; 95% CI, 2.52–7.81); being male (OR 2.44; 95% CI, 1.50–4.03); Caucasian race (OR 2.09; 95% CI, 1.32–3.33); having a certified respirator (OR 1.99, 95% CI, 1.20–3.28); and having participated in mold clean-up (OR 1.82; 95% CI,1.00–3.41). Interventions to improve respirator donning should be considered in planning for influenza epidemics and disasters.

Many respirators certified by the National Institute for Occupational Safety and Health (NIOSH), particularly disposable N95 filtering facepiece respirators (N95 FF respirators [Figure 1]), are available to the public. The certification indicates that the respirator material will perform at a given filter efficiency (*1*). Because proper fit is also necessary for respirator function, US regulations state that an employer who requires workers to wear respirators must establish a respiratory protection program that covers respirator selection and maintenance, fit testing, and worker instruction (*2*). Although nonoccupational respirator use has not been well studied, members of the public who use respirators may be less likely than workers in a respiratory protection program to achieve a proper fit, given lack of formal training (*3*).

Public health agencies have recommended N95 FF respirators to members of the public for some situations. Such occasions have included after major floods, for potential heavy exposure to bioaerosols in water-damaged buildings (Grand Forks, North Dakota, 1997; eastern North Carolina after Hurricane Floyd, 1999) (*4*), and for settings that pose a risk for airborne transmission of infection, such as during the severe acute respiratory syndrome (SARS) epidemic (for select patients at risk of acquiring the infection and for persons visiting patients with SARS) (*5–7*). There is also a longstanding recommendation for N95 FF respirator use for visitors of hospitalized patients with tuberculosis (*8*). The US Department of Health and Human Services (HHS) currently recommends that persons living in or visiting an area affected by avian influenza A (H5N1) wear N95 FF respirators when in contact with birds in an enclosed environment (*9*).

In the fall of 2005, after the unprecedented flooding in New Orleans, Louisiana caused by Hurricanes Katrina and Rita, public health officials recommended that members of the public use N95 FF respirators when cleaning or remediating mold-contaminated buildings (*10*). A survey of 159 New Orleans area residents 7 weeks after Katrina found that 68% of those interviewed were aware of the recommendation (*11*) and that at least 30% of those participating in remediation activities had used a NIOSH-certified respirator (*12*). Despite these levels of awareness and experience, subsequent anecdotal reports suggested that some New Orleans residents were not properly donning N95 FF respirators. Improper donning would promote the entry of

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Figure 1. Noncertified masks and certified respirators. A surgical mask (upper left) and a dust mask (lower left) are examples of disposable masks that are not designed to filter small particles and that are not certified by the National Institute for Occupational Safety and Health (NIOSH). The disposable N95 filtering facepiece respirators pictured on the right (with exhalation valve, upper right; without exhalation valve, lower right) are made of material certified by NIOSH to filter 95% of 0.3-μm diameter particles and bear the NIOSH name and "N95" filter identification. The European FFP2 respirator is most analogous to the N95 filtering facepiece respirator. NIOSH also certifies more expensive reusable respirators (not pictured), which can be fitted with disposable cartridges that filter particles. Reusable respirators may cover the face from the bridge of the nose to the chin (half-face) or from the forehead to the chin (full-face).

unfiltered air through leaks or gaps between the respirator and the skin, compromising the protection offered (*13*). To better understand respirator use by the public, we investigated the nonoccupational use and donning of N95 FF respirators in post-hurricane New Orleans.

Methods

Participants

Using geographic information system mapping software, we randomly selected homes in Orleans Parish (city of New Orleans). To focus on residential areas, we eliminated 6,345 of the parish's 10,181 census blocks (US Census, 2000). The eliminated blocks were likely to be sparsely populated or to contain industrial buildings, commercial centers, or parks (blocks with ≤20 housing units and blocks in the lower 2.5% of housing unit density); to contain mostly high-rise apartment buildings or public housing units that would be difficult to access or remain uninhabited (blocks in the upper 2.5% of housing unit density); and to be in uninhabited neighborhoods (blocks in the Lower Ninth Ward). We randomly generated 600 waypoints (unique locations based on latitude and longitude) across the remaining 3,836 census blocks.

Each waypoint served as a starting point to locate eligible participants. Using a global positioning system device, a survey team navigated to a waypoint and identified the nearest home. To be eligible for participation, a person had to be an English-speaking adult (≥18 years of age) associated with a home as its owner, current occupant, or relative/friend of the owner/occupant. Because we were interested in nonoccupational respirator use by the general public, persons at a home as paid employees (e.g., remediators) were not eligible. However, residents encountered at their own homes who were employed as remediators were not excluded. If unable to conduct an interview at the first encountered home, the team proceeded in a systematic fashion to the next home. Once 1 interview was conducted at a waypoint, the team navigated to the next waypoint and repeated the process.

Questionnaire and Evaluation of Respirator Donning

From March 4 to March 11, 2006, survey team members interviewed participants with a 10-minute questionnaire that collected information on experience with residential flooding, water damage, and mold growth; participation in mold clean-up activities; lifetime and post-Katrina experiences with respiratory protection (both noncertified dust masks and surgical masks, hereafter "masks"; and NIOSH-certified respirators, including disposable and reusable types, hereafter "certified respirators"); and nonidentifying demographic factors, including self-identified ethnicity and race. Each team used the same photographs and actual examples of masks and certified respirators during the interviews.

Each interview included an evaluation of respirator donning. Interviewers were trained before the survey on proper donning (Figure 2), including the following: proper orientation of the respirator; use of both straps; proper placement of straps; need for tightening of the nose clip; and need for removal of facial hair (*14*,*15*). A participant with an N95 FF respirator that appeared to be in good condition was permitted to use this respirator for the evaluation. Otherwise, the participant was asked to choose between 2 models then available from New Orleans retailers. The interviewer asked the participant to put on the N95 FF respirator as he or she would for participating in mold clean-up activities. Written and pictorial manufacturer's instructions were included with the respirator packaging, but no additional instructions were given until the evaluation was complete (*16*). The interviewer recorded whether the participant referred to the manufacturer's instructions

Figure 2. Properly donned disposable N95 filtering facepiece respirator. To be properly donned, the respirator must be correctly oriented on the face and held in position with both straps. The straps must be correctly placed, with the upper strap high on the head and the lower strap below the ears. For persons with long hair, the lower strap should be placed under (not over) the hair. The nose clip must be tightened to avoid gaps between the respirator and the skin. Facial hair should be removed before donning. Photo used with permission.

and, once the participant indicated that the respirator was donned, noted any observed "donning errors" that could contribute to an insufficient fit.

Statistical Analyses

For calculations of frequencies of donning errors, we included all participants who had at least 1 error. However, because 2 errors, visible gap and facial hair, could reflect aspects of study design (size and shape of respirators offered and lack of opportunity to shave before evaluation, respectively) rather than participants' donning technique, they were not considered in analyses of factors associated with proper donning. For these analyses, participants with at least 1 of the other donning errors were categorized as improperly donning the respirator; the remaining participants were categorized as properly donning the respirator.

To identify factors associated with proper donning, we used contingency tables and simple logistic regression. We included significant factors ($p \leq 0.05$) in multiple logistic regression models, applying stepwise logistic regression. We used the likelihood ratio χ^2 test and calculated odds ratios (OR) with 95% likelihood confidence intervals (CI). We conducted analyses with SAS (version 9.1) and JMP (version 5.1) software packages (SAS Institute, Cary, NC, USA).

Results

Participants

We conducted 553 interviews at the 600 visited waypoints, for a response rate of 92%. Half of the participants were male, with a median age of 50 years, and about half identified their race as Caucasian and half as African-American or black (Table 1). Most described previously using a mask or respirator, but few reported ever having a respirator fit test. Most had participated in mold clean-up activities since Hurricane Katrina.

Respiratory Protection Use during Mold Clean-up Activities

Overall, of the 553 participants interviewed, 42% (n = 233) had used a certified respirator, and 35% (n = 192) had used an N95 FF respirator, specifically, for mold clean-up activities since Katrina. Among the 368 who reported participating in mold clean-up activities, most (n = 315, 86%) reported using a mask or certified respirator during those activities, most frequently the N95 FF respirator (Table 2). A minority (n = 60, 19%) of the 315 reported referring to the manufacturer's instructions. More commonly (n = 129, 41%), participants stated that they used respiratory protection without any instruction.

Evaluation of Respirator Donning

A total of 538 (97%) participants agreed to put on an N95 FF respirator. Most of these (n = 489, 91%) used 1 of the 2 models offered by the interviewers. Twelve (2%) referred to the manufacturer's directions.

Overall, 433 (80%) of the participants who donned an N95 FF respirator were noted to have at least 1 donning error that could contribute to a poor fit. More than half of these did not tighten the nose clip, and half incorrectly placed the 2 straps; in addition, 22% put the respirator on upside down, and 21% used only 1 strap (Table 3). While 31% (n = 135) made 1 error, 34% (n = 146) made 2, and 35% (n = 152) made $\geq$3.

Characteristics Associated with Proper Donning

For 24 participants, the only noted donning error was a visible gap (n = 6) or facial hair (n = 18). When these 24 persons who otherwise demonstrated proper donning were included, 129 (24%) of the participants properly donned the N95 FF respirator. In simple logistic regression analyses of all participants, proper donning was significantly associated with several personal factors: being male, being Caucasian, and being a nonrenter (i.e., a homeowner or as-

Table 1. Characteristics of Orleans Parish participants, March 2006*

Characteristic	Values
Age in y, median, range (N = 547)	50, 18–89
Male, n/N (%)	292/553 (53)
Hispanic, n/N (%)	21/548 (4)
Race, n/N (%)†	
Caucasian	241/548 (44)
African-American or black	296/548 (54)
Asian	20/548 (4)
American Indian or Alaska Native	21/548 (4)
Native Hawaiian or other Pacific Islander	5/548 (1)
Relationship to home, n/N (%)‡	
Owner	415/553 (75)
Renter	80/553 (14)
Other (includes relatives, friends, other associates)	58/553 (10)
Smoking status, n/N (%)	
Current	127/551 (23)
Former	123/551 (22)
Never	301/551 (55)
Physician-diagnosed asthma, n/N (%)	68/553 (12)
Flood level in feet,‡ median, range (N = 527)	4, 0–18
Water entry due to roof or window damage,‡ n/N (%)	300/547 (55)
Mold extent,‡ n/N (%)	
None	143/550 (26)
<50% of walls and ceilings	213/550 (39)
≥50% of walls and ceilings	179/550 (33)
Do not know	15/550 (3)
Employed in mold remediation, n/N (%)	45/553 (8)
Ever used mask or respirator, n/N (%)	439/553 (79)
Ever had respirator fit test,§ n/N (%)	80/543 (15)
Activities in water-damaged/moldy home since Katrina	
Been inside, n/N (%)	467/551 (85)
Participated in clean-up, n/N (%)	372/551 (68)
No. of homes cleaned (N = 368), median, range	2, 1–50
No. with mold extent ≥50% (N = 367), median, range	1, 0–25
Still participating in clean-up activities, n/N (%)	183/358 (51)

*Data for some characteristics were missing for some participants.
†Participants could select >1 racial category; total >100%.
‡Home at which participant was encountered and interviewed.
§"Fit test" was defined in the questionnaire as "a test in which a technician measures how well the respirator fits your face during activities such as talking and moving your head. It could involve smelling smoke, tasting something sweet or bitter, or a special machine that counts particles."

sociate) in the home at which the interview occurred. In addition, proper donning was associated with post-hurricane experiences: having been inside a water-damaged or moldy home and having participated in mold clean-up. Finally, proper donning was associated with several factors related to respirators: ever having used a mask or certified respirator, ever having had a respirator fit test, having at the time of the interview a mask or certified respirator, and having at the time of the interview a respirator confirmed by the interviewer to be NIOSH certified (Table 4).

For the subset that had participated in clean-up activities, proper donning was also associated with use of respiratory protection during clean-up, including having specifically used an N95 FF respirator. For the subset that had used a mask or certified respirator during clean-up, having obtained that mask or respirator from the workplace and having obtained information on how to use the mask or respirator from the workplace were also significant factors. Proper donning was not associated with age, Hispanic ethnicity, level of floodwater, water incursion due to roof or window damage, extent of mold coverage, current employment in mold remediation, asthma diagnosis, smoking status, or respirator brand.

When multiple logistic regression was used, the factors significantly associated with proper donning for all participants

Table 2. Orleans Parish participants' experiences with respiratory protection during mold clean-up activities since Hurricane Katrina, March 2006*

Experience	n/N (%)
Used mask or respirator	315/368 (86)
Type of mask or respirator used†	
Noncertified mask (dust or surgical)	143/315 (45)
Certified respirator, type†	233/315 (74)
Disposable N95 filtering facepiece	192/233 (82)
Reusable half-face with cartridges	87/233 (37)
Reusable full-face with cartridges	4/233 (2)
Source of mask or respirator†	
Store	207/315 (66)
Nongovernmental organization‡	73/315 (23)
Workplace	27/315 (9)
Relative or friend	24/315 (8)
Other source	16/315 (5)
Main source of information on use of mask or respirator	
Manufacturer's instructions	60/315 (19)
Media	20/315 (6)
Instructions given at work	51/315 (16)
Store employee/clerk	5/315 (2)
Relative or friend	16/315 (5)
Internet site§	10/315 (3)
Other source	24/315 (8)
No information used	129/315 (41)
Conditions that would prompt replacing mask or respirator†	
When it became dirty	163/312 (52)
When it became damaged	34/312 (11)
When it became harder to breathe through	25/312 (8)
Other¶	131/312 (42)

*372 (68%) of 553 survey participants reported participating in mold clean-up activities since Hurricane Katrina. Data for some characteristics were missing for some participants.
†Participants could choose >1 response; total >100%.
‡Includes Red Cross, Salvation Army, volunteer groups, and church groups.
§In 4 cases, Internet site was specified by name: Channel 6, Federal Emergency Management Agency, city of New Orleans, and National Institute for Occupational Safety and Health.
¶Write-in responses included various time intervals (e.g., every 3 h, daily, weekly, never) and other conditions such as when smelling moldy odor or feeling sick.

Table 3. Errors observed among Orleans Parish participants donning disposable N95 filtering facepiece respirators, March 2006

Error	n (%)*
Nose clip not tightened	303 (71)
Both straps used, but straps incorrectly placed	221 (52)
Visible gap between respirator and skin†	136 (32)
Respirator donned upside down	94 (22)
Only 1 of 2 straps used	91 (21)
Facial hair†	48 (11)
Respirator donned sideways or tilted	11 (3)
Other‡	5 (1)

*N = 427; 433 participants were noted to have at least 1 donning error; for 427 participants, the nature of the error(s) was documented.
†Among those participants with only 1 observed donning error, 6 had a visible gap between the respirator and skin, and 18 had facial hair. These 24 participants are included in calculations of frequencies of donning errors. For analyses of factors associated with proper donning, they were considered to have properly donned the respirator.
‡The "other" category was used for 2 participants who were noted to be unable to put on the respirator and for 1 participant who did not use either of the 2 straps. For 2 other participants, the "other" category was indicated, but the interviewer did not further specify the nature of the errors.

were as follows: ever having used a mask or certified respirator (OR 5.28; 95% CI, 1.79–22.64), ever having had a respirator fit test (OR 4.40; 95% CI, 2.52–7.81), being male (OR 2.44; 95% CI, 1.50–4.03), being Caucasian (OR 2.09; 95% CI, 1.32–3.33), having a certified respirator at the time of the interview (OR 1.99, 95% CI 1.20–3.28), and having participated in mold clean-up activities (OR 1.82; 95% CI,1.00–3.41). For the subset that participated in mold clean-up, the significant factors were as follows: having used a certified respirator during clean-up (OR 5.17; 95% CI, 2.75–10.24); ever having had a respirator fit test (OR 3.38; 95% CI, 1.75–6.61); being Caucasian (OR 3.38, 95% CI, 1.97–5.91); and being male (OR 2.80; 95% CI, 1.58–5.13). These same factors were also significant for the subset that used a mask or certified respirator during clean-up.

Discussion

The protection afforded by a certified respirator depends on its fit, and a fundamental component of achieving a good fit is proper donning (*13*,*17*). In post-hurricane New Orleans, public concern about adverse health effects of exposure to mold was near universal (*11*,*12*). Yet our investigation demonstrated that, despite this high level of motivation, most of participants did not properly don an N95 FF respirator.

Our investigation benefited from several strengths. We used a random selection process to obtain our sample, and comparisons with existing population-based surveys suggest we achieved a representative cross-section (*18*,*19*). Given inconsistencies in respiratory protection terminology, we facilitated effective communication by using photographs and actual examples of masks and certified respirators. Finally, of the few prior field investigations that have addressed N95 FF respirator donning (*20*–*22*), none have focused on nonoccupational use.

Table 4. Characteristics associated with proper donning of disposable N95 filtering facepiece (FF) respirators in simple logistic regression analyses of all Orleans Parish participants and subsets of participants

	Proper donning (%)*			
Characteristic	With characteristic	Without characteristic	OR (95% CI)†	p value
All participants (N = 538)‡				
Male	34	12	3.84 (2.47–6.12)	<0.001
Caucasian (N = 533)	29	20	1.66 (1.12–2.49)	0.01
Relationship to interview home (nonrenter vs. renter)	26	13	2.29 (1.19–4.87)	0.01
Ever used mask or respirator	29	5	8.31 (3.57–23.44)	<0.001
Ever had respirator fit test	58	18	6.10 (3.69–10.21)	<0.001
Been inside water-damaged/moldy home (N = 536)	26	14	2.11 (1.12–4.35)	0.02
Participated in clean-up (N = 536)	29	14	2.58 (1.60–4.31)	<0.001
Mask or respirator at interview	38	18	2.74 (1.80–4.15)	<0.001
Confirmed certified respirator at interview	44	19	3.48 (2.23–5.43)	<0.001
Participated in clean-up§ (N = 363)¶				
Used mask or respirator during clean-up	31	16	2.42 (1.15–5.74)	0.02
Used certified respirator during clean-up	39	12	4.54 (2.59–8.42)	<0.001
Used N95 FF respirator during clean-up	35	22	2.02 (1.27–3.25)	<0.01
Used a mask or respirator during clean-up# (N = 312)**				
Workplace source of mask or respirator	48	29	2.22 (1.00–4.95)	0.05
Workplace source of information	47	28	2.29 (1.24–4.23)	<0.01

*Proportion of participants with characteristic demonstrating proper donning, followed by proportion of participants without characteristic demonstrating proper donning. For the first characteristic, 34% of males and 12% of females properly donned the respirator.
†Unadjusted odds ratio (OR) and 95% confidence interval (CI) for proper donning of disposable N95 FF respirator by participants with characteristic compared to participants without characteristic.
‡538 of 553 participants donned an N95 FF respirator for the interviewer. For some analyses, N is <538 (as noted) because of missing data.
§The following variables were also statistically significant in simple logistic regression analyses for this subset: male, Caucasian, ever used mask or respirator, ever had respirator fit test, had mask or respirator at time of interview, and had certified respirator at time of interview.
¶367 of 372 who participated in clean-up donned an N95 FF respirator for the interviewer. N is <367 because of missing data.
#The following variables were also statistically significant in simple logistic regression analyses for this subset: male, Caucasian, ever had respirator fit test, had mask or respirator at time of interview, had certified respirator at time of interview, used certified respirator during clean-up, and used disposable N95 FF respirator during clean-up.
**312 of 315 who used a mask or respirator during clean-up donned an N95 FF respirator for the interviewer.

An important limitation is that we did not confirm the observed proper donnings with respirator fit testing. While we cannot estimate the relative contribution of each donning error to declining protection without quantitative measurements, those participants who put on the respirator improperly would clearly have failed a standard fit test. However, the outcome in those who appeared to properly put on the respirator is less certain because even a properly donned respirator may have leaks that limit its effectiveness. A study of 18 different N95 FF respirator models found that, overall, in the absence of fit testing, 74% of proper donnings would provide the full protection possible with an N95 FF respirator (range 31%–99%, depending on the model) (*23*). Thus, the proportion of our participants who would have achieved the full protection possible with an N95 FF respirator is likely to be lower than the proportion who demonstrated proper donning. Ultimately, designing models with good fit characteristics would be beneficial.

Our findings have implications for the use of N95 FF respirators by members of the public to prevent the transmission of communicable diseases. Both experimental and epidemiologic studies suggest that airborne transmission of influenza (by small particles <10 μm) can occur and may result in more severe disease than transmission by large droplets or fomites (*24*–*27*). A recent review argues that airborne transmission may play an important role in the spread of a pandemic strain (*28*). While formal recommendations for N95 FF respirator use by the public do not exist—beyond the HHS recommendation regarding potential exposures to infected birds—a recent Institute of Medicine (IOM) report notes that "a properly fitted N95 FF respirator is likely to be both the least expensive and the most widely available NIOSH-certified respirator for protecting... the public against airborne [influenza] infection" (*29*). Our results suggest that much of the public may have difficulty achieving a proper fit because of improper donning. Given the observed role of experience in proper donning, and the high frequency of recent experience with respirators reported by our survey participants, one could argue that the overall performance in post-Katrina New Orleans is likely to be superior to that of virtually any other locale.

The World Health Organization anticipates use of respiratory protection by the public will occur spontaneously in the event of pandemic influenza (*30*,*31*). Indeed, N95 FF respirators are currently being marketed to the public as "bird flu masks" (*32*). While uncertainty remains about the level of protection needed against influenza and that offered by an N95 FF respirator, an improperly donned N95 FF respirator will provide less protection than a properly donned one. Our results suggesting that workplace training increased proper donning among the public indicate that educational efforts could have a positive effect. Since few of our participants reported, or were observed, referring to manufacturers' instructions, consideration also should be given to incorporating instructions onto the respirator itself, such as arrows or simple words ("nose," "chin") to indicate orientation. The IOM report's authors could find no simple modification of N95 FF respirators that would prevent the need for fit testing (*29*). Short of mass fit testing, proper donning will be the vital step to ensuring that members of the public using N95 FF respirators derive the greatest possible benefit from them.

Even under workplace conditions, respirator donning may be imperfect. An observational study of 62 healthcare workers in 3 California hospitals found that 40 (65%) improperly put on N95 FF respirators before entering the room of a patient in isolation for tuberculosis. Errors included use of only 1 strap, incorrectly placed straps, and presence of facial hair (*21*). The results of that study, in terms of the proportion who demonstrated improper donning and the nature of the errors, are similar to our findings. The impact of the 2005 US policy that suspended enforcement of annual fit testing of healthcare workers who use respirators for occupational exposure to tuberculosis is unknown (*33*). Yet N95 FF respirators will clearly be part of healthcare workers' defense in the event of pandemic influenza (*34*,*35*). HHS, as part of procurement of essential medical supplies for pandemic influenza, has stockpiled 20 million N95 FF respirators and plans to acquire 87 million more through September 2007 (*36*). The pandemic plans of other countries, including Australia and France, recommend use of N95 (or FFP2) respirators (*28*). Further evaluation of respirator donning among healthcare workers therefore may be warranted.

In summary, this population-based survey of nonoccupational respirator use found that a minority of participants demonstrated proper donning of an N95 FF respirator. Our findings are of particular importance to public health agencies planning for future events, from floods to pandemic influenza, in which use of N95 FF respirators by the public will be recommended or is anticipated. A unique opportunity exists to enhance protection of the public through interventions, such as educational campaigns, training sessions, and respirator design modifications, aimed at improving the public's ability to don a respirator correctly. Infection control officers and the healthcare workers they protect also may benefit from the insights gained from this survey.

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Apoptosis and Pathogenesis of Avian Influenza A (H5N1) Virus in Humans

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The pathogenesis of avian influenza A (H5N1) virus in humans has not been clearly elucidated. Apoptosis may also play an important role. We studied autopsy specimens from 2 patients who died of infection with this virus. Apoptosis was observed in alveolar epithelial cells, which is the major target cell type for the viral replication. Numerous apoptotic leukocytes were observed in the lung of a patient who died on day 6 of illness. Our data suggest that apoptosis may play a major role in the pathogenesis of influenza (H5N1) virus in humans by destroying alveolar epithelial cells. This pathogenesis causes pneumonia and destroys leukocytes, leading to leukopenia, which is a prominent clinical feature of influenza (H5N1) virus in humans. Whether observed apoptotic cells were a direct result of the viral replication or a consequence of an overactivation of the immune system requires further studies.

The pathogenesis of avian influenza A (H5N1) virus in humans is not well understood. Although several studies have shown some aspects of this pathogenesis in animal models, direct evidence of pathogenic mechanisms in humans has been limited to only a few autopsy studies (*1*–*3*). We previously demonstrated in an autopsy case that alveolar epithelial cells are the major target cell type of this virus (*3*). The case in that study, as well as other previous autopsy reports, died late in the disease. Some of the findings may not reflect the actual pathogenesis at the acute period but may be consequences of secondary events. We performed an autopsy of a patient who died on day 6 of onset of illness. The findings in this case are more likely to reflect viral pathogenesis in the acute phase of the disease.

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Apoptosis has been implicated in the pathogenesis of influenza. Infection of epithelial cells and lymphocytes has been shown to induce apoptosis in vitro (*4*–*8*). Several modes of apoptosis induction and responsible viral genes have been proposed (*8*–*13*). Infection with virulent influenza (H5N1) virus was also shown to induce lymphopenia and lymphocyte apoptosis in vivo (*14*). However, whether and to what extent apoptosis contributes to the highly virulence property of influenza (H5N1) viruses are not clear. In this report, we studied apoptotic activity in 2 patients who died of avian influenza.

Materials and Methods

Patients

The study was approved by the Siriraj Ethics Committee. The first patient (patient A) was a 48-year-old man who had progressive viral pneumonia. He had fever, cough, running nose, myalgia, and chest pain at the onset of illness. Dyspnea developed on day 2 of illness, and a chest radiograph showed interstitial infiltrations at right upper and left middle lung fields and a masslike infiltration at the right middle lung field. The diagnosis of avian influenza was suspected on day 4 of illness after a history of direct contact with dying chickens was revealed. Respiratory secretions were then sent to national laboratories and confirmed positive for influenza (H5N1) virus. The patient died on day 6 of illness.

An autopsy was conducted by using standard techniques and precautions to minimize risk for transmission of infection. Tissues obtained were prepared for routine histologic analysis and samples were stored at –70°C for further study.

The other autopsy case (patient B) has been previously reported (*3*). This patient was a 6-year-old boy who had progressive viral pneumonia that led to acute respiratory distress syndrome and death 17 days after onset of illness.

RNA, Antigen, and Apoptosis Analyses

Lung, trachea, liver, spleen, colon, and bone marrow tissues were tested for viral RNA. For reverse transcription–PCR (RT-PCR), fresh unfixed specimens were minced into small pieces in lysis buffer of an RNA extraction kit (RN easy; QIAGEN, Valencia, CA, USA). Total RNA was then extracted according to the manufacturer's protocol. RT-PCR for hemagglutinin 5 (H5) was then performed on the extracted RNA by using the One-Step RT-PCR Kit (QIAGEN) with an H5-specific primer pair. Strand-specific RT-PCR was performed by using a method similar to the RT-PCR for viral RNA detection except that only 1 primer was added at the reverse transcription step. Tumor necrosis factor-α (TNF-α) mRNA was detected in RNA extracted from lung, trachea, liver, spleen, colon, and bone marrow tissues by an RT-PCR as previously described (*3*).

Tissue sections of lung, trachea, liver, spleen, and colon were stained for influenza A virus antigen. The sections were deparaffinized and rehydrated. Antigenic sites were identified by digestion with 0.5% trypsin for 15 min at 37°C. Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 for 15 min at 37°C. Sections were incubated with 2.5% bovine serum albumin (Dako, Roskilde, Denmark) for 15 min at room temperature and subsequently incubated with a monoclonal antibody to influenza A virus nucleoprotein at a dilution of 1:40 (B.V. European Veterinary Laboratory, Woerden, the Netherlands) overnight at 4°C. Slides were rinsed 3 times in 1× phosphate-buffered saline (PBS) plus 0.05% Tween-20 and incubated with horseradish peroxidase–conjugated goat antimouse immunoglobulins at dilutions of 1:400 (Dako) for 30 min at room temperature. The slides were washed as above and developed with diaminobenzidine (Dako).

Lung, liver, spleen, colon, and bone marrow sections were analyzed for apoptosis by using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay. After digestion with 0.5% trypsin as described above, sections were treated with TUNEL reaction mixture by using the In Situ Cell Death Detection Kit (Boehringer, Mannheim, Germany) for 1 h at 37°C in the dark. Slides were then rinsed 3 times with 1× PBS and incubated with alkaline phosphatase–conjugated fluorescein isothiocyanate–labeled antibody for 30 min at 37°C. Sections were then washed and developed with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate p-toluidine.

Results

Pathologic Findings

Pathologic findings in patient A were not identical to the previously reported findings in patient B (*3*). Common findings in both patients were diffuse alveolar damage and positive staining for influenza A virus antigen in alveolar epithelial cells (Figure 1). In patient A, lungs showed an earlier stage of exudative phase of diffuse alveolar damage than the damage found in patient B. Patient A also showed some atypical pneumocytes with large bizarre and clumping nuclei. Bronchiolitis and pleuritis were also observed. No superimposed bacterial or fungal infection was identified. Hemophagocytic activity was found in lungs, liver, and bone marrow. The liver showed some cholestasis but was otherwise unremarkable.

Sites of Viral Replication

Viral RNA was detectable in the lung, trachea, and liver of patient A (Figure 2A). To test whether virus replicated outside the lung, we tested the trachea and liver by using a strand-specific RT-PCR and found that both tissues contained positive-stranded viral RNA, which suggested active viral replication in these organs. This was in contrast to our previous report (patient B), in which positive-stranded viral RNA was detectable in lung and intestine (*3*). Whether this was a result of a difference in tissue tropism of the viruses is unclear. It is also possible that this reflected different

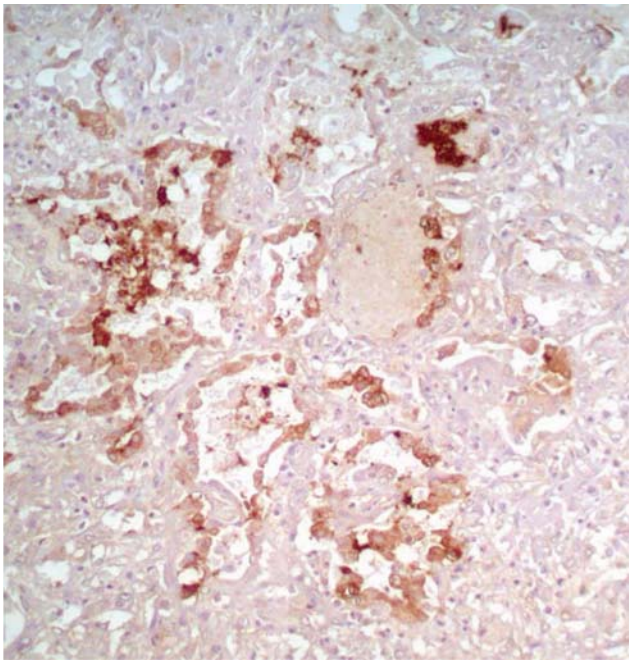

Figure 1. Immunohistochemical staining of viral antigen in alveolar epithelial cells of patient A infected with avian influenza A (H5N1) virus (magnification ×100).

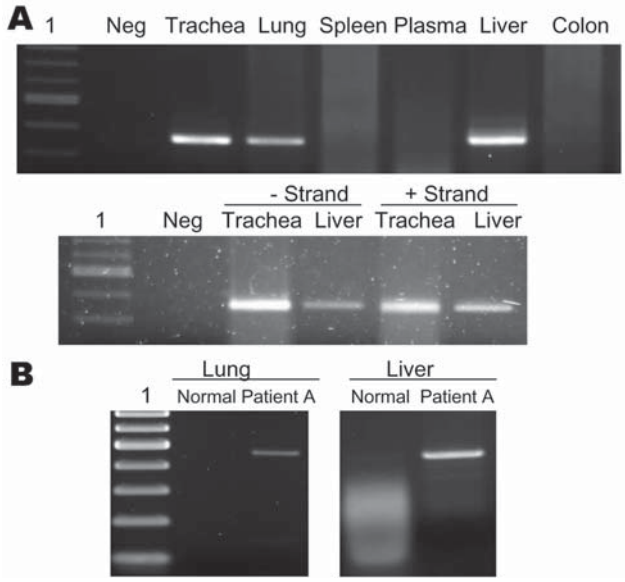

Figure 2. A) Detection of viral RNA in lung, trachea, and liver by reverse transcription–PCR (RT-PCR) (upper panel) and detection of positive- and negative-stranded viral RNA in trachea and liver by strand-specific RT-PCR (lower panel). Lane 1, 100-bp ladder; Neg, negative. B) RT-PCR showing overexpression of tumor necrosis factor-α in lung and liver tissues of patient in A compared with normal tissues.

phases of the disease course between the 2 patients. The finding also indicated that viral replication was maintained in the lung throughout the course of the disease. This was in agreement with the finding in both patients that viral antigen was detectable by immunohistochemical analysis only in alveolar epithelial cells of the lungs, which indicated that this cell type is the major target cell in humans.

Apoptosis

Apoptosis was frequently observed in hyperproliferating alveolar epithelial cells (Figure 3A) in lung tissues from patient B. In patient A, apoptosis was prominent in leukocytes that infiltrated the lung, but apoptotic alveolar epithelial cells were less frequent partly because epithelial cells were mostly absent, which left alveolar surface denuded (Figure 3B). We have previously shown that TNF-α mRNA was upregulated in the lung of patient B (*3*), which may be 1 of the mechanisms leading to apoptosis. Similarly, we tested the lung of patient A and found upregulation of TNF-α mRNA. We also tested trachea, liver, spleen, colon, and bone marrow tissues of this patient; TNF-α mRNA was detectable only in the liver (Figure 2B).

To compare these findings with those of viral pneumonia caused by human influenza virus, we searched archival pathologic specimens for lung tissues with histopathologic findings compatible with viral pneumonia. The specimens were then tested for influenza A virus by RT-PCR. A virus-positive specimen was stained for apoptosis. This lung specimen was from a 47-year-old man who sought medical attention because of fever and hemoptysis. A lung biopsy was performed to investigate the cause of hemoptysis. Pathologic examination showed bronchiectasis and interstitial pneumonia. The patient responded to supportive care and recovered. In comparison with lungs infected with influenza (H5N1) virus, no apoptotic alveolar epithelial cells were detected in this lung tissue that contained human influenza virus. However, apoptotic leukocytes infiltrating the lung were as prominent as in patient A (Figure 3C).

Apoptotic lymphocytes were abundant in the red pulp and occasionally observed in the white pulp (Figure 3D) of the spleens of both patients. In contrast, a normal spleen specimen showed only a minimal number of apoptotic cells (Figure 3E). We also observed apoptotic cells in intestinal epithelial cells of patients A and B. Liver samples from patient A did not show large numbers of apoptotic cells despite the presence of replicating viral RNA and TNF-α mRNA. The lack of apoptosis in the liver and other organs and cell types indicated that the observed apoptotic cells in lungs, spleens, and intestines were specific to the pathologic process and not due to postmortem changes.

Because leukopenia and thrombocytopenia are prominent clinical features of infection with influenza (H5N1) virus (*15*), we investigated whether bone marrow failure plays a role in addition to increased destruction by apoptosis of leukocytes in the lung. We stained bone marrow samples from both patients for a proliferation marker, Ki-67, and an apoptotic marker. These samples showed normal levels of Ki-67+–proliferating cells when compared with normal bone marrow samples. The number of apoptotic cells in bone marrow did not increase. This finding suggested that bone marrow may maintain normal function during infection with influenza (H5N1) virus and is likely not responsible for the leukopenia and thrombocytopenia.

Discussion

Loss of alveolar epithelial is probably 1 of the pathogenic mechanisms of pneumonia caused by influenza (H5N1) virus, and apoptosis is at least partly responsible. The hyperproliferation of pneumocytes observed in patient B was likely the regenerative process in the late phase of the disease. Whether the apoptosis of alveolar epithelial cells was a direct result of infection in those cells or an indirect consequence caused by cytokine dysregulation is not yet clear. Expression of influenza viral genes has been shown to induce apoptosis in infected cells (*8–13*). These viral genes likely play a role in induction of apoptosis. However, the role of cytokines in apoptosis in pneumocytes cannot be excluded.

Lymphopenia has been shown to be a predictive marker for acute respiratory distress syndrome and death (*15*).

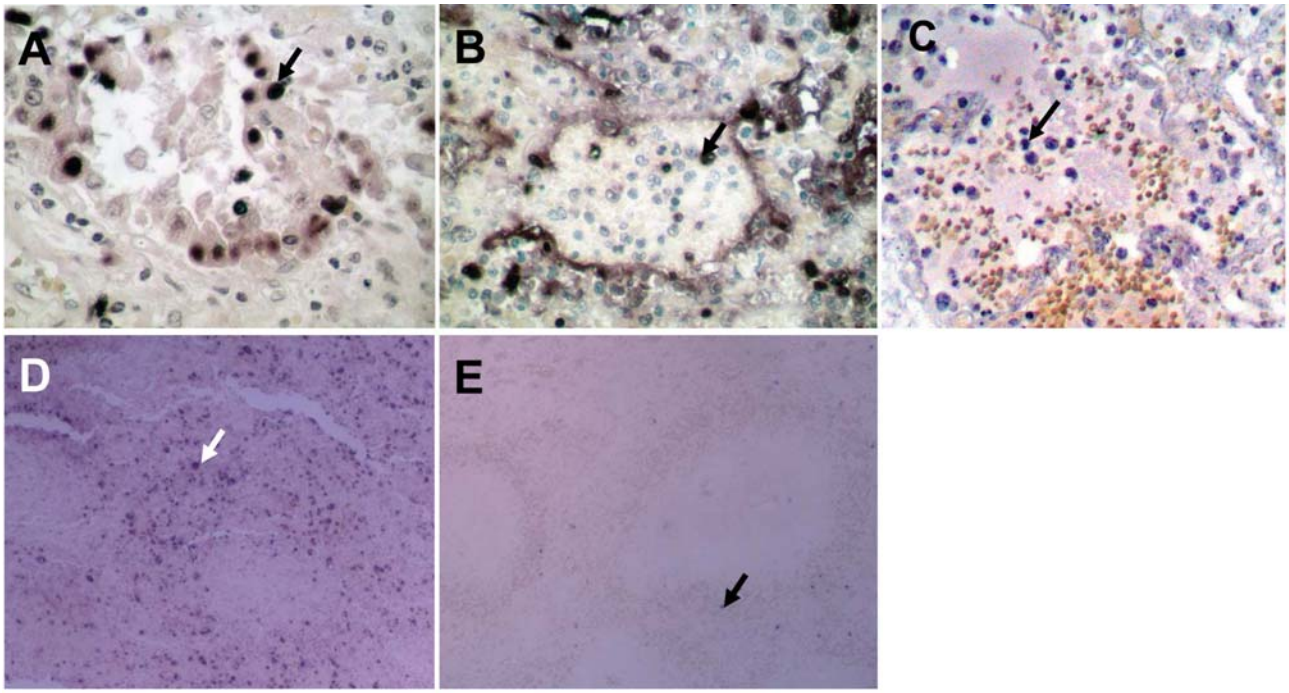

Figure 3. Terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end-labeling staining showing numerous apoptotic alveolar epithelial cells in lung of patient B (A) and leukocytes in lung of patient A (B). C) Lung tissue from a patient with pneumonia caused by human influenza A (H5N1) virus showing apoptosis only in leukocytes. D) Spleen of patient B showing numerous apoptotic cells. E) Normal spleen tissue showing only a minimal level of apoptosis. Apoptotic cells are stained dark blue, and an apoptotic cell in each panel is indicated by an arrow. Magnification ×400 in A, B, and C; ×100 in D and E.

Infection of primary human lymphocytes in vitro has been shown to induce apoptosis (*7*). Increased apoptosis leading to severe lymphopenia was likely evidence of more active viral replication and higher viral load. Because we did not find evidence of viral replication in the spleen of patient A, which contained many apoptotic cells, lymphocyte apoptosis may not be a direct consequence of infection in these cells. Apoptosis of lymphocytes may have been caused indirectly by cytokine dysregulation and overactivation of the immune response.

Because we also found numerous apoptotic leukocytes in lungs of a person infected with influenza virus and leukopenia is not a prominent clinical feature of human influenza, apoptosis of infiltrating leukocytes in inflamed tissue alone may not be sufficient to cause leukopenia. Conversely, systemic cytokine dysregulation during infection with influenza (H5N1) virus may cause massive apoptosis in lymphoid organs, which leads to lymphopenia. Another possible explanation is that apoptosis was induced locally while lymphocytes were circulating through the infected lung, Determining if apoptotic lymphocytes can be directly detected in patients' blood and can be a predictive marker for disease outcome requires further studies.

Apoptosis could not be detected in the liver despite the presence of viral RNA and TNF-α mRNA. The liver did not show severe inflammation as observed in the lung, which suggests that without apoptosis viral infection would not cause severe tissue damage. This finding underscores the role of apoptosis in viral pathogenesis. Conversely, absence of apoptotic cells in the liver may indicate that viral replication in this organ was not sufficient to induce apoptosis and pathologic changes. The absence of apoptosis in the liver, despite the presence of TNF-α mRNA, suggests that expression of TNF-α alone may not be the major mechanism responsible for induction of apoptosis in pathogenesis of influenza virus.

We did not detect viral antigen in other organs, despite the presence of viral RNA. This finding suggested that although other cell types were permissive for replication of viral RNA, this replication in these cells was inefficient. This is in contrast to the widespread presence of viral antigen in animal tissues and probably reflects the interspecies barrier and incomplete adaptation of influenza (H5N1) virus to the human host. Although a recent reported showed that upper airway epithelium of humans lacks the α-2,3–linked sialic acid receptor for avian influenza virus (*16*), other in vitro data suggest that α-2,3–linked sialic acid is expressed in ciliated columnar epithelial cells of the airway (*17*,*18*). Recent in vitro experiments showed that the α-2,3–linked sialic acid receptor specific for avian influenza virus replicates and spreads poorly in cultured differentiated human tracheobronchial epithelial cells (*19*,*20*). This finding is

consistent with our data, which showed no detectable viral antigen in the trachea and probably reflects inefficient infection of influenza (H5N1) virus in this tissue.

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Genome Analysis Linking Recent European and African Influenza (H5N1) Viruses

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To better understand the ecology and epidemiology of the highly pathogenic avian influenza virus in its transcontinental spread, we sequenced and analyzed the complete genomes of 36 recent influenza A (H5N1) viruses collected from birds in Europe, northern Africa, and southeastern Asia. These sequences, among the first complete genomes of influenza (H5N1) viruses outside Asia, clearly depict the lineages now infecting wild and domestic birds in Europe and Africa and show the relationships among these isolates and other strains affecting both birds and humans. The isolates fall into 3 distinct lineages, 1 of which contains all known non-Asian isolates. This new Euro-African lineage, which was the cause of several recent (2006) fatal human infections in Egypt and Iraq, has been introduced at least 3 times into the European-African region and has split into 3 distinct, independently evolving sublineages. One isolate provides evidence that 2 of these sublineages have recently reassorted.

The first cases of human infection with highly pathogenic avian influenza (HPAI) strain H5N1 occurred in Hong Kong in 1997; it was brought under control by massive culling of the chicken population (*1*,*2*). An antigenically distinct strain emerged in 2002, in the same location, and has since spread to hundreds of millions of birds (*3*,*4*). More alarming has been the growing number of human influenza (H5N1) infections; by September 2006, 251 human cases had been reported, resulting in 148 deaths (*2*). From late 2005 to early 2006, HPAI (H5N1) was detected for the first time in birds in eastern Europe, the Middle East, and northern Africa, indications that the virus was spreading, possibly aided by wild bird migration. Human cases were reported beginning in January 2006 in Egypt, Iraq, Turkey, Djibouti, and Azerbaijan.

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Methods

We sequenced and analyzed the genomes of 36 recent isolates of highly pathogenic influenza (H5N1) viruses collected from Europe, northern Africa, the Middle East, and Asia. We used high-throughput methods described previously (5).

Sample Collection

Samples primarily consisting of pooled trachea and lung tissue, pooled intestines, or tracheal and cloacal swabs collected from dead or moribund animals were processed for attempted virus isolation as described (*6*). Hemagglutinating isolates were typed either by reverse transcription–PCR (RT-PCR) or by serologic methods (*7*). RNA was extracted with the High Pure Extraction Kit (Roche, Indianapolis, IN, USA), according to manufacturer's instructions.

Primer Design

Sequences from recent human and avian influenza (H5N1) isolates were downloaded from GenBank and were aligned with MUSCLE (*8*). Degenerate primers were designed on the basis of consensus sequences generated with

BioEdit (*9*). An M13 sequence tag was added to the 5′ end of each primer to be used for sequencing. Four of the reactions were analyzed by electrophoresis on an agarose gel for quality control purposes. Primer design was optimized by analysis of the sequence success rate of each primer pair. Primers that did not perform well were redesigned and replaced in the primer set. Primers were designed to produce ≈500-nt overlapping amplicons to provide 2× coverage of each genomic segment. Additionally, a second set of primers was designed to produce 500-nt amplicons offset ≈250 nt from the original primer pair, which gave at least 4× sequence coverage of each segment.

cDNA Synthesis

Amplicons tiling the genome of the influenza isolates were generated with a OneStep RT-PCR kit (QIAGEN, Valencia, CA, USA). They were treated with shrimp alkaline phosphatase-exonuclease I (U.S. Biologicals, Swampscott, MA, USA) before sequencing.

Sequencing and Assembly

Sequencing reactions were performed as described previously (*5*). After sequencing, each segment was downloaded, trimmed to remove amplicon primer-linker sequence as well as low-quality sequence, and assembled. A small genome assembler called Elvira, based on the open-source Minimus assembler (http://cbcb.umd.edu/software), has been developed to automate these tasks. The Elvira pipeline delivers exceptions, including failed reads, failed amplicons, insufficient coverage of a reference sequence (as obtained from GenBank), ambiguous consensus sequence calls, and low-coverage areas. Additional sequencing and targeted RT-PCR were conducted to close gaps and to increase coverage in low-coverage or ambiguous regions.

All sequence data used in this study are available from GenBank and also from ftp.cbcb.umd.edu/pub/data/flu. GenBank accession numbers are available in the supplementary data (online Technical Appendix 1, available from www.cdc.gov/EID/content/13/5/713-app1.txt).

Phylogenetic Analysis

Multiple sequence alignments of nucleotide data were performed by using MUSCLE (*8*) with default parameters. Most alignments of segments within a subtype lack internal gaps. Leading and trailing gaps were not considered in tree-length calculations, but all nucleotide positions were considered.

The phylogenetic trees for Figures 1, 2A, and online Appendix Figures 1–3 (available from www.cdc.gov/EID/content/13/5/713-appG1.htm, www.cdc.gov/EID/content/13/5/713-appG2.htm, and www.cdc.gov/EID/content/13/5/713-appG3.htm) were constructed by using the neighbor-joining method as implemented in PAUP* version 4.0b10 (*10*,*11*) using the F84 distance between nucleotide sequences and the default parameters. The phylogeny of 71 complete genomes (avian isolates) and 3 hemagglutinin (HA) sequences (human isolates) in Figure 2B comprises isolates chosen because they formed the European-Middle Eastern-African (EMA) clades and the Russian and Chinese sister clades in a larger analysis of 759 influenza (H5N1) isolates from the locales and host range of all H5N1 sequences published since 1996. The figure includes every member of the EMA clade for which the complete genome sequence is currently available, except chicken/Nigeria/1047–62/2006 and chicken/Kurgan/05/2005, which appear to be reassortants.

To find optimal phylogenetic trees for Figure 2B, we used a combination of tree search algorithms available in the "new technology" heuristic strategies in the TNT (*12*) software package (available from www.zmuc.dk/public/phylogeny/TNT). These strategies include a successive combination of hill-climbing techniques (branch swapping) followed by simulated annealing (ratcheting), divide-and-conquer (sectorial searches), and genetic algorithms (tree fusion). Figure 2B depicts a strict consensus based on 286 minimal-length trees resulting from a parsimony search of 1,000 replicates in TNT under the command "xmult = lev5." Each component tree had a tree length of 1,613 steps. Gaps were treated as a fifth state, and all edit costs

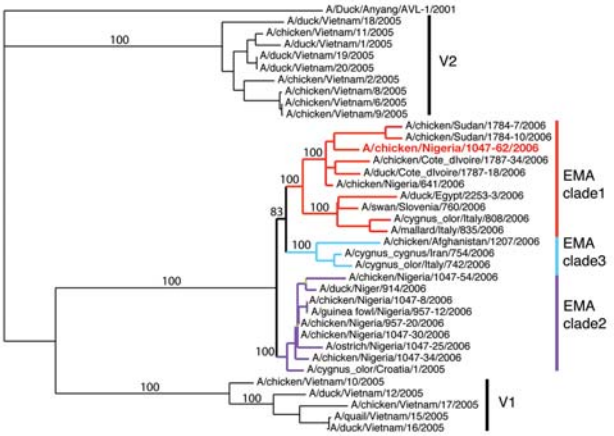

Figure 1. Phylogenetic tree of hemagglutinin (HA) segments from 36 avian influenza samples. A 2001 strain (A/duck/Anyang/AVL-1/2001) is used as an outgroup at top. Clade V1 comprises the 5 Vietnamese isolates at the bottom of the tree, and clade V2 comprises the 9 Vietnamese isolates near the top of the tree. The European-Middle Eastern-African (EMA) clade contains the remaining 22 isolates sequenced in this study; the 3 subclades are indicated by red, blue, and purple lines. The reassortant strain, A/chicken/Nigeria/1047–62/2006, is highlighted in red. Note that 4 segments including HA from this reassortant fall in EMA-1; the other 4 fall in EMA-2, as shown in online Appendix Figure 1. Bootstrap values supporting the 3 distinct EMA clades are taken from a consensus tree based on concatenated whole-genome sequences, excluding the reassortant strain. The consensus tree is provided as online Appendix Figure 2.

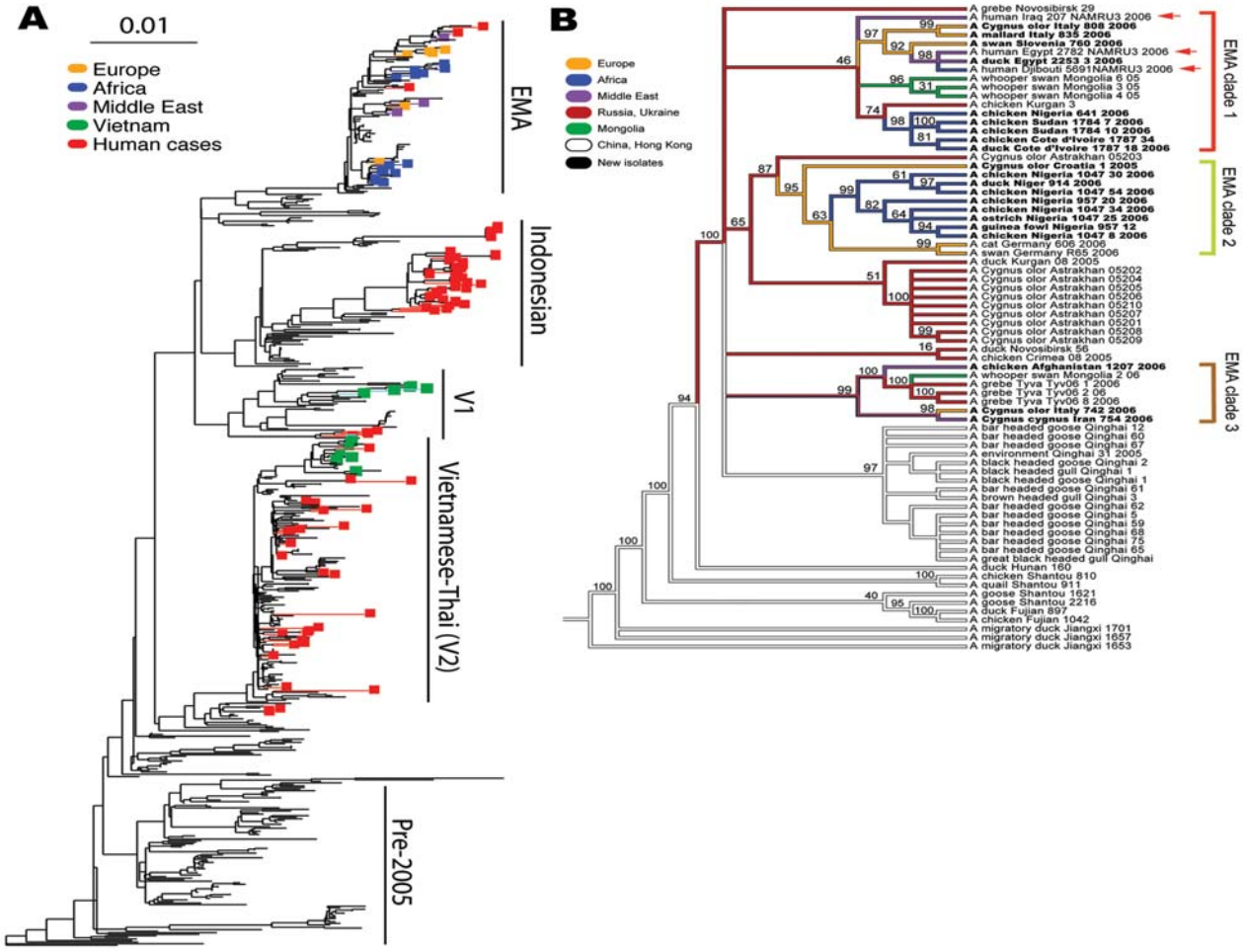

Figure 2. A) Phylogenetic tree relating the influenza A (H5N1) hemagglutinin (HA) segments of 589 avian, feline, and human viruses. The tree includes all HA segments isolated since 2000 from humans (82 isolates, minimum sequence length 1,000 nt), birds (503 isolates, minimum length 1500 nt), and cats (4 isolates). The 36 newly sequenced genomes are highlighted in color. Human cases, which occur in all 4 of the major influenza (H5N1) clades, are highlighted in red. The scale bar indicates an F84 distance of 0.01. A full-scale version of this tree is provided as online Appendix Figure 3. B) Phylogeny of 71 complete genomes (avian isolates, all 8 segments concatenated) and 3 HA sequences (human isolates, marked with red arrows) from Europe, the Middle East, Africa, Russia, and Asia. Bootstrap values represent the percentage of 1,000 bootstrap replicates for which the partition implied by the edge was observed; see Methods for further details. The 3 European-Middle Eastern-African (EMA) subclades from Figure 1 are indicated with the same color scheme. Isolates from human hosts are found only in EMA-1. Colors indicate locales. The names of the isolates newly sequenced in this study are shown in boldface text.

were given equal weights under the parsimony criterion. The heuristic tree strategy was run until a stable strict consensus was achieved. This strict consensus is a conservative estimate of the phylogenetic relationship between the isolates, where an edge is included only if it was observed in all 286 optimal trees. Separately, RAxML (*13*) was run over the same data for maximum likelihood analyses under the general time-reversible (GTR) mixed model of nucleotide substitution. This likelihood analysis produced a tree with the same clade contents as the parsimony tree, preserving the 3 EMA clades. Branches were traced with colors to represent the locale of isolation of the virus.

Results and Discussion

The 36 new isolates reported here greatly expand the amount of whole-genome sequence data available from recent avian influenza (H5N1) isolates. Before our project, GenBank contained only 5 other complete genomes from Europe for the 2004–2006 period, and it contained no whole genomes from the Middle East or northern Africa. Our analysis showed several new findings. First, all European, Middle Eastern, and African samples fall into a clade that is distinct from other contemporary Asian clades, all of which share common ancestry with the original 1997 Hong Kong strain. Phylogenetic trees built on each of the

8 segments show a consistent picture of 3 lineages, as illustrated by the HA tree shown in Figure 1. Two of the clades contain exclusively Vietnamese isolates; the smaller of these, with 5 isolates, we label V1; the larger clade, with 9 isolates, is V2. The remaining 22 isolates all fall into a third, clearly distinct clade, labeled EMA, which comprises samples from Europe, the Middle East, and Africa. Trees for the other 7 segments display a similar topology, with clades V1, V2, and EMA clearly separated in each case. Analyses of all available complete influenza (H5N1) genomes and of 589 HA sequences placed the EMA clade as distinct from the major clades circulating in People's Republic of China, Indonesia, and Southeast Asia.

The influenza (H5N1) viruses isolated in Europe, the Middle East, and Africa show a close relationship, despite the fact that they were collected from a widely dispersed geographic region, including Côte d'Ivoire, Nigeria, Niger, Sudan, Egypt, Afghanistan, Iran, Slovenia, Croatia, and Italy. The shared lineage of the viruses suggests a single genetic source for introduction of influenza (H5N1) into western Europe and northern and western Africa; our analysis places this source most recently in either Russia or Qinghai Province in China (Figure 2B; online Appendix Table [available from www.cdc.gov/EID/content/13/5/713-appT.htm]). The broad dispersal of these isolates throughout these countries during a relatively short period, coupled with weak biosecurity standards in place in most rural areas, implicates human-related movement of live poultry and poultry commodities as the source of introduction of influenza (H5N1) into some of these countries. The virus' presence in wild birds leaves open the alternative possibility that migratory birds may have been the primary source, with secondary spread possibly caused by human-related activities.

A phylogenetic tree containing 589 isolates from 2001 through 2006 (Figure 2A and online Appendix Figure 3) shows the relationship of the 36 recent isolates from this study to previous isolates and shows the 3 major lineages of influenza (H5N1) that are now circulating in Asia plus the fourth lineage, EMA, that has spread west into Europe and Africa. Figure 2B depicts a consensus view of the parsimony-based analysis of 74 isolates of complete genomes from the EMA lineage. The EMA clade contains all known European, Middle Eastern, and North African cases (which began appearing in late 2005), as well as cases from China, Russia, and Mongolia in 2005 and 2006. Some of the EMA clade isolates appear in clusters of influenza (H5N1) infection that were reported in geese in Qinghai Province, China (*14*), and in mute swans in Astrakhan (*15*), both of which are possible sources of spread through migration.

The evolutionary relationships shown in Figure 2B provide clear evidence that 3 distinct clades, labeled EMA 1–3, are circulating in the European and African region. These clades clearly share a common ancestor in Asia. The 3 clades may represent separate introductions or, alternatively, a single introduction from Asia into Russia, Europe, or another western site that has subsequently evolved into 3 lineages. More data will be required to pinpoint when and where the 3 clades split apart. All previously reported European and Middle Eastern isolates belong to EMA-1.

Our results show that EMA-2 has spread to Europe and that EMA-3 has spread to both Europe and the Middle East. These results agree in part with a recent study (*16*) that reported 3 distinct introductions of influenza (H5N1) into Nigeria. Our analysis, based on all available HA sequences (online Appendix Figure 3), indicates that the Nigerian isolates fall into just 2 clades, EMA 1–2, that likely resulted from at least 2 introductions of influenza (H5N1).

European countries have been affected by each of the 3 introductions of the EMA strains. For example, the Italian sequences can be segregated into 2 subgroups (Figure 2B). Two isolates in EMA-1 (Co/Italy/808/06 and Md/Italy/835/2006) are closely related in all segments and likely share a common ancestor with isolates found in Slovenia (Sw/Slovenia/760/2006), Bavaria, and the Czech Republic (Co/Czech Republic/5170/2006). The third Italian strain from our study (Co/Italy/742/2006) falls into EMA-3, along with our newly sequenced isolates from Iran (Co/Iran/754/2006) and Afghanistan (Ck/Afghanistan/1207/2006). EMA-2 contains 1 European isolate, from a swan in Croatia, and multiple isolates from domesticated birds in Nigeria and Niger. This group shares a common ancestor with a group of isolates from Astrakhan and Kurgan (Russia).

Of the 22 EMA isolates newly sequenced in this study, 20 have the amino acid lysine (K) at position 627 of the polymerase basic protein 2 (PB2), while only 2 have glutamic acid (E). (These last 2 are both from Italy and both in EMA-1.) The 627K mutation is associated with virulence in mice and adaptation to mammalian hosts (*17*) and with increased host range (*18*). Lysine at this position is common in human viruses: all 65 human influenza (H5N1) isolates from 2001 through 2006 for which the PB2 sequence is available have lysine at position 627. Before the analysis of our collection, the PB2 627K was a relatively rare finding in avian influenza (H5N1) viruses: it was present in only 42 of 385 isolates previously collected from 2001 through 2006. Our analysis shows that all 42 of these fall in the EMA clade (Figure 2 and supplementary data available in online Technical Appendix 2, available from www.cdc.gov/EID/content/13/5/713-app2.txt). Excluding our current European, Middle Eastern, and African isolates, this mutation appears primarily in isolates obtained from wild birds in Astrakhan (*15*) and at Qinghai Lake (*14*,*17*). This mutation also occurs in the recent isolate A/Guinea fowl/Shantou/1341/2006 and in a mouse-adapted 2001 Asian isolate, A/pheasant/Hong Kong/Fy155/01-MB. This find-

ing is in keeping with current knowledge of the acquisition of such mutations.

Our study increases current knowledge on strains circulating in Asia before the westward spread of influenza A (H5N1). The Vietnamese samples fall into 2 clusters, the larger of which (V2 in Figure 1) is the same strain responsible for multiple cases in Southeast Asia since 2004, particularly in Vietnam and Thailand. These isolates all seem to derive from earlier Hong Kong samples (including 2 cases of human infection) in 2002 and 2003. The second cluster, V1, which contains 5 samples, significantly expands our understanding of this distinct Vietnamese influenza (H5N1) lineage. The only other isolate from this cluster was recently reported in a Vietnamese duck (A/duck/Vietnam/568/2005) and labeled a "recent Vietnam introduction" (*4*). This sample groups with the V1 clade when shown in the context of a larger tree of HA sequences (online Appendix Figure 3). The 5 newly sequenced isolates in clade V1 show the same phylogenetic relationship for all segments except PB2 (online Appendix Figure 1). The isolates in clade V1 appear to have undergone the same reassortment as was suggested (*4*) for the 1 previous example of this Vietnamese clade, A/duck/Vietnam/568/2005; i.e., they have acquired a new PB2 segment. This PB2 is similar to older (1996–2002), A/duck/Guangdong/1/96-like viruses from China. V1 clade isolates are associated with a distinct set of human cases, from China's Anhui and Guangxi Provinces in 2005, a finding that provides additional support to the hypothesis that this group of influenza (H5N1) viruses was introduced into Vietnam from China (*4*).

Although EMA has split into 3 independently evolving clades, 1 isolate, A/chicken/Nigeria/1047–62/2006, shows clear evidence of reassortment. In this genome, 4 segments—HA, (nucleocapsid protein, nonstructural protein, and PB1—belong to EMA-1, as seen in Figure 1 and online Appendix Figure 1. The other 4 segments—neuraminidase, matrix protein, PA, and PB2—belong to EMA-2 (online Appendix Figure 1). Individual segment trees based on all available sequences in GenBank corroborate this pattern and consistently split the 8 segments of this Nigerian isolate into 2 distinct clades. Reassortment events such as this can only be discovered by sequencing multiple virus segments.

The presence of all 3 EMA sublineages in the same geographic region creates ample opportunities for reassortment. Isolate A/chicken/Nigeria/1047–62/2006 is the most recent of the Nigerian isolates, consistent with the hypothesis that this reassortant was generated in Africa. Additional surveillance will be necessary to determine if this reassortant strain spreads further in the avian population and to assess its ability to infect mammals.

As shown in Figure 2A, the EMA clade is a distinct lineage evolving independently of the 3 exclusively Asian lineages. All 3 human influenza (H5N1) cases that have been sequenced outside east Asia—from Iraq (*19*), Djibouti, and Egypt—belong to the EMA lineage. The human sequences A/Djibouti/5691/NAMRU3/06 and A/Egypt/2782/NAMRU3/06 group closely together and consistently fall in EMA-1. The placement of A/Iraq/207/NAMRU3/06 is slightly less certain; it also groups with EMA-1 (Figure 2B) but with lower bootstrap support. EMA viruses isolated from humans are thus quite distinct from the recent large clusters of human cases in Indonesia and China, which fall into separate clades containing none of our samples. The EMA isolates are also distinct from other human cases in Southeast Asia, which fall into the clades (V1 and V2) containing our Vietnamese samples.

The emergence of 3 (or more) substrains from the EMA clade represents multiple new opportunities for avian influenza (H5N1) to evolve into a human pandemic strain. In contrast to strains circulating in Southeast Asia, EMA viruses are derived from a progenitor that has the PB2 627K mutation. These viruses are expected to have enhanced replication characteristics in mammals, and indeed the spread of EMA has coincided with the rapid appearance of cases in mammals—including humans in Turkey, Egypt, Iraq, and Djibouti, and cats in Germany, Austria, and Iraq. Unfortunately, the EMA-type viruses appear to be as virulent as the exclusively Asian strains: of 34 human infections outside of Asia through mid-2006, 15 have been fatal (2).

Analyses of the complete HA tree (Figure 2A and online Appendix Figure 3) suggest that the earliest sequenced relatives of the EMA clade are from the Yunnan region of China (A/duck/Yunnan/6255,6445/2003), Hong Kong, (A/chicken/Hong Kong/WF157/2003), and South Korea (A/chicken/Korea/ES/2003, A/duck/Korea/ESD1/2003), which were part of a regional outbreak in 2003 (*20*). Experiments on the 2 Korean isolates showed them to be infectious but not fatal in mice (*21*).

These findings show how whole-genome analysis of influenza (H5N1) viruses is instrumental to the better understanding of the evolution and epidemiology of this infection, which is now present in the 3 continents that contain most of the world's population. This and related analyses, facilitated by global initiatives on sharing influenza data (*22*,*23*), will help us understand the dynamics of infection between wild and domesticated bird populations, which in turn should promote the development of control and prevention strategies.

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Pet Rodents and Fatal Lymphocytic Choriomeningitis in Transplant Patients

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In April 2005, 4 transplant recipients became ill after receiving organs infected with lymphocytic choriomeningitis virus (LCMV); 3 subsequently died. All organs came from a donor who had been exposed to a hamster infected with LCMV. The hamster was traced back through a Rhode Island pet store to a distribution center in Ohio, and more LCMV-infected hamsters were discovered in both. Rodents from the Ohio facility and its parent facility in Arkansas were tested for the same LCMV strain as the 1 involved in the transplant-associated deaths. Phylogenetic analysis of virus sequences linked the rodents from the Ohio facility to the Rhode Island pet store, the index hamster, and the transplant recipients. This report details the animal traceback and the supporting laboratory investigations.

Lymphocytic choriomeningitis virus (LCMV) is a rodentborne arenavirus endemic in house mice (*Mus musculus*) worldwide (*1–3*). Lymphocytic choriomeningitis (LCM) in immunocompetent persons usually is a mild, self-limited, viral syndrome or is asymptomatic; aseptic meningitis also can occur, but the infection is rarely fatal (*4–6*). In immunocompromised persons, LCM may result in serious systemic infections and death. LCM during pregnancy can cause spontaneous abortion or severe birth defects, including hydrocephalus, chorioretinitis, blindness, or psychomotor retardation (*7*). Congenital LCMV infection is likely greatly underreported as a cause of poor pregnancy outcomes (*7*). Human infection occurs most commonly through exposure (by direct contact or inhalation of infectious aerosol) to secretions or excretions of infected animals (*8*). To our knowledge, person-to-person transmission has not been reported, except for transmission from mother to fetus (*7*) and 1 previous cluster in December 2003 of infection through organ transplantation (*9*,*10*).

In early April 2005, 4 recipients of solid-organ transplants in 3 hospitals in Rhode Island and Massachusetts became gravely ill shortly after transplantation; 3 subsequently died (*10*). All 4 recipients shared a common donor. Tissue and blood samples from the donor and recipients were sent from the Rhode Island Department of Health and the Massachusetts Department of Public Health to the Centers for Disease Control and Prevention (CDC), where LCMV was identified as the etiologic agent (*10*). Viral sequences from the organ recipients were identical to those from a pet hamster acquired by the donor's household 17 days before organ donation (*10*). Here we report the results of an epidemiologic and environmental investigation to identify the origin of the index hamster and the source of the virus.

Methods

Epidemiologic Investigation

Thorough epidemiologic investigations were conducted at the Rhode Island pet store where the index

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hamster was purchased, the Ohio distribution facility that supplied the pet store, and the primary breeding facility in Arkansas. These investigations focused on interviews; review of invoices, shipping records, and US Department of Agriculture inspection reports; and on-site environmental assessments.

Rodent Sample Collection

All available rodent species known to be competent hosts for LCMV (capable of becoming chronically infected and shedding virus for up to 9 months) (*6*,*11*,*12*) were collected from the remaining rodent stock at the Rhode Island pet store. These species included Syrian hamsters (*Mesocricetus auratus*), "fancy" mice (*M. musculus*), and guinea pigs (*Cavia porcellus*). Although they have not been shown to be competent reservoirs for LCMV, "fancy" rats (*Rattus norvegicus*) and gerbils (*Meriones unguiculatus*) also were sampled because of their exposure to the infected rodents. Rodents were sampled and euthanized following approved CDC Animal Care and Use Committee protocols.

With a known population size and a LCMV prevalence estimate, the hypergeometric probability distribution was used to determine the minimum sample size needed to provide a 95% chance of detecting at least 1 LCMV infected rodent at each site. The LCMV prevalence was estimated to be 4.7% in Ohio and 4.3% in Arkansas. The Ohio prevalence was based on 4 infected of 85 tested at the Rhode Island pet store; the revised prevalence for Arkansas was based on 9 of 211 positives after data from the Ohio samples were incorporated.

The population sizes (Table 1) included only dwarf hamsters and did not distinguish between the Chinese and Roborovsky dwarf hamsters (*Cricetulus curtatus* and *Phodopus roborovskii*, respectively). An agreement was reached with the owner in which ≈10% of the total population of 140 Roborovsky's dwarf hamsters was sampled. In this case, the probability of detecting at least 1 positive rodent was 36.5%.

Laboratory Investigation

The index hamster, the organ recipients, the animals from the pet store, and rodents from the Ohio and Arkansas facilities were tested for LCMV with a combination of assays that included serology, immunohistochemistry (IHC), reverse transcription–PCR (RT-PCR), TaqMan (Applied Biosystems, Foster City, CA, USA), and virus isolation. Genetic sequences obtained from the respective samples were used in the phylogenetic analysis to identify the LCMV strain and epidemiologic link leading to transplant-associated deaths.

Virus Isolation

Virus isolation was conducted by using Vero E-6 cells. For blood or serum, 100 μL of sample was used as inoculum. For tissues, a 10% cell suspension was prepared in a viral support medium (Hank's balanced salt solution with 5% heat-inactivated fetal bovine serum) and clarified by centrifugation. A100-μL aliquot of the supernatant fluid was used as the inoculum. Flasks were incubated for 1 hour, fed with maintenance medium, and observed for 2 weeks. Cells from flasks were tested for replicating LCMV by immunofluorescent antibody assay (IFA) on 1 of days 4–7 (depending on supplemental information made available through other testing) and again on day 14. If no reactivity was detected by IFA from days 4 to 7 or on day 14, the flask was considered negative for virus.

Molecular Detection of LCMV in Rodents

Highly sensitive real-time RT-PCR TaqMan assay was performed as described previously (*10*). RNA isolated from rodent blood, serum specimens, or tissue was subjected to TaqMan real-time assay, and samples with cycle threshold (Ct) values <40 were scored as LCMV-positive. TaqMan-positive specimens were further analyzed by traditional RT-PCR to produce a 232-nt product within the RNA polymerase (L) gene and sequences were obtained by using previously described primers (*10*). The sequences of LCMV from the transplant recipients, index hamster, and rodents from the Rhode Island pet store and Ohio distribution center were then compared with those obtained for other characterized LCMV strains by using GCG Version 11.1.1 (Accelrys, San Diego, CA, USA) and PAUP (Sinauer Associates Inc., Sunderland, MA, USA). Further evidence of a genetic link between LCMV detected in the rodents and the human cases investigated was obtained by analyzing the viral S RNA segment. A 611-nt S segment PCR

Table 1. Estimated population sizes and samples taken from 2 rodent distribution facilities

Location	Species	Population size	Projected prevalence, %	Sample size	Probability of detecting a positive, %
Ohio	Syrian hamsters	5,000	5.0	116	99.8
Arkansas	Fancy rats	>10,000	3.4	125	98.7
Arkansas	Fancy mice	200	3.4	75	96.5
Arkansas	Gerbils	2,500	3.4	125	98.8
Arkansas	Dwarf hamsters	3,750	3.4	113*	98.1
Arkansas	Roborovsky dwarf hamster	140	3.4	12†	36.5

*Sample size after removal of 12 Roborovsky hamsters from requested sample size.
†Sample size represents an agreed-upon portion of the total population.

product was amplified by using 1-step RT-PCR protocols with a generic primer set (*13*) capable of amplifying Old World arenaviruses including LCMV. The 1-step RT-PCR was carried out by using the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity as described by the manufacturer (Invitrogen, Carlsbad, CA, USA).

Serologic and Immunohistochemical Detection of LCMV in Rodents

An ELISA was used to evaluate serum samples collected from rodents for immunoglobulin class G (IgG) antibodies that reacted with LCMV antigens produced in Vero E-6 cells. The assay was run as described in Fischer et al. (*10*), except that a protein A/protein G conjugate (Immunopure, Pierce Biotechnology Inc., Rockford, IL, USA) was used. A subset of the samples was also tested by IFA, using infected Vero E-6 cells. Immunohistochemical tests were carried out on a variety of tissues from the index hamster (blood, adrenal gland, salivary gland, pancreas, liver, spleen, kidney, lung, heart, bone marrow, cerebrum, cerebellum, brain stem, spinal cord) as previously described by Fischer et al. (*10*).

Results

Rhode Island Traceback Investigation

Physical inspection of the pet store where the donor's hamster was purchased produced no evidence of wild rodent infestation. The store maintained live-capture traps in areas likely to harbor rodents (e.g., near feed bags); the trapping log showed no captures in the preceding 3 months.

Although other rodents had been housed in the same area of the pet store with the index hamster, detailed records were not available and these specific rodents were not identified. Invoices dated from February through March 2005 confirmed that all rodents sold at the pet store had come from the Ohio facility.

Biosecurity in the store was limited, with opportunity for interspecific and intraspecific cross-infection, particularly due to a lack of employee hand hygiene between handling of individual rodents. As a precautionary measure, all rodents were quarantined at the store and further sales were prohibited by the Rhode Island Department of Health after LCMV was identified in the organ recipients and the index hamster.

A total of 85 animals (55 hamsters, 8 guinea pigs, 10 mice, 7 gerbils, and 5 rats) were sampled from the remaining quarantined rodent stock at the Rhode Island pet store. Of these, 1 guinea pig and 2 hamsters were found positive for LCMV by several methods (Table 2). LCMV antibodies were detected in 1 hamster by IFA, but not ELISA. LCMV isolates were obtained from either blood or kidney and immunohistochemical stains were positive in at least 1 organ in each of the 3 rodents. All 3 rodents were positive for LCMV RNA with the L-gene–specific TaqMan primer/probe set. The L-gene sequences obtained from these rodents were identical to one another and differed from the index hamster and transplant recipients by only 1 nt (Figure, panel A). Further evidence confirming the presence of viruses of the same genetic lineage in this episode was gathered by RT-PCR amplification of a product from the S segment. The 611-nt S segment sequences of the index hamster and the transplant recipients were 100% identical, thereby reconfirming the previously established genetic link (*10*). In addition, the S segment sequences obtained from the 2 Rhode Island pet store hamsters were identical, and they differed by only 2 nt from the guinea pig sequence (Figure, panel B). These results indicate the same LCMV virus strain was present in the hamsters and guinea pig in the Rhode Island pet store.

Ohio Traceback Investigation

The Ohio facility served as a distribution/staging area for rodents destined for sale in the northeastern United States. Records indicated that it received most of its hamsters from its parent breeding facility in Arkansas. Both facilities, owned by the person, routinely received shipments of rodents from smaller breeders. The Arkansas facility

Table 2. Results of laboratory testing on the index hamster and traceback rodents associated with organ transplantation transmission of LCMV*

Rodent†	IFA	ELISA	IHC	RT-PCR/ TaqMan	Virus isolation	Sequence (L gene), bp
Index hamster	ND	Neg	Pos	Pos	Pos	232
Pet store hamster 1	Pos	Neg	Pos	Pos	Pos	232
Pet store hamster 2	Neg	Neg	Pos	Pos	Pos	232
Pet store guinea pig 1	ND	Neg	Pos	Pos	Pos	232
Ohio hamster 1	Pos	Neg	Pos	Pos	Pos	232
Ohio Hamster 2	Pos	Neg	Pos	Pos	Pos	NA
Ohio hamster 3	Pos	Neg	ND	Neg	Neg	NA
Ohio hamster 4	Neg	Neg	Pos	Pos	Pos	NA
Ohio hamster 5	Neg	Neg	Neg	ND	Pos	NA

*LCMV, lymphocytic choriomeningitis virus; IFA, immunofluorescent antibody assay; IHC, immunohistochemistry; RT-PCR, reverse transcription–PCR; ND, no data; Neg, negative; Pos, positive; NA, no amplification (could not get traditional PCR primers to amplify for sequencing).
†The table includes only those rodents that tested positive with ≥1 test.

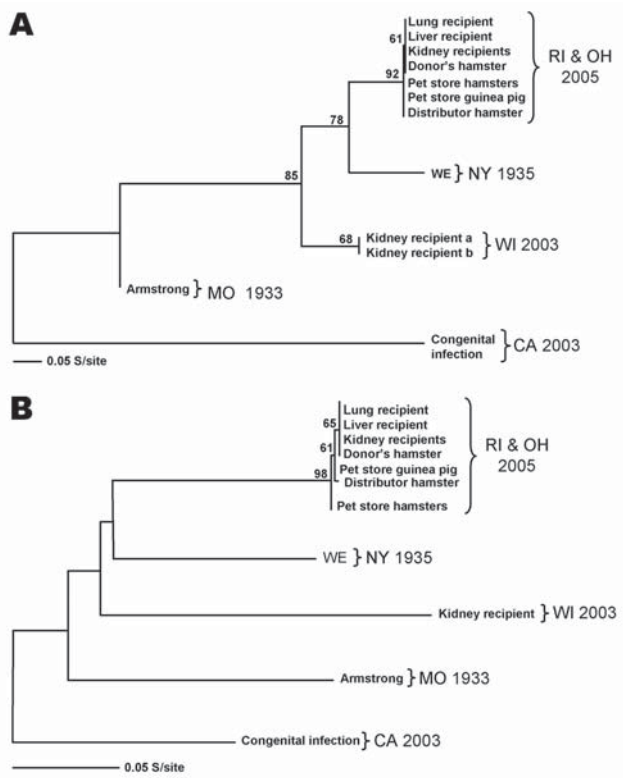

Figure. Lymphocytic choriomeningitis virus (LCMV) phylogenetic analysis of L- and S-segment sequence differences. A) Maximum likelihood analysis of a 232-nt fragment of the L segment was completed, and bootstrap numbers were generated based on analysis of 500 replicates. The graphic representation was outgrouped to the California (CA) LCMV sequence. GenBank nos. for the included sequence are as follows: Rhode Island (RI) and Ohio (OH) transplant recipients strain 200501927 (DQ182703), Rhode Island pet store and Ohio distributor rodents strain 200504261 (DQ888889), New York (NY) strain WE (AF004519), Wisconsin (WI) transplant recipients strain 810362 (DQ182706), Missouri (MO) strain Armstrong (J04331), and the CA congenital infection strain 810366 (DQ182707). B) Maximum likelihood analysis of a 611-nt fragment of the S segment NP gene was completed as mentioned above. The GenBank nos. are as follows: RI and OH transplant recipients strain 200501927 (DQ888890), RI pet store guinea pig strain 200502048 (DQ888891),OH distributor hamster strain 200504261 (DQ888893),RI pet store hamsters strain 200501966 (DQ888892), NY strain WE (M22138), WI transplant recipient strain 810362 (DQ182704), MO strain Armstrong (NC_004294), and the CA congenital infection strain 810366 (DQ182705).

was the largest distributor in North America, with sales to many states.

Breeding operations at the Ohio facility had been suspended in February 2005 by order of the United States Department of Agriculture (USDA) for multiple violations of the Animal Welfare Act (AWA) (*14*); the suspension included all rodent species other than mice and rats. Interviews and a review of prior USDA facility inspection records indicated ongoing AWA violations and poor biosecurity practices: escaped mice and wild *Mus* spp. that entered the facility were routinely captured and added to captive breeding populations; sick rodents shared common airspace with healthy rodents, attending veterinary services were sporadic, rodents were shipped to Ohio without necessary permits and veterinary inspections, and rodents from different sources shared cages for breeding purposes without adequate quarantine practices.

With the assistance of the Ohio Departments of Agriculture and Health, the Ohio facility was quarantined and inspected on July 18, 2005. During this inspection, several examples of poor biosecurity were found: escaped rodents ran free and entered other holding bins; evidence of a wild rodent infestation was found among the feed sacks; and rodents from disparate sources were housed in adjacent racks, with opportunity for large and small particle cross-contamination.

Shipping records were inadequately maintained, making it difficult to accurately account for the individual shipments of rodents. However, hamsters arriving at the Ohio facility from either Arkansas or other outside breeders were placed in tubs labeled with the location of origin and the date of arrival. According to employees at the facility, these hamsters were not mixed with other shipments.

A sample of 126 rodents (116 hamsters, 9 mice, and 1 guinea pig) was collected. This total comprised the statistically necessary 75 (taken from the general population) plus escaped, sick, and dead rodents. Of the specimens examined, 5 hamsters were positive for LCMV by at least 1 method (Table 2): 3 were positive by IFA, but not by ELISA. Three were positive by IHC; these same 3 were LCMV RNA–positive as evidenced by L-segment–specific TaqMan assay. Virus was isolated from kidney tissues of these 3 hamsters, and sequences were obtained from 1 virus isolate. L-segment sequence comparison of the Ohio specimen found exact identity to the Rhode Island pet store hamster virus (Figure, panel A). Additionally, sequences obtained from the 611-nt S segment of the Ohio hamster differed by only 3 nt from the index hamster sequence, thus showing 99.5% identity (Figure, panel B). These sequences were compared to sequences from other previously identified LCMV strains such as the laboratory strains LCMV-Armstrong and WE, and other isolates from clinical material (Table 3). Differences in L-segment sequences in the viruses from the index hamster and pet store/distribution center rodents were <0.5%, while LCMV-Armstrong and WE differed by 18.1% and 13.4%, respectively. Comparison of the S-segment sequences between the index hamster and pet store/distribution center viruses showed <1% difference. LCMV-Armstrong and WE differed by 14.1% and 14.7%, respectively, from the index hamster virus.

Table 3. Comparison of nucleotide identity differences among LCMV strains and isolates*

State/sample	1	2	3	4	5	6	7	8	9	10	11	12	13
Rhode Island													
Lung recipient 1		0.0	0.0	0.0	0.0	0.4	0.4	0.4	0.4	13.4	15.8	18.1	22.9
Liver recipient 2	0.0		0.0	0.0	0.0	0.4	0.4	0.4	0.4	13.4	15.8	18.1	22.9
Kidney recipient A 3	0.0	0.0		0.0	0.0	0.4	0.4	0.4	0.4	13.4	15.8	18.1	22.9
Kidney recipient B 4	0.0	0.0	0.0		0.0	0.4	0.4	0.4	0.4	13.4	15.8	18.1	22.9
Donor's hamster 5	0.0	0.0	0.0	0.0		0.4	0.4	0.4	0.4	13.4	15.8	18.1	22.9
Pet store hamster 1 6	0.3	0.3	0.3	0.3	0.3		0.0	0.0	0.0	12.9	15.3	18.5	23.4
Pet store hamster 2 7	0.3	0.3	0.3	0.3	0.3	0.0		0.0	0.0	12.9	15.3	18.5	23.4
Pet store guinea pig 8	0.2	0.2	0.2	0.2	0.2	0.2	0.2		0.0	12.9	15.3	18.5	23.4
Ohio													
Distributor hamster 9	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2		12.9	15.3	18.5	23.4
New York													
WE 10	12.4	12.4	12.4	12.4	12.4	12.1	12.1	12.3	12.3		16.2	18.5	24.1
Wisconsin													
Kidney recipients 11	14.4	14.4	14.4	14.4	14.4	14.4	14.4	14.2	14.4	14.6		19.5	22.5
Missouri													
Armstrong 12	14.1	14.1	14.1	14.1	14.1	13.7	13.7	13.9	14.1	15.2	13.7		21.7
California													
Congenital infection 13	14.7	14.7	14.7	14.7	14.7	14.4	14.4	14.6	14.7	13.3	14.6	14.4	

*LCMV, lymphocytic choriomeningitis virus. The percentage of differences for each pair of sequences was calculated with PAUP as uncorrected distances. Values on the upper diagonal represent differences in the L fragment (232 nt) and values on the lower diagonal represent differences on the S fragment (611 nt).

Arkansas Traceback Investigation

Several attempts were made to sample the rodents in the Arkansas facility. The initial sample was to include only Syrian hamsters and guinea pigs, but these species were destroyed by the owner when LCMV was found in the Ohio facility. Other remaining rodent species were then sampled as a proxy measure. With the assistance of the Arkansas Department of Health, 450 rodents were sampled, including 125 fancy rats, 125 gerbils, 75 fancy mice, 113 Chinese dwarf hamsters, and 12 Roborovski dwarf hamsters. One fancy rat was IgG positive for LCMV by ELISA. All other test results, including virus isolation, were negative.

Discussion

This report documents the animal traceback investigation that linked a major pet rodent distribution operation to the recent outbreak of lymphocytic choriomeningitis in 4 organ transplant recipients in Rhode Island and Massachusetts. This investigation demonstrates the ways in which classic epidemiology, laboratory diagnostics, and molecular biology can complement one another in the investigation of disease clusters. LCMV was not found in the organ donor's tissues; however, the viral isolate from the pet store hamster was sequenced and matched to the sequences of the isolates from the recipients (*10*). The near-complete sequence match between the virus found in the index hamster and the virus sequenced from the Ohio hamster indicates that the genotypes share a common lineage that is distinct from previously identified strains. It is unlikely that this genotype would be as similar to a genotype found in wild house mice in Rhode Island. Thus, the animal traceback, coupled with the molecular phylogenetic evidence, supports the hypothesis that the index hamster's infection came from the rodent distribution center in Ohio, rather than from wild *M. musculus* populations around the home of the donor or pet store.

Sequence and phylogenetic data provided strong support for the presence of the same LCMV lineage in hamsters and guinea pigs in the Rhode Island pet store and the Ohio distribution center and established the epidemiologic link of that particular lineage of LCMV to transplant-associated deaths. Comparison of LCMV genotypes obtained from this investigation with previously identified strains LCMV-Armstrong and WE and other isolates from clinical material found considerable differences (Figure, Table 3). While the differences in S-segment sequences between the index hamster and pet store/distribution center viruses were <1% (≤3-nt difference), LCMV-Armstrong and WE differed from index hamster sequences by 14.1% (76-nt difference) and 14.7% (86-nt difference), respectively. Similarly, the differences in L-segment sequences in the viruses from the index hamster and pet store/distribution center rodents were <0.5% (3-nt difference), while LCMV-Armstrong and WE differed by 18.1% and 13.4% (41 nt and 30 nt), respectively. These analyses indicate an overall close identity among LCMV strains implicated in this investigation, and wide differences from previously published sequences of strains Armstrong and WE. This molecular evidence corroborates the epidemiologic data implicating LCMV transmission within the commercial pet trade.

After the identification of LCMV in the Ohio facility, all states that had received animals from this facility in the previous 5 months were notified. Many potentially infected animals still remained in stores; their disposition was deter-

mined by individual states. Actions taken ranged from sale or adoption with an information leaflet or informed consent to issuing stop sale orders on specific rodent species from the Ohio facility.

Upon notification of the Ohio sample test results, the Ohio Department of Agriculture informed the proprietor of the LCMV contamination in his facility and requested that a written plan for decontaminating the facility and a second plan for keeping the facility LCMV-free in the future be provided. The proprietor responded by depopulating the Ohio facility. The quarantine was lifted after the building was disinfected, but the facility was never reopened.

A direct link between the Arkansas and Ohio facilities was established by the discovery of the marked tubs in Ohio. This enabled the Arkansas Departments of Health and Agriculture (Livestock and Poultry) to issue a Joint Quarantine and Inspection Order. However, several days after depopulating the Ohio facility, the proprietor also destroyed all the Syrian hamsters and guinea pigs at the Arkansas facility. Although efforts were made to sample the remaining rodents at the Arkansas facility in an attempt to pinpoint the source of the virus found in Ohio, the owner refused to allow access to the rodents on several occasions and >4 months elapsed between the initial and the ultimately successful attempts to conduct sampling. Virus isolation on all samples was unsuccessful.

Several factors may have contributed to the lack of virus in the Arkansas breeding facility, including the following: 1) the virus was never there, 2) the destruction of the Syrian hamsters and guinea pigs eliminated the virus from the facility, 3) the elapsed time allowed for the removal of infected animals and subsequent decontamination of the facility, and 4) a complete replacement of the rodent stock was accomplished within the facility. Although the facility was under quarantine for 3 of the 4 months between the first and last attempts at sampling, only sporadic surveillance of the facility was carried out by a governmental authority. Elimination of the virus from the population did not likely occur naturally because LCMV can chronically infect mice and will lead to persistent colonial transmission (*6*,*11*).

Since the time of our investigation, the proprietor's license has been suspended by the USDA for 5 years for violations of the AWA unrelated to this investigation. The Rhode Island pet retailer who sold the index hamster reportedly ceased business relations with the distributor shortly after the infection was linked to his facilities.

To our knowledge, the Rhode Island outbreak represents the first documented case of fatal LCMV infection involving a pet animal (*10*). In the previous cluster of transplant-related LCMV deaths, no rodent exposure was identified (*9*). Several rodent species that are sold as pets, including hamsters, mice, and guinea pigs, can be incidental hosts of LCMV. These species become infected through contact with infected wild mice, and can pass the infection to humans. Most human LCMV infections are associated with exposure to wild house mice (*6*,*15*); however, several outbreaks have been attributed to laboratory and pet mice and hamsters (*5*,*6*,*16*,*17*). One example is the 1974 outbreak associated with pet hamsters sold by a single distributor. A total of 181 symptomatic cases (46 requiring hospitalization) in persons with hamster contact were identified in 12 states; no deaths occurred (*5*). The outbreak was brought under control by voluntary cessation of sale and destruction of the infected breeding stock.

Prevention

LCMV surveillance should be a primary concern in the pet rodent industry to avoid entry of this virus into pet trade populations. Because of the ubiquitous distribution of the house mouse, eliminating the natural reservoir of LCMV is not practical. Steps can be taken, however, to exclude wild house mice from homes and businesses. Immunocompromised persons and pregnant women should be advised to avoid close contact with all rodents and infested areas. Educational materials should address the risk from exposure to wild mice as well as pet rodents. The virus is not naturally present in pet rodent species and the ease of transmission of the virus from pet rodents to humans may be greater than from wild mice when one considers the nature of the relationship between pet rodents and their owners (i.e., close physical contact). Therefore, every effort should be made to eliminate the virus from pet populations when it is discovered.

LCMV is already actively excluded from laboratory rodent populations, because the infection can be an occupational hazard to laboratory workers who work around infected rodents (*5*), and because inapparent infection can interfere with experimental results in rodent studies (*18*). Economic considerations may prohibit such rigorous biosecurity measures like those used for laboratory animals; however, sentinel surveillance (*19*), adequate veterinary care, exclusion of wild rodents (*20*), and good infection control practices can substantially reduce the opportunity for introduction and spread of LCMV and other pet rodent pathogens in commercial pet populations. Efforts to increase such practices within the pet trade are under way. Adherence to regulations that are already in place for obtaining permits and veterinary inspection of commercial rodent populations can also reduce the likelihood of infection and improve animal welfare.

Further efforts to reduce risk for LCM in pet owners are ongoing. Education is critical in preventing LCM and other pet-related infections. Potential pet owners should choose pets appropriate to their household (*21*). Pregnant women and immunocompromised persons should avoid pet rodents altogether (*22*). Additionally, pet owners should be advised

of the possibility of acquiring zoonotic diseases from any pet and of precautions that should be taken to prevent acquiring pet-related infections such as LCM, tularemia (*23*), salmonellosis (*24*), and others. Persons can reduce risk for infection from pet rodents by being attentive to proper hand hygiene and environmental cleaning. Additional information on LCMV is available from the CDC website (*25*).

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International Spread of Multidrug-resistant *Salmonella* Schwarzengrund in Food Products

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We compared 581 *Salmonella enterica* serotype Schwarzengrund isolates from persons, food, and food animals in Denmark, Thailand, and the United States by antimicrobial drug susceptibility and pulsed-field gel electrophoresis (PFGE) typing. Resistance, including resistance to nalidixic acid, was frequent among isolates from persons and chickens in Thailand, persons in the United States, and food imported from Thailand to Denmark and the United States. A total of 183 PFGE patterns were observed, and 136 (23.4%) isolates had the 3 most common patterns. Seven of 14 isolates from persons in Denmark had patterns found in persons and chicken meat in Thailand; 22 of 390 human isolates from the United States had patterns found in Denmark and Thailand. This study suggests spread of multidrug-resistant *S.* Schwarzengrund from chickens to persons in Thailand, and from imported Thai food products to persons in Denmark and the United States.

Salmonella enterica is a common cause of human gastroenteritis and bacteremia worldwide, and a wide variety of animals, particularly food animals, have been identified as reservoirs for nontyphoidal *Salmonella* spp. (*1*–*3*). Human infections with nontyphoidal *Salmonella* are commonly caused by ingestion of food that has been contaminated by animal feces (*3*). Although >2,500 serovars of *S. enterica* have been identified, most human infections are caused by a limited number of serovars. *S.* serovar Typhimurium and serovar Enteritidis are the most common causes of human salmonellosis worldwide, although other serovars have been reported to be more prevalent in some regions (*3*–*7*). Shifts in prevalence of specific strain types and serovars can reflect the influence of international travel and trade of animals and food products, and can therefore serve as useful epidemiologic markers.

We recently reported an increase in the prevalence of *S.* serovar Schwarzengrund in broiler chickens in Thailand and an increase in the proportion of human *Salmonella* infections caused by *S.* Schwarzengrund in Thailand (*6*), from 0% in 1992 to 2.4% in 2001. This serovar was also recently reported as causing more illness in Denmark (www.germ.dk) and the United States, where several isolates have shown multidrug resistance (*8*,*9*).

In recent years, an increase in antimicrobial drug resistance, including resistance to nalidixic acid, among *Salmonella* spp. has been observed in many countries, particularly in Asia (*10*–*17*). Nalidixic acid–resistant and ciprofloxacin-resistant *S.* Schwarzengrund has been reported in Taiwan (*18*) and the United States (*9*), the US cases linked to patients previously hospitalized in the Philippines. The emergence of antimicrobial drug resistance is a matter of concern. Persons with infections caused by antimicrobial drug–resistant *Salmonella* spp., particularly nalidixic acid–resistant *Salmonella* spp., are more likely to die, are more likely to be hospitalized, and are hospitalized for longer periods than patients with infections caused by susceptible strains (*18*–*20*).

Antimicrobial drug susceptibility profiles and genetic strain typing methods are useful epidemiologic tools to determine the sources of infections, including potential links between food animals and persons. Pulsed-field gel electrophoresis (PFGE) is highly discriminatory and useful in epidemiologic studies (*21*,*22*). To our knowledge, no molecular studies on *S.* Schwarzengrund have been previously described.

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This study was conducted to determine the clonality and molecular variation of *S.* Schwarzengrund from persons, food products, and animals in Denmark, Thailand, and the United States. In addition, antimicrobial drug resistance profiles were determined for some of the isolates. The implications of the findings in relation to the global spread of new serovars and the potential international spread by imported food products are discussed.

Materials and Methods

Bacterial Isolates

A total of 581 *S.* Schwarzengrund isolates were included, 73 from Denmark, 105 from Thailand, and 403 from the United States. All available isolates were selected from the strain collections at the Danish Institute for Food and Veterinary Research (n = 59) and Statens Serum Institut in Denmark (n = 14); the World Health Organization International *Salmonella* and *Shigella* Centre in Thailand (n = 105); and the US Food and Drug Administration (n = 7) and Centers for Disease Control and Prevention (CDC) in the United States (n = 396) (Table).

The 73 isolates from Denmark were isolated from 1995 to 2004: 14 from ill persons, ≥2 of whom reported travel to Thailand in the 30 days before specimen collection; 22 from pigs on farms; 20 from chicken meat; 9 from turkey meat; 4 from pork; and 4 from other food sources. Of the 20 chicken meat products tested in Denmark, ≥13 were imported, and 10 of these were known to be from Thailand. The origin of the remaining 7 chicken meat products was not known. The 105 isolates from Thailand were isolated from 1994 to 2003 and included 57 from ill persons at 17 different medical facilities and 48 from chicken meat. The 403 isolates from the United States were isolated from 1998 to 2005: 390 from ill persons, 4 from turkey meat, 3 from chicken meat, 2 from pigs on farms, 1 from a turkey on a farm, 1 from a squid roll imported from Taiwan, 1 from a catfish imported from Thailand, and 1 from a dehydrated whole chili imported from Thailand. Most isolates from Denmark and Thailand, but only a limited number of isolates from the United States, were available for susceptibility testing.

Antimicrobial Drug Susceptibility

Of the 581 isolates obtained, 204 were tested for antimicrobial drug susceptibility: 69 from Denmark, 90 from Thailand, and 45 from the United States. Susceptibility to antimicrobial agents was performed as MIC determinations by using a commercially prepared, dehydrated panel (Sensititer; TREK Diagnostic Systems Ltd., East Grinstead, UK), according to the Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards (*23*) for the following antimicrobial agents: ampicillin, ceftiofur, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, sulfamethoxazole, and tetracycline. Reduced susceptibility to ciprofloxacin was defined as MIC ≥0.125 mg/L and resistance as MIC ≥4 mg/L.

Table. Origin and occurrence of resistance among *Salmonella enterica* serovar Schwarzengrund isolates from humans, food, and food animals in Denmark, Thailand, and the United States

Country/source	No. isolates tested/ total no. isolates	No. isolates resistant to antimicrobial drugs*							
		AMP	CHL	CIP	GEN	NAL	STR	SUL	TET
Denmark									
Humans	14/14	8	3	1	6	8	11	9	8
Pigs on farm	22/22	0	2	0	0	0	4	2	2
Pork	4/4	0	0	0	0	0	0	0	0
Chicken meat of unknown origin	7/7	4	1	0	4	5	5	6	4
Imported chicken	13/13	11	0	0	11	11	12	13	13
Imported turkey	9/9	1	0	0	1	1	4	4	4
Others	0/4	–	–	–	–	–	–	–	–
Thailand									
Humans†	46/57	30	13	10	27	42	45	41	35
Chicken meat	44/48	23	16	2	28	39	39	36	23
Turkey meat	2/4	0	0	0	0	0	0	0	0
United States									
Humans	38/390	13	08	16	4	17	4	20	22
Chicken meat	0/3	–	–	–	–	–	–	–	–
Turkey on farm	0/1	–	–	–	–	–	–	–	–
Pigs on farm	2/2	0	0	0	0	0	0	1	1
Imported food	3/3	1	2	1	2	3	2	3	3
Total	204/581	91	45	30	83	126	126	135	115

*AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline.
†One human isolate from Thailand was resistant to ceftiofur.

Pulsed-Field Gel Electrophoresis

All 581 isolates were analyzed for genetic relatedness by PFGE by using *Xba*I according to the CDC PulseNet protocol (*24*). Electrophoresis was performed with a CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA, USA) by using 1% SeaKem agarose in 0.5× Tris-borate-EDTA at 180 V. Running conditions consisted of 1 phase from 2.2 to 63.8 s for a run time of 22 h.

All isolates from Denmark and Thailand and 7 of the food isolates from the United States were typed at the Danish Institute for Food and Veterinary Research; the remaining isolates from the United States were typed by PulseNet-participating state and local health departments, with the PFGE patterns submitted to the PulseNet database. One representative of each PFGE type identified in Denmark was sent to CDC for comparison with the PulseNet database. Comparison of the PFGE profiles was performed by using Bionumerics software v3.5 (Applied Maths, Sint-Martens-Latem, Belgium).

Results

Antimicrobial Drug Susceptibility

Among the 69 isolates from Denmark, nalidixic acid resistance was found in 8 (57%) of 14 human isolates, including those from both patients with known recent travel to Thailand, and in 16 (80%) of 20 chicken isolates, including all 10 isolates from chicken imported from Thailand. Nalidixic acid resistance was not found in pig, pork, or turkey meat isolates (Table). All nalidixic acid–resistant isolates from Denmark also displayed reduced susceptibility to ciprofloxacin. Nalidixic acid resistance was common among isolates from Thailand, including 42 (91%) of 46 human isolates and 39 (89%) of 44 chicken meat isolates. All nalidixic acid–resistant isolates from Thailand also exhibited reduced susceptibility to ciprofloxacin; 12 isolates (10 from persons and 2 from chickens) were resistant to ciprofloxacin. Ten of the ciprofloxacin-resistant isolates from Thailand contained 2 single-base substitutions in the *gyrA* gene at codons 83 [(T**C**C (Ser) → T**T**C (Phe))] and 87 [(**G**AC (Asp) → **A**AC (Asn))]. (Mutated bases are shown in boldface.)

Among the 45 isolates from the United States for which susceptibility results were available, nalidixic acid resistance was found in 17 (45%) of 38 human isolates and 3 (43%) of 7 food and food animal isolates, including all 3 isolates from imported food from Thailand and Taiwan. All nalidixic acid–resistant isolates from the United States also showed decreased susceptibility to ciprofloxacin; 16 of the 17 nalidixic acid–resistant isolates from persons and the isolate from an imported dehydrated whole chili from Thailand were resistant to ciprofloxacin.

Pulsed-Field Gel Electrophoresis

A total of 180 unique PFGE patterns were observed among the 581 isolates, with 136 (23%) isolates having the 3 most common patterns (online Appendix Figure, available from www.cdc.gov/EID/content/13/5/726-appG.htm). The most common pattern (58 isolates, JM6X01.0001) represented only human isolates from the United States; 5 of these isolates were susceptibility tested, and all were resistant to sulfamethoxazole and tetracycline and susceptible to all other antimicrobial agents tested. The second most common pattern (44 isolates, JM6X01.0091) included isolates from persons in Denmark, chicken meat imported from Thailand to Denmark, and persons and chickens in Thailand; all 44 isolates were susceptibility tested, and 42 (95%) were nalidixic acid resistant. The third most common pattern (34 isolates, JM6X01.0015) included isolates from persons and chicken meat in Denmark, persons and chicken meat in Thailand, persons in the United States, and catfish imported from Thailand to the United States; 20 of these isolates were tested for susceptibility, and 15 (75%) were nalidixic acid resistant.

Two additional patterns are noteworthy. One pattern (18 isolates, JM6X01.0059) included 1 isolate from a person in Denmark, 5 isolates from chicken and 9 isolates from persons in Thailand, 2 isolates from persons in the United States, and the isolate from the dehydrated whole chili imported from Thailand to the United States. Of the 9 isolates that underwent susceptibility testing, 7 (78%) were ciprofloxacin-resistant. Another pattern (14 isolates, JM6X01.004) included only human isolates from the United States; 10 of these isolates were susceptibility tested, and all were ciprofloxacin-resistant. This is the pattern from the previously described outbreak of ciprofloxacin-resistant *S.* Schwarzengrund infections in medical facilities in Oregon (*9*).

Twenty-six different PFGE types were found among the 73 isolates from Denmark (online Appendix Figure). Of these, 11 were found among 14 human isolates, 10 among the 26 isolates from pigs and pork, 7 among the 19 chicken meat isolates, and 4 among the 9 turkey meat isolates. Seven (50%) human isolates belonged to types that were also found in food, including 6 PFGE types found in chickens. Forty-three different PFGE types were found among the 105 isolates from Thailand; 25 of these were found among the 57 human isolates and 25 among the 48 chicken meat isolates. Thirty-five (61%) of the human isolates and 30 (63%) of the chicken isolates belonged to PFGE types that were found in both sources.

Four of the 6 PFGE types, involving 7 of the 14 isolates found among persons and chickens in Denmark, were also observed among persons and chicken meat in Thailand. Both ill persons in Denmark who reported travel to Thailand in the 30 days before specimen collection were infected with 1 of

the PFGE types common in Denmark and Thailand. These 4 PFGE types included the most common type among persons and chickens in Denmark and the 2 most common types among persons and chickens in Thailand.

Among the 403 isolates from the United States, 121 different PFGE types were found; 116 of these were found among the 390 human isolates and 12 among the 13 food and food animal isolates. Seven PFGE types were found in both persons and food; 73 (19%) of the human isolates and 8 (62%) of the food isolates belonged to types found in persons and food. All 8 food isolates with types also found in persons were found in foods imported to the United States, including food imported from Thailand. Twenty-two (6%) of the 390 human isolates from the United States matched PFGE patterns found in persons and chickens in Denmark or Thailand. Of the 19 isolates from persons in Denmark and the United States that belonged to PFGE types found in both countries and Thailand, 4 were tested for resistance and 3 (75%) of these were multidrug resistant.

Discussion

Foodborne diseases caused by nontyphoidal *Salmonella* spp. represent an important public health problem worldwide. In Denmark alone, the costs related to foodborne cases of salmonellosis were estimated to be US $10.4 million to $25.5 million in 2001 (*25*). In the United States nontyphoidal *Salmonella* spp. are responsible for an estimated >1.4 million illnesses, almost 16,000 hospitalizations, and >500 deaths every year (*26*) at an estimated annual cost of up to $2.3 billion. (*27*).

Historically, *Salmonella* serotypes Enteritidis and Typhimurium have been the most important causes of nontyphoidal salmonellosis. *S.* Schwarzengrund is a less common cause of human salmonellosis worldwide. In recent years, however, the relative incidence of this serovar seems to have increased (*6*,*8*). It now ranks among the 20 most frequently identified *Salmonella* serovars in several countries, including Slovakia, New Zealand, Venezuela, and Thailand; is among the 40 most frequently identified serovars in Denmark and the United States; and was the fifth most common serovar isolated from retail meat in the United States in 2004, associated exclusively with poultry products. Other studies also suggest that poultry could be the most common reservoir (*6*,*28*,*29*).

This study showed a high frequency of antimicrobial drug resistance, including an unusually high prevalence of nalidixic acid resistance, among *S.* Schwarzengrund isolates from chickens in Denmark, persons and chickens in Thailand, and food products imported into the United States. In contrast, the frequency of resistant isolates from pigs and turkey meat in Denmark was low. The prevalence of resistance among isolates from persons in Denmark was intermediate compared with the high level in persons and chickens in Thailand and the low level in Danish food animals. Along with the PFGE data, these resistance data support a hypothetical transmission of *S.* Schwarzengrund from chickens to persons in Thailand, and transmission from chickens, pigs, and turkeys to persons in Denmark.

Ciprofloxacin resistance was detected in 29 (24%) of 123 nalidixic acid–resistant *S.* Schwarzengrund isolates. Fluoroquinolone (ciprofloxacin) resistance among *Salmonella* spp. has recently emerged in several countries (*30*–*32*). Ten ciprofloxacin-resistant isolates tested in this study contained double mutations in *gyrA* at codons 83 (Ser → Phe) and 87 (Asp → Asn). These positions, located in the quinolone resistance–determining region of *gyrA*, are commonly reported among numerous bacterial species, including *Salmonella* isolates with a high-level of ciprofloxacin resistance (*30*–*32*). A ciprofloxacin-resistant strain of *S.* Schwarzengrund recently caused a nosocomial outbreak involving 2 nursing homes and 1 hospital in Oregon (*9*). The index patient in the Oregon outbreak was initially hospitalized in the Philippines. In many countries, including Denmark, fluoroquinolones are the drugs of choice for treating complicated gastrointestinal infections. Thus, resistance to this group of antimicrobial agents is especially critical, both for management of salmonellosis and because of the association of resistance with increased illness and death (*19*,*20*).

To our knowledge, this is the first study of the molecular epidemiology of *S.* Schwarzengrund. The study demonstrated a substantial diversity in PFGE patterns of this serotype with the presence of several common international clones. The PFGE types of isolates from persons and chicken meat in Thailand formed overlapping populations, with more than half of the isolates from both sources belonging to shared types. This supports the involvement of chicken meat as a reservoir for human *S.* Schwarzengrund infections in Thailand.

The epidemiology of *S.* Schwarzengrund infections in Denmark is complicated. Although some PFGE types were only found among isolates from pigs, several PFGE types were shared among isolates from persons and pigs; persons, chicken meat, and turkey meat; and persons and chicken meat. This suggests that chicken meat, pork, and turkey meat are sources of *S.* Schwarzengrund infections for persons in Denmark. Because modern trade and distribution of food products makes it difficult to determine the country of origin of meat samples sold retail in Denmark, the sources of the chicken meat, pork, and turkey meat included in this study are not completely known. However, Denmark has been importing an increasing amount of chicken meat, and much of this imported chicken is from Thailand. In addition, *S.* Schwarzengrund has, to our knowledge, not been detected in the Danish production of chicken. In this study, ≥13 of the 20 chicken meat products tested in Denmark

were imported, and 10 of these were from Thailand. These data, with the identification of identical PFGE types from persons and chickens in both Denmark and Thailand, support the possibility that some persons in Denmark acquired *S.* Schwarzengrund from imported chicken meat from Thailand. Another less frequent means of acquiring the infections is travel by persons from Denmark to Thailand; ≥2 of the 14 case-patients in Denmark had recently returned from travel to Thailand before they became ill.

A limited number of isolates from food and food animals in the United States were included in this study. The prevalence of *S.* Schwarzengrund in retail meat was low in 2003, with only 3 isolates recovered. In addition, PFGE data on isolates from food animals were not available for comparison in this study. The study would have benefited from additional isolates, especially from food and food animals in the United States. Nevertheless, a high proportion of these available isolates from food and food animals shared PFGE patterns with human isolates. In addition, several patterns found among human isolates in the United States were also present among human isolates in Denmark and Thailand, which suggests an international spread of these clones. Specifically, 1 PFGE type that was frequently ciprofloxacin resistant was found in a person in Denmark, in persons and chicken meat in Thailand, and in persons and the dehydrated whole chili imported from Thailand to the United States. Another PFGE type that was nalidixic acid–resistant was found in persons and chicken meat in Denmark, persons in the United States, and in catfish imported from Thailand to the United States.

In our study and other studies, *Salmonella* isolates from imported food in Denmark had a higher frequency of resistance than was found in domestically produced meats (*33*). A study from the United States also reported a high frequency of antimicrobial drug resistance among *Salmonella* isolates from imported food (*34*). Food is an important vehicle for the national and international dissemination of *Salmonella* spp. and antimicrobial drug resistance genes from food animals to persons (*35*–*38*).

This study supports the conclusion that multidrug-resistant, including nalidixic acid–resistant, *S.* Schwarzengrund was likely disseminated internationally by chicken products from Thailand. Because antimicrobial drug resistance among *Salmonella* isolates from food animals commonly reflects antimicrobial drug use in food animals, efforts are needed to ensure appropriate use of antimicrobial agents in food animals and to improve food safety to reduce dissemination of *Salmonella* spp. worldwide.

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Rudolf Virchow and the Recognition of Alveolar Echinococcosis, 1850s

Dennis Tappe* and Matthias Frosch*

Alveolar echinococcosis, which is caused by the larval stage of the fox tapeworm *Echinococcus multilocularis*, is one of the most dangerous parasitic diseases. It is endemic in many parts of the Northern Hemisphere and an emerging health problem in the People's Republic of China. In Europe and North America, human cases are rare, but concomitant with an increase in the population of the final host, the red fox, an increase of human infections is expected. Rudolf Virchow, the father of the concept of cellular pathology, determined in the 1850s that an *Echinococcus* sp. was the causative agent of this enigmatic emerging disease. In his famous publication in 1855, he described the clinical course of the disease, its macroscopic aspects, and histopathologic findings in detail. He also identified the disease formerly known as alveolar colloid of the liver to be an infection with the larval stage of an *Echinococcus* sp.

Rudolf Virchow (1821–1902, Figure 1), the originator of the concept of cellular pathology, also concerned himself extensively with the pathology of infectious diseases (*1*). In 1848, when he was working as a military physician at the Charité Hospital in Berlin, he distributed oppositional political pamphlets and was thereupon suspended from work. On the condition that he would no longer get involved with political activities, he was offered a position as a professor of pathology at the University of Würzburg, Lower Franconia, Germany. He stayed only 7 years in Würzburg, but it was during these years, from 1849 to 1856, that he made some of his major discoveries. Virchow initially worked in the Theatrum Anatomicum (Figure 2), a baroque but rather small pavilion in the center of the city. He shared the site with Albert Kölliker (1817–1905), a contemporary anatomist who once called the place "a gaunt dive" (*2*).

In Würzburg, Virchow soon became the secretary of the local scientific society, Physicalisch-Medicinische Gesellschaft, (Physico-Medical Society). In 1855, the society's scientific journal Verhandlungen der Physicalisch-Medicinischen Gesellschaft (Proceedings of the Physico-Medical Society) published his observations and conclusions about a disease that we know today as alveolar echinococcosis. The title of the article was "Die multiloculäre, ulcerirende Echinokokkengeschwulst der Leber" (The multilocular, ulcerating *Echinococcus*-tumor of the liver [*3*], Figure 3). He was the first to conclude and publish that the enigmatic emerging disease called alveolar colloid was caused by the larval stage of a previously unknown member of the genus *Echinococcus* and was thus not a form of cancer, as was generally believed. He presented his findings and assumptions during meetings of the scientific society on March 10, 1855, and May 12, 1855.

Courtesy of the Institute of Pathology, University of Würzburg.

Figure 1. Rudolf Virchow. Photograph taken during his 7 years in Würzburg, Germany (1849–1856), as professor of pathology.

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Previous Studies by Buhl and Zeller

Previous to Virchow's report, Frans Buhl had described the first 2 cases of a strange new condition of the liver, which he called alveolar colloid, in 1852 and 1854, respectively (*4*,*5*). Buhl had found hepatic lesions consisting of many alveoles that contained a gelatinous mass. He believed that the lesions were not a form of a gelatinous cancer (gallertkrebs) as proposed by Meyer (*6*), but rather degenerating tissue (*5*). In 1854, Zeller had reported a similar case (*7*). Both authors provided a detailed description of the new disease, but they were unable to determine its origin or the causative agent.

Virchow's Findings

In the introduction of his 1855 article, Virchow pays deference to Buhl and Zeller for the correct description of their histopathologic findings before he describes his own examinations. Virchow states that he is able to add a fourth case to the 3 existing ones. However, he argues that his interpretation of the cases is completely different. Virchow describes how he repeatedly examined the histologic specimens and that the laminated aspect of the gelatinlike membranes and the concentric calcified bodies he observed were in concordance with what Buhl and Zeller had reported about the alveolar colloid. Virchow continues with the statement "already the first glance at the [macroscopic] specimen evoked the imagination of many little echinococcal vesicles." This opinion seems a bit advanced at this early stage of his investigations, but it nonetheless underscores his interpretation of the alveolar colloid that he would soon rename. After many days of looking at the histologic preparations under the microscope, Virchow was even able to find fully developed protoscolices (third larval stage of the tapeworm) in the vesicles.

From today's point of view, this finding must be considered a rarity because in human infections with the larval stage of *Echinococcus multilocularis*, hardly any protoscolices are found (*8*). This contrasts with the findings in rodents, the natural intermediate host. This fortunate circumstance guided Virchow to the correct diagnosis, but he stated that he would have come to the same conclusion even without finding any of the "young animals" or their hooklets. In this context, he again mentions observations by Zeller (*7*), who had also found protoscolices in his case of alveolar colloid but had refused to categorize the disease as an echinococcosis. Zeller focused on the differences between the alveolar colloid and the colloidal cancer of the liver. During the time Virchow conducted this study, only the causative agent of hydatid disease, the dog tapeworm *E. granulosus* was known. The larval stage of this species develops protoscolices regularly in both humans and its natural intermediate host. The developmental cycle of *E. granulosus*, which involves dogs as definitive hosts and sheep as intermediate hosts, was previously elucidated by Carl Theodor Ernst von Siebold in 1853 and 1854 (*9*,*10*).

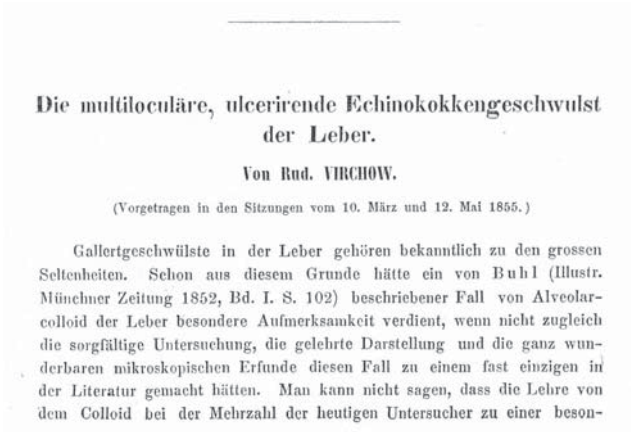
Courtesy of the Institute of Pathology, University of Würzburg.

Figure 2. Baroque pavilion in the backyard of the Juliusspital in Würzburg. Originally a garden pavilion, it was later remodeled to form the Theatrum Anatomicum, where Virchow worked from 1849 to 1853 in the right wing. Kölliker, an anatomist, occupied the left wing. Years later, it was converted into a greenhouse. It is a conference room and exhibition hall today.

In his report, Virchow continues with a detailed description of the clinical course of the disease. The patient was a 38-year-old man in whom upper abdominal pain, a short episode of diarrhea, and jaundice developed. The right upper abdomen showed a protrusion, and his physician diagnosed a markedly enlarged liver. The patient was apathetic, his urine was brown, and his stool was the color of sand. Later petechiae, hematemesis, and bloody diarrhea developed. He became rapidly cachectic and died ≈3 months after the onset of jaundice.

Virchow then gives a pictorial description of the autopsy he performed on March 5, 1855. An adherent mass was seen on the surface of the enlarged liver. After sectioning the liver, he observed in the middle of the lesion a large cavum, which was filled with a yellow, puslike fluid. Virchow extensively characterized the cavum, depicting the large lesion as ulcerative. Some peripheral areas of the mass extended into thin rootlike structures, and others formed thick protrusions. The pathologist saw the beginning infiltration of blood vessels by the lesion by which "a further spread would have been possible." Virchow had described the classic infiltrative aspect and the potential metastasis of lesions of alveolar echinococcosis.

Under the microscope, the lesion consisted of small alveolar vesicles containing gelatin. "The composition of those gelatin-structures, even in concordance with the skin of *echinococcus*, is in many parts so peculiar, that I could hardly resolve my doubts about their nature. Only after some time I was able to find the young animals." Virchow was about to find protoscolices within the vesicles, "I only found them in the portal parts of the tumor, where the



Die multiloculäre, ulcerirende Echinokokkengeschwulst der Leber.

Von Rud. VIRCHOW.

(Vorgetragen in den Sitzungen vom 10. März und 12. Mai 1855.)

Gallertgeschwülste in der Leber gehören bekanntlich zu den grossen Seltenheiten. Schon aus diesem Grunde hätte ein von Buhl (Illustr. Münchner Zeitung 1852, Bd. I. S. 102) beschriebener Fall von Alveolarcolloid der Leber besondere Aufmerksamkeit verdient, wenn nicht zugleich die sorgfältige Untersuchung, die gelehrte Darstellung und die ganz wunderbaren mikroskopischen Erfunde diesen Fall zu einem fast einzigen in der Literatur gemacht hätten. Man kann nicht sagen, dass die Lehre von dem Colloid bei der Mehrzahl der heutigen Untersucher zu einer beson-

Figure 3. Reproduction of the beginning of Virchow's original publication (*3*) of a case of hepatic multilocular echinococcosis and his proof that the disease was caused by an *Echinococcus* sp.

biggest alveoles were present... They were rounded or of a heart-like shape... The hooklets were retracted... Some of the animals had no hooklets at all and looking at the smaller ones, I would believe that they were still juvenile." Virchow concluded that the entire tumor consisted of a multitude of exceptionally small echinococcal vesicles, thus the name "multilocular." He stated that the lesion could therefore no longer be named alveolar colloid. However, he was confused by finding only few protoscolices. Virchow speculated that the protoscolices may have perished in the smaller vesicles or been transformed into a cystic form, which he called acephalocysts. He stated that "there is no longer any doubt about the existence of sterile echinoccocal vesicles in human beings and it seems likely that they developed from immature, hookless [protoscolices]." We know today that in most human patients with alveolar echinococcosis, the parasitic tissue has only sterile vesicles and that protoscolices obtained from lesions of the rodent intermediate host can transform into vesicles in cell culture (*11*). Virchow further concludes that the state of the huge lesion could not be caused by a massive invasion of the liver but must rather be the result of a production of new offspring within the liver. The enormous production of echinococcal vesicles originates from only 1 or a few small oncospheres, which are the invasive larval stage of the parasite.

After Virchow had sent his findings and conclusions to Buhl, Buhl congratulated Virchow for his investigations in a letter (*12*). However, Buhl also wrote that he himself had come to the same conclusions some months before but that he had unfortunately delayed his own publication until it was too late. Buhl stated that he had contacted von Siebold, who had sent him a specimen of a similar echinococcal tumor of an animal liver from his worm collection. This statement led Virchow to conclude that alveolar echinococcosis had a zoonotic aspect. As a pathologist, Virchow read the publications of parasitology and zoology at that time. However, it was believed that the causative organism of alveolar echinococcosis was an aberrant *Echinococcus* sp. and not a separate species. Therefore, other definitive and intermediate hosts than those described by von Siebold for the dog tapeworm were not considered to be involved in the parasite's life cycle at that time. Buhl's letter was later presented during a meeting of the scientific society in August 1855 and published in the same volume of the journal as Virchow's article.

Further Studies by Others

Soon after Virchow's article was published, many new cases of this emerging disease were reported (*13*–*17*). Without Virchow's work, those articles would have described the disease as further cases of alveolar colloid. Thus, the disease had not only been newly recognized but had also been on the increase. In 1863, Leuckart postulated that the newly discovered "multilocular ulcerative *echinococcus*-tumor" is caused by an independent species, which he named *E. multilocularis* (*18*). It took another 91 years until the natural cycle involving foxes as definitive hosts and rodents as intermediate hosts was described by Rausch and Schiller in Alaska (*19*) and by Vogel in the Swabian Alb in Germany (*20*). In 1954 and 1955, respectively, it was finally proven that alveolar echinococcosis is caused by the morphologically and biologically distinct species *E. multilocularis*.

Today's Situation

Since the 1990s, *E. multilocularis* has been spreading, and increased rates of infection in red foxes have been observed in Europe. Today, the range of the parasite extends from central to eastern Europe (*21*). As a consequence, the rate of newly diagnosed alveolar echinococcosis has already doubled in Germany (*22*). In North America, *E. multilocularis* is present from Alaska to the Hudson Bay and from southern Canada to the central United States. The parasite will likely spread further since suitable definitive and intermediate hosts are found throughout North America (*23*). Most human cases of alveolar echinococcosis are reported in the People's Republic of China, where the disease is a serious emerging public health problem (*23*) and a ≤15% prevalence of human alveolar echinococcosis has been reported in some areas (*24*).

More than 150 years after Virchow's discovery, many questions about alveolar echinococcosis remain unanswered. Why do some persons become infected and exhibit symptoms of this disease, whereas others never show any symptoms of the disease despite being seropositive? It is generally acknowledged that humans contract this disease by ingesting fecally contaminated fruit, such as wood strawberries, in areas where *E. multilocularis* is highly prevalent

in red foxes. However, the risk factors for acquisition of this disease are still not fully elucidated (*25*). Even today, only a few treatment options exist. Antiparasitic chemotherapy with albendazole or mebendazole has only a parasitostatic effect, and patients have to rely on surgery to cure alveolar echinococcosis, if the disease is diagnosed in time.

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Fatal Disseminated *Acanthamoeba lenticulata* Infection in a Heart Transplant Patient

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We report a fatal case of disseminated acanthamebiasis caused by *Acanthamoeba lenticulata* (genotype T5) in a 39-year-old heart transplant recipient. The diagnosis was based on skin histopathologic results and confirmed by isolation of the ameba from involved skin and molecular analysis of a partial 18S rRNA gene sequence (DF3).

Acanthamoeba is 1 of 3 genera of free-living amebae that commonly cause disease in humans (*1*). These protozoa have been implicated in local infections, such as amebic keratitis, mainly in immunocompetent contact lens wearers, and in the mostly fatal, granulomatous amebic encephalitis in immunocompromised patients with HIV/AIDS and immunosuppressant-treated patients, including organ transplant recipients (*2–4*). Disseminated acanthamebiasis (DA), which is defined as widespread extracerebral disease, is extremely rare, but its incidence has increased in recent years (*5*). Among DA reported, only 5 occurred in solid organ (3 lung and 2 kidney) transplant recipients (*4*). We report a fatal case of DA in a heart transplant recipient and identify *Acanthamoeba lenticulata* (genotype T5) as the cause of life-threatening disease.

The Case

A 39-year-old man from Martinique had received a second heart transplant in March 2004 because of chronic rejection. He had received his first transplant 14 years earlier because of alcohol-related dilated cardiomyopathy. Skin complications included epidermoid carcinoma on the right leg in 1995 and diffuse viral warts on the trunk in 2003. Maintenance immunosuppression after the second heart transplant in 2004 included cyclosporine (220 mg/day), prednisone (20 mg/day), and mycophenolate mofetil (500 mg/day). The latter drug was withdrawn because of pancytopenia. Postsurgery complications included acute refractory bleeding (aortic anastomosis), cytomegalovirus infection of the gut, bacterial pulmonary infection, and postoperative renal failure that required chronic hemodialysis that prolonged his stay in the intensive care unit (ICU) to 5 months.

In January 2006, after a short visit to Martinique, the patient was transferred to our institution because of fever, dyspnea, and acute costal and back pain, with suspected osteitis underlying cutaneous lesions. Two months earlier, 4 trunk and leg abscesses or carbunclelike skin lesions had developed. Despite oral antistaphylococcal therapy, these lesions spread and became ulcerated and painful. Three ulcerated, violaceous plaques with undermined deep-infiltrated margins were present: 1 on the trunk (largest diameter 5 cm) (Figure, panel A) and 1 on each thigh. Three subcutaneous abscesses were present on the trunk and their puncture yielded a brown liquid. The differential diagnosis included pyoderma gangrenosum, neutrophilic dermatoses, mycobacteriosis, cutaneous bacterial infection, and calciphylaxis (chronic hemodialysis).

The first histologic examination of a periulcerated skin lesion (punch biopsy specimen) showed diffuse dermal and hypodermal neutrophil infiltration and sparse histiocytelike cells (Figure, panel B). No infectious elements were identified. Biologic data indicated an inflammatory syndrome (C-reactive protein 250 mg/L [normal <5 mg/L], procalcitonin 25 ng/mL [normal <1 ng/mL]), with increased elevated circulating neutrophil counts (10.9×10^9 cells/L) and anemia (hemoglobin 7 g/dL). Cultures of blood, abscess fluid, and involved skin were repeatedly negative for bacterial,

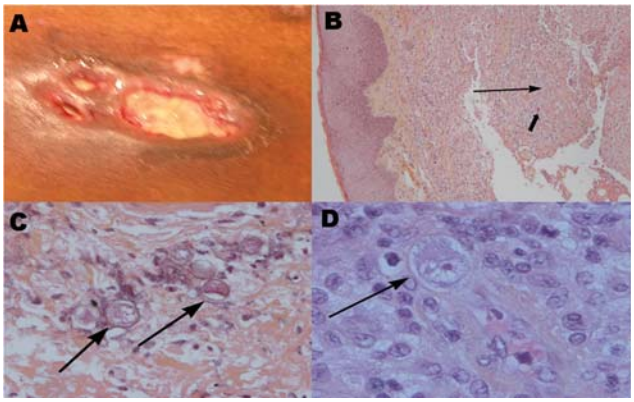

Figure. A) Ulcerated, violaceous plaque on the trunk of the patient with undermined infiltrated peripheral walls. B) Section of the lesion in A showing diffuse dermal-hypodermal necrosis with neutrophil infiltration (thin arrow) and sparse histiocytelike cells (thick arrow) (hematoxylin and eosin–stained, magnification ×10). C) Surgical skin biopsy specimen showing amebic cysts (arrows) in the dermal-hypodermal junction (hematoxylin and eosin–stained, magnification ×20). D) Surgical skin biopsy specimen showing intravascular amebic trophozoite (arrow) characterized by acanthopodia, cytoplasmic vacuoles, and a prominent nucleolus (hematoxylin and eosin–stained, magnification ×40).

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mycotic, or parasitic agents. A computed tomographic body scan showed a massive abscess under the left kidney associated with pulmonary nodules without cutaneous calciphylaxis. Positron emission tomography scan confirmed those abnormalities and showed extensive and severe bone osteomyelitis.

Atypical pyoderma gangrenosum with visceral involvement was considered and treated with 3 intravenous prednisolone pulses. After minor initial improvement, the patient's condition deteriorated, and 10 days later septic shock associated with multiorgan failure developed. Surgical periulcer skin biopsy specimens were obtained in the ICU and specific parasite investigations were conducted. DA was then diagnosed. Hematoxylin and eosin staining of histologic sections showed cysts and trophozoites (30 μm diameter) in the dermal-hypodermal junction within polymorphous inflammatory granulomas associated with ischemic necrosis (Figure, panels C and D). The presence of *Acanthamoeba* sp. was confirmed by culturing amebae from involved skin on agar plates coated with *Escherichia coli*. In an indirect assay, the patient's serum showed weak immunofluorescence labeling against his own cultured cysts or vegetative amebae.

Molecular identification of DNA extracted from the isolated amebae was made by using the UNSET method (*6*). A diagnostic small subunit rDNA fragment (ASA.S1) was amplified by using JDP1 and JDP2 primers, and its differentiating fragment (DF3) was sequenced by using an internal 892c primer (*7*). The sequence of the DF3 subset that contains the highly variable and informative section in the ASA.S1 region of the *Acanthamoeba* isolate was visually compared with those of different published genotypes (T1–T15) (Table). Genotype T5 was identified (European Molecular Biology Laboratory accession no. AM411530). No drug-of-choice exists for treating DA. Despite treatment with pentamidine, 5-fluorocytosine, and itraconazole, the infection was rapidly fatal. Although analysis of cerebrospinal fluid obtained on days 5 and 18 after admission to the ICU showed no biochemical or parasite data suggestive of granulomatous amebic encephalitis, callus corpus necrosis was observed on a computed tomographic brain scan on day 18. The patient died of multiorgan failure on day 23. Family members refused to allow an autopsy.

Conclusions

Protozoan infections are rare in heart transplant recipients, unlike in lung transplant recipients (*8*,*9*). To our knowledge, our patient, whose DA involved skin, bones, lungs, intraabdominal organs, and perhaps the brain, represents the first case to be reported in a heart transplant recipient. In a recent review of the literature, Duarte et al. (*4*) reported 5 cases of DA in lung (60%) or kidney (40%) transplant recipients. DA was difficult to diagnose in the patient, with 60% of the diagnoses made postmortem, which is similar to 74% of the diagnoses in 23 HIV/AIDS patients (*2*). The patient's clinical picture was atypical because his lesions were pyodermalike ulcers with subcutaneous abscesses, whereas the most frequently reported clinical skin manifestations were painful nodules, purpura, and pustules (*10*). Furthermore, the first histologic examination did not identify cysts. *Acanthamoeba* trophozoites with characteristic acanthopodia, cytoplasmic vacuoles, and a prominent nucleolus, especially in dermal vessels, were observed only after staining of the second biopsy specimen with hematoxylin and eosin in a context of strong clinical suspicion of DA. When reexamined retrospectively, the first skin biopsy specimen contained some pathogens, but trophozoites had been misidentified as histiocytelike cells.

Another important finding was the identification of the DA-causative agent as genotype T5, which is commonly

Table. rDNA sequences of *Acanthamoeba* isolate (2/533) from the patient, a keratitis isolate (GAK1), 3 environmental T5 subtypes, and 4 other genotypes from persons with nonkeratitis infections

Genotype (strain)	DF3 sequence (5′→3′)*
T5 (2/533)	CAAAACACCGC**C**GTTAATCCTTT**TT---**CGGGGGTTAA**CG**GTTGGTGAAT
T5 (GAK1)	CAAAACACCGC**C**GTTAATCCTTT-----CGGGGGTTAA**TG**GTTGGTGAAT
T5 (72/2)†	CAAAACACCGC**C**GTTAATCCTTT-----CGGGGGTTAA**TG**GTTGGTGAAT
T5 (PD2S)‡	CAAAACACCGC**T**GTTAATCCTTT-----CGGGGGTTAA**TA**GTTGGTGAAT
T5 (FLAIV)§	CAAAACACCGC**C**GTTAATCCTTT**T-CAA**CGGGGGTTAACGGTTGGTGAAT
T4	CAAAACACCAATCGGCGCGGTCGTCCTTGGCGTCGGTCCTTCACGGGGCCGGCGCGAGGGCGGCTTAGCCCG GTGGCACC
T1	CAAAACACCACCATCAGGCAGTGGGGTCGTGCTTCGCTTTTCCGGCAACGGGGAAGTGGAGGCGGTCTCATT CCCCTGATGG
T10	CAAAACACCATCCATTTAGCAYGGTCGTTTTCAAATATTCCTTTTTGCGAAGGTTGTTTGGGAACGATTCGT CCTGATGGATC
T12	CAAAACACCACCATTAACACGATCGTTTTTTGCAAATATGCCACATGCGCAAGTGTGTGGTTGTGTTTGAAG GAACGATTTG

*Sequence differences are shown in **boldface**.
†Five isolates at European Molecular Biology Laboratory (EMBL).
‡Eight isolates at EMBL.
§One isolate at EMBL.

found in the environment (*11*) and corresponds to *A. lenticulata*. This species has been isolated from nasal mucosa of persons without documented amebic infection (*12*). Although *A. lenticulata* has been shown to be pathogenic (*12*), genotype T5 was only recently isolated from a patient with keratitis (*13*). To our knowledge, our patient has the first case in which genotype T5 is the etiologic agent of a nonkeratitis, life-threatening DA infection.

Acanthamoeba spp. are free-living amebae found in soil, water, air, humans, and various animals (*14*). Depending on the molecular methods used (i.e., nuclear 18S rRNA or 16S rRNA mitochondrial gene amplification), 15 genotype sequences have been identified in environmental and human strains (T1–T15, Table). While genotype T4 is the most prevalent (79% of isolates) (*15*), only 1 *A. lenticulata* strain isolated from a patient with ocular keratitis had genotype T5 (*13*).

This case should alert physicians to a rare but life-threatening infection with *A. lenticulata* (genotype T5) in a heart transplant recipient. In organ transplant patients, when sterile cutaneous ulcers or subcutaneous abscesses develop that fail to respond to antibacterial treatments and pulse corticosteroids, histologic analysis should emphasize identifying *Acanthamoeba* spp.

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Leptospirosis in Urban Wild Boars, Berlin, Germany

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We found antibodies to leptospires in 25 (18%) of 141 wild boars from Berlin (95% confidence interval 12–25). Seropositivity was associated with chronic interstitial nephritis (odds ratio 10.5; p = 0.01), and leptospires were detected in kidney tissues. Wild boars represent a potential source for human leptospirosis in urban environments.

Leptospira spp. are endemic to many domestic and wild animals, which may shed the pathogen in urine (*1*). Humans may acquire potentially fatal leptospirosis through contact with urine-contaminated water or soil. In Germany, ≈50 cases of leptospirosis are reported each year, mostly related to recreational and residential exposures (*2*). Among wildlife species, rodents are considered to be the most important reservoirs for leptospirosis in rural and urban environments (*3*,*4*). Contact with water contaminated with rodents' urine (usually inadequately treated sewage) is a well-known risk factor for leptospirosis. In wild boars (*Sus scrofa*) in Europe and the United States, antibodies against *Leptospira* spp. serovar Pomona—the main serogroup that infects domestic swine—have been frequently detected (*5*,*6*). During the past decades, the population density of this game species has increased substantially (*7*,*8*). Subsequently, boar migration to urban areas and close contact with humans has been noted. At present, an estimated 5,000 wild boars live in urban and suburban areas of Berlin. Although boars are known to be susceptible to leptospirosis, data on the prevalence of the disease in synanthropic wild boars and the possible implications for human health are absent. Our objective was to assess the potential role of wild boars as a reservoir for leptospirosis in an urban environment. In addition, we examined their role in transmission of *Leptospira* spp. to city residents with occupational exposure to wild boars.

The Study

The survey was conducted in Berlin, which has an area of 891.7 km^2 and a population of 3.4 million. The study area is mostly urban (56%) and contains industrial, commercial, and residential buildings; the other portions are forest (17.9%), green space (14.5%), and water (6.6%). The southwestern study area partially merges with the adjacent city of Potsdam.

Serum and kidney tissue samples were collected from wild boars killed in the study area for population control during fall and winter 2005–06. Wild boars were categorized according to age (determined by teeth; shoats <1 year, yearlings 1–2 years, adults >2 years), sex, and location of death. For antibody detection, microscopic agglutination test was conducted with a panel of 12 leptospiral serovars; a titer ≥100 was considered positive (Table 1). For histologic investigation, the tissues were fixed in 10% formalin; embedded in paraffin; and stained with hematoxylin and eosin, Masson trichrome, or Warthin-Starry silver according to standard protocols. Chronic interstitial nephritis and resultant renal fibrosis, the characteristic lesions of renal leptospirosis in animals (*9*), were the criteria used to classify renal infection. Selected tissue samples were further studied by classic PCR targeting the leptospiral outer membrane lipoprotein LipL32 (*10*). The amplification products were confirmed by direct sequencing.

To assess seroprevalence among city residents, in April 2006 we enrolled 84 municipal hunters from Berlin. A standardized questionnaire was used to assess demographic information (age, sex), frequencies of contact with wild boars, and use of gloves during boar evisceration. Serum samples were collected from all hunters and analyzed as described above. Univariate odds ratios (OR), prevalence ratios (PR), and 95% confidence intervals (95% CI) were calculated by using SPSS 14 software (SPSS Inc., Chicago, USA). A p value <0.05 was considered significant.

During the 2005–06 hunting season, municipal hunters in the study area shot 294 wild boars. A total of 219 (74%) blood samples and 77 (26%) kidney specimens were collected from the boars. Of these, 78 (36%) blood samples had to be excluded from analysis because of insufficient quality (i.e., clotting). Of the 141 remaining serum samples, antibodies against pathogenic leptospires were found in 25 (18%) (95% CI 12–25). Of these 25 positive samples, 10 demonstrated cross-reactivity with antigens of other serovars. Among the samples without cross-reactivity, *Leptospira* spp. serovar Pomona (n = 6) and serovar Bratislava (n = 4) were most frequently identified (Table 1). Titers of *Leptospira*-positive serum samples varied from 100 through 800. Although not statistically significant, seropositivity was highest in animals from the southwestern part of the study area (Figure 1; Table 2). Seropositivity was also higher in adult animals (p = 0.02) but was unrelated to sex (p = 0.6). A total of 29 kidney specimens were examined histologically, 17 of which were

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Table 1. Characteristics and titers of antibodies to leptospires of 25 seropositive wild boars, Berlin, fall/winter, 2005–06

Boar characteristics			*Leptospira* spp. serovar*						
Sex†	Age, y‡	Place of death§	Australis	Autumnalis	Bratislava	Copenhageni	Grippotyphosa	Pomona	Pyrogenes
M	Adult	1	–	–	100	–	–	–	100
M	Adult	1	–	–	100	–	–	–	–
F	Adult	1	–	–	100	–	–	–	100
M	Adult	1	–	100	800	200	200	800	–
M	Yearling	1	–	–	200	–	100	100	–
M	Adult	1	–	–	200	–	–	200	–
M	Yearling	1	–	–	200	–	100	400	–
F	Yearling	1	–	–	100	–	–	100	–
M	Shoat	1	–	–	–	–	–	400	–
M	Adult	2	–	–	–	–	–	100	–
F	Shoat	2	–	–	–	–	–	100	–
F	Yearling	3	–	–	100	–	–	–	–
F	Adult	3	–	–	–	–	–	200	–
F	Yearling	3	–	–	–	–	100	–	–
F	Yearling	3	–	–	100	–	100	400	–
F	Adult	3	–	–	–	–	–	–	100
F	Adult	3	100	–	100	–	–	–	100
M	Yearling	3	–	–	–	–	–	400	–
NK	Yearling	4	–	–	100	–	–	–	–
F	Shoat	4	–	–	–	–	–	–	100
F	Adult	5	–	–	100	–	–	–	–
M	Shoat	6	–	–	100	–	–	–	100
F	Shoat	7	400	–	–	–	–	–	–
F	Shoat	7	–	–	–	–	–	800	–
M	Adult	7	–	–	—	—	–	–	100

*Microscopic agglutination test panel included *Leptospira interrogans* serovars Australis, Autumnalis, Bataviae, Bratislava, Copenhageni, Hardjo, Icterohaemorrhagiae, Pomona, and Pyrogenes; *Leptospira borgpetersenii* serovar Tarassovi; and *Leptospira kirschneri* serovar Grippotyphosa. A titer of ≥100 or above was considered positive. The highest titers are shown.
†NK, not known.
‡Shoat, <1 y; yearling, 1–2 y; adult >2 y.
§Numbers correspond to areas shown in Figure 1.

from seropositive boars. Of these 17, 15 (88%) showed moderate to severe chronic lymphoplasmacytic interstitial inflammation (Figure 2), compared with 5 (42%) from the 12 seronegative boars (OR 10.5; 95% CI 1.3–110.4; p = 0.01). Leptospires were detected by silver staining in 3 (30%) of 10 specimens from seropositive wild boars with chronic interstitial nephritis (5 of the 15 specimens were unsuitable for silver staining) and were confirmed by PCR in 2 of the leptospire-positive samples. However, among 84 municipal hunters (96% males, mean age 51 years) antibodies to leptospires were not detected. Sixty-one (73%) hunters shot >10 wild boars per season. Of these, 51 (72%) used gloves, at least on occasion, compared with 6 of the 17 hunters (35%) who shot ≤10 boars per year (PR 2; 95% CI 1.2–3.4; p<0.05).

Conclusions

Our study describes a newly discovered urban focus of leptospirosis among wild boars in Berlin. The high frequency of porcine *Leptospira* spp. serovars Pomona and Bratislava, the association of chronic interstitial nephritis with positive results of the microscopic agglutination test, and the demonstration of leptospires in kidney specimens all indicate that wild boars act as a maintenance host for *Leptospira* spp. in this urban area. A relatively high prevalence of leptospirosis was observed in the southwestern districts of the city, which are particularly rich in freshwater

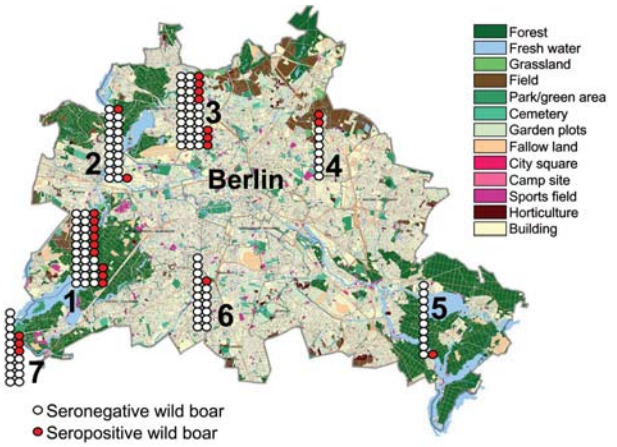

Figure 1. Map of Berlin showing the regional distribution, numbers of wild boars killed during the 2005–06 hunting season (n = 141), and numbers of wild boars seropositive for antibodies against *Leptospira* spp. (red). Districts are numbered from 1 to 7 (with permission from the Senate Department of Urban Development, Berlin) and correspond to numbers in Tables 1 and 2.

Table 2. Locations of wild boars seropositive for *Leptospira* spp., Berlin, fall/winter, 2005–06*

District†	% Boars	95% CI
1. Zehlendorf/Wannsee	27	15–44
2. Spandau	10	3–29
3. Tegel/Reinickendorf	21	11–38
4. Pankow/Mahrzahn/Hellersdorf	22	6–55
5. Köpenick	9	2–38
6. Lichterfelde/Steglitz/Charlottenburg	16	1–27
7. Potsdam	18	6–41
Total	18	12–25

*Seropositivity determined by microscopic agglutination test. CI, confidence interval.
†Numbers correspond to areas shown in Figure 1.

lakes intensively used for recreational activities by urban inhabitants. The relevance of this finding to human health was demonstrated by a recent case of severe leptospirosis in this area of Berlin; the patient had had contact with fresh water, which was most likely contaminated by wild boar urine (*11*).

In contrast to findings of a study from Austria (*12*), we found no antibodies to leptospires in hunters. Although this finding may be related to the regular use of gloves by highly exposed persons, it also indicates that the transmission of leptospirosis from wild boars to humans, although present, occurs at a considerably low rate. However, the epidemic potential of infections like leptospirosis that have a basic reproduction number close to 0 (i.e., that are minimally transmissible within human populations) is largely determined by the number of introductions from the animal hosts. Thus, among other contributing factors (e.g., human population expansion and encroachment, transmission to sympatric populations of susceptible domestic animals), the ongoing increase in wild boar populations, and the shift from sylvatic to synanthropic occurrence of this game species might lead to increased leptospirosis in humans.

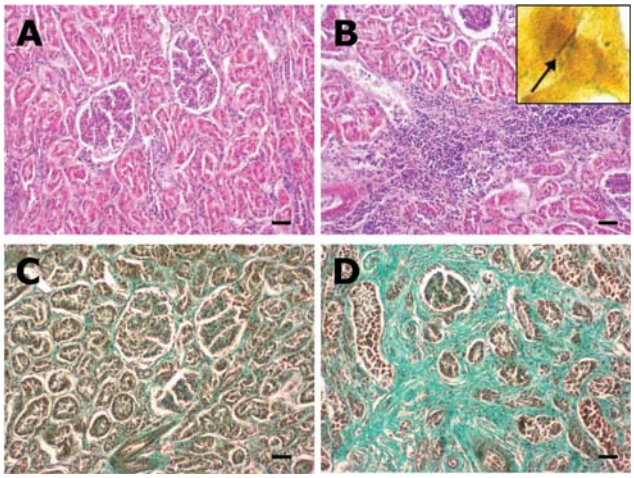

Figure 2. A) Normal renal parenchyma from wild boar seronegative for *Leptospira* spp. (hematoxylin and eosin [HE] staining). B) Kidney from a seropositive wild boar, showing chronic interstitial nephritis (HE staining). Inset: silver-stained leptospire (arrow) within the tubulus epithelium of the kidney (Warthin-Starry, oil ×1,000). C) Normal renal parenchyma (Masson trichrome staining). D) Kidney with severe interstitial fibrosis (green) as a result of chronic interstitial nephritis in a wild boar seropositive for *Leptospira* spp. (Masson trichrome staining). Scale bars represent 50 μm.

From a public health perspective, surveillance of leptospirosis incidence, prevalence, and serovar distribution in wild boars and humans (especially in potential high-risk groups with recreational freshwater contact) is necessary to establish the direction and the significance of this newly discovered potential exposure route. Additionally, physicians and public health authorities should be aware that bodies of fresh water in areas populated with wild boars may be contaminated with *Leptospira* spp., even if typical indicators, like rat infestations or contamination with sewage, are absent.

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Mycobacterium liflandii Infection in European Colony of *Silurana tropicalis*

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Mycobacterium liflandii causes a fatal frog disease in captive anurans. Here we report, to our knowledge, the first epizootic of mycobacteriosis in a European colony of clawed frogs (*Silurana tropicalis*), previously imported from a United States biologic supply company. Our findings suggest the emerging potential of this infection through international trade.

Many species of nontuberculous mycobacteria inhabit the environment. *Mycobacterium fortuitum*, *M. chelonae, M. marinum,* and *M. xenopi* are some of the mycobacteria that infect amphibians, causing subcutaneous nodules, edema, and chronic wasting (*1*).

The aquatic, pipid frog, *Silurana tropicalis*, is an emerging laboratory model for genetic and embryologic/ontogenetic research. Although smaller than the related *Xenopus laevis*, *S. tropicalis* has research advantages: diploidy and brief maturation time make this species ideal for genetic analyses over multiple generations (*2*).

In 2004, Trott et al. characterized a new mycobacterial pathogen in pipid frog colonies (*3*), now named *M. liflandii* (*4*). On Middlebrook 7H11 agar supplemented with oleic acid, albumin, dextrose, and catalase, *M. liflandii* form rough, nonpigmented, slightly buff-colored colonies. Visible colonies develop after 30 to 35 days at 28°C on Löwenstein-Jensen (LJ) medium (*3*). This *M. ulcerans*–like mycobacterium produces a plasmid-encoded toxin, mycolactone E, which is less cytopathogenic than mycolactone A/B, produced by African *M. ulcerans* (*4*). *M. liflandii* infection in frogs manifests as cutaneous lesions, coelomitis, and bloating, with a high death rate (*3*).

We investigated an epizootic of *M. liflandii* in a colony of African tropical clawed frogs (*S. tropicalis*) in a European research laboratory. With the rising popularity of this vertebrate laboratory model and the foreseen establishment of stock centers for mutant or transgenic animals, the epizootiology of this emerging disease must be defined so that preventive measures may be instituted.

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The Study

In November 2004, we began to study an epizootic mycobacteriosis in a colony of imported captive *S. tropicalis,* the African tropical clawed frog. The Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology of Ghent University, Belgium, had imported *S. tropicalis* frogs from a supplier in the United States in September 2004. Within 5 weeks, some animals became lethargic with signs similar to those described by Trott et al.: loss of diving reflex, bloating, and ulcerative skin lesions (*3*). An average number of 2 deaths each week were reported in a colony of 300 specimens. Preliminary examination of 2 affected animals did not show chytridiomycosis; iridoviral infection; common bacterial infections of liver, lungs and kidneys; chlamydophila infection; or intestinal parasites.

From November 2004 through April 2005, 19 visually affected and visually unaffected specimens of *S. tropicalis,* 2 tadpoles, and 4 tank water samples were selected for detailed examination for mycobacteria. The frogs were euthanized and dissected, and selected organs and fluid were removed aseptically (liver, lungs, gallbladder, gastrointestinal tract, spleen, kidneys, fat body, ovary, oviduct, tibia, and coelomic fluid). Each of the specimens was divided into 2 equal parts, half for histopathologic analysis and half for preparation of decontaminated suspensions for culture and microscopic examination (*3*,*5*–*7*). Water samples were concentrated by filtration as described by Iivanainen et al. (*7*), and suspensions were made from the complete tadpoles (*6*). Further analyses were performed as described for the decontaminated frog suspensions. DNA for genetic analyses was extracted from the suspensions and pure cultures as described previously (*6*,*8*). *M. liflandii* was identified by IS*2404* nested PCR and sequence analysis of 16S rRNA gene (*9*,*10*). A combination of 4 genetic typing assays (including 3 previously investigated in *M. ulcerans)* was used to type *M. liflandii* (*3*,*9*,*11*,*12*). A flowchart of the performed tests is shown in Figure 1.

All visually affected specimens showed positive results for at least 1 organ and for at least 2 of the following tests: microscopy (Ziehl-Neelsen staining), in vitro cultivation (LJ medium and charcoal medium), IS*2404* nested PCR, and histopathologic examination. Of note, all ovarian tissue of the 11 visually affected specimens showed positive results for at least 2 tests. Three of 8 visually unaffected specimens showed positive results for at least 1 organ (including the ovary) and for 1 test. All tadpoles and water samples showed negative results for all tests.

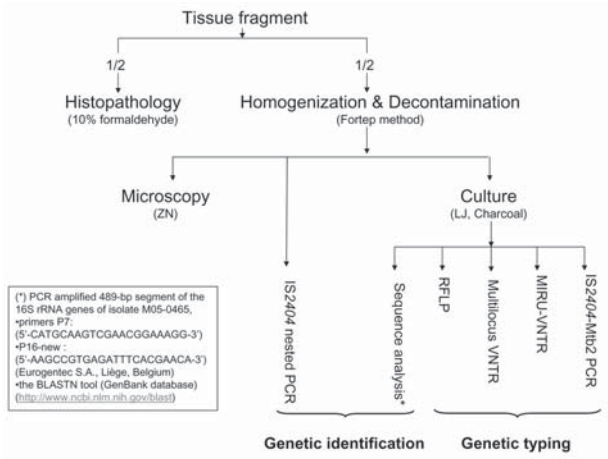

Figure 1. Flowchart of performed tests. ZN, Ziehl-Neelsen staining; LJ, Löwenstein-Jensen medium; Middlebrook 7H11 acidified with phosphoric acid, supplemented with sheep blood and charcoal; RFLP, restriction fragment length polymorphism; VNTR, variable number of tandem repeats; MIRU, mycobacterial interspersed repetitive unit.

Histopathologic evaluation showed many acid-fast bacilli (AFB) in the oviduct lumen (Figure 2). Numerous AFB were found in the kidney tubules, on the surface epithelium, and in the lumens of the gallbladder, stomach, intestine, and oviduct. Papillary hyperplasia of the gallbladder mucosa was marked, and the lamina propria was expanded by heterophils and many AFB (Figure 2). Lung parenchyma, liver, femur, and tibia were normal and free of AFB.

We identified the causative pathogen as *M. liflandii* in all frogs: by growth on charcoal medium, by restriction fragment length polymorphism, or by sequence analysis. Isolate M05–0456 had a similarity value of 100% with *M. liflandii* (GenBank accession no. AY845224.1). Growth on charcoal medium can be considered as an additional identification criterion for *M. liflandii* because growth on charcoal is better than on LJ medium (*3*), differentiating *M. liflandii* from *M. ulcerans*. Clinical isolates of *M. ulcerans* are grown readily on LJ medium but never on charcoal medium. The antibiogram of strain M04–2878 showed resistance to isoniazid, ethambutol, rifampin, clarithromycin, and ethionamide. In each of the genotyping assays, *M. liflandii* produced profiles that were distinct from those of *M. ulcerans* (data not shown). None of the laboratory staff who handled the anurans exhibited any signs of a mycobacterial disease.

Conclusions

The first epizootic of *M. liflandii* infection was reported by Trott et al. in 2004 in pipid frog colonies in the United States (*3*). To our knowledge, our report is the first account of *M. liflandii* disease in a colony of captive *S. tropicalis* frogs in Europe. We do not know the prevalence of *M. liflandii* infection in the colony, but we believe it was very high because 3 of 8 clinically healthy frogs were positive for *M. liflandii* by at least 1 test.

The genetic and phenotypic identification of *M. liflandii* as causative agent of the epizootic, the fact that cases of *M. liflandii* infection have not been reported in Europe to date, the strikingly similar signs and disease progress (*3*), and the probability that the frogs were imported from the same supplier (*3*) all suggest that some members of the imported *S. tropicalis* colony were infected with *M. liflandii* before arrival in Europe. Crowding and stress associated with captivity may have contributed to spread of infection within the colony. How and where the imported frogs became infected remains unknown (*3*). Additionally, during an extensive study in the Democratic Republic of Congo, Portaels (*13*) isolated 956 mycobacterial strains from the environment from Buruli ulcer–endemic regions. Among the unknown species, none was characterized as a *M. ulcerans*–like mycobacterium. To our knowledge, no *M. liflandii* infection, in humans or wild anurans, has been reported from Africa. We have confirmed that all isolates from Buruli ulcer patients and environmental samples analyzed by our laboratory were true *M. ulcerans* infections and not IS*2404* PCR-positive *M. ulcerans*–like mycobacteria (unpub. data).

The apparently enzootic character of *M. liflandii* infection in different *S. tropicalis* breeding companies in

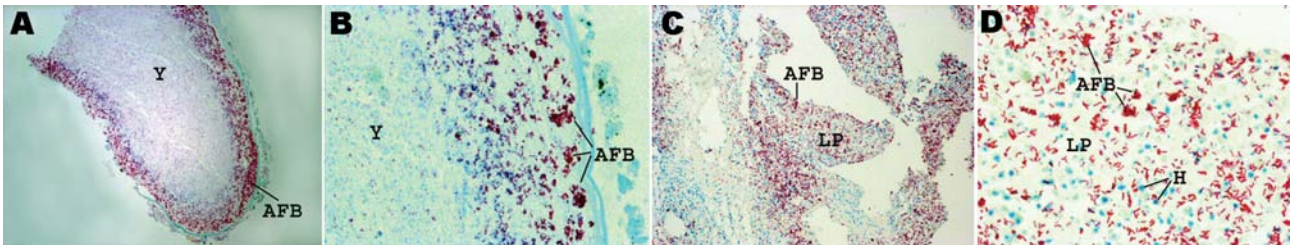

Figure 2. A) Oviduct, focally expanded by collections of macrophages and yolk (Y) material. Note acid-fast bacilli (AFB) throughout specimens, but concentrated at the periphery (Ziehl-Neelsen [ZN] stain ×25). B) High-power magnification of periphery of oviduct containing macrophages and Y material; AFB are concentrated at the periphery (ZN stain ×300). C) Gallbladder with papillary hyperplasia of the mucosa. Note masses of AFB in the lamina propria (LP) of the mucosa (ZN stain ×50). D) High-power magnification of the LP of the gallbladder mucosa showing large numbers of AFB and heterophils (H) (ZN stain ×300).

the United States (*3*,*4*) and the exchange of transgenic or mutant *S. tropicalis* lines between research laboratories, may pose a serious threat for the international research community working with this emerging laboratory model. Difficulties in detecting the pathogen in visually unaffected specimens and the high infection rate call for urgent efforts in the management of this epizootic disease. Thus far, no preventive measures or treatment for this amphibian mycobacteriosis are known (*3*,*4*,*14*). Resistance to antimycobacterial agents by environmental mycobacteria is not unusual and has been reported previously (*15*).

We propose examining the oocytes of newly imported frogs as an intervening noninvasive screening method on a regular basis, noting that all affected frogs reported in both intercontinental epizootics were females (*3*), oocytes from living adult *S. tropicalis* are easily obtained for research purposes (*2*), and ovarian tissue was positive for all visually affected specimens and for 1 of 3 positive visually unaffected specimens. However, further studies are needed to determine the role of oocytes in the epizootics of this emerging frog disease, especially in the evaluation of our proposed screening method. To prevent the infection of existing stocks with wild-caught frogs of unknown origin, we further recommend the importation of only certified pathogen-free laboratory-bred specimens from recognized biologic suppliers. Recently, Tarigo et al. reported a frog mycobacteriosis in an adult female, albino South African clawed frog (*X. laevis*) in a research colony at North Carolina State University (*14*). The etiologic agent was identified as *M. marinum* complex on the basis of mycobacterial culture, but genetic analyses were not performed to exclude *M. liflandii* infection. To avoid further spread of this disease, every new outbreak of *M. liflandii* infection in pipid frogs or other anuran species should be reported to relevant authorities and research communities. Until more is known about this epizootic and its prevention and treatment, caution must be exercised in transportation, husbandry, and human contact with these animals (zoonotic potential). We do not know at this stage whether the importation of frogs contaminated by *M. liflandii* represents a danger for wild or autochthonous frogs. Further investigation is required to establish this.

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Environmental Source of *Candida dubliniensis*

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We isolated *Candida dubliniensis* from a nonhuman source, namely, tick samples from an Irish seabird colony. The species was unambiguously identified by phenotypic and genotypic means. Analysis of the 5.8S rRNA gene showed that the environmental isolates belong to *C. dubliniensis* genotype 1.

The ever-increasing number of immunosuppressed humans has led to a marked rise in opportunistic infections, particularly those caused by fungi (*1*). *Candida albicans* is the yeast species most commonly associated with oropharyngeal and systemic candidiasis in immunocompromised persons. However, the last 2 decades have seen an increase in infections by other *Candida* species, including *C. dubliniensis,* which was first recognized as distinct from *C. albicans* in 1995 in Ireland (*2*,*3*). *C. dubliniensis* has been recovered mainly from the oral cavities of HIV-infected persons (*4*) but also from lungs, vaginas, blood, and feces; occasionally this organism causes fatal systemic infections (*5*). Isolates are assigned to 4 genotypes, defined by the sequence of the internal transcribed spacer regions of the rRNA gene (*6*).

C. dubliniensis is globally distributed. In HIV-infected patients, the oral prevalence is 1.5%–32% (*5*). In healthy persons not infected with HIV, *C. dubliniensis* is absent or rare, but 14% of healthy Caucasians had oral *C. dubliniensis* in a South African study (*7*). Like *C. albicans*, *C. dubliniensis* may be a member of the normal oral microbial flora of humans, and oral candidosis may result from overgrowth of resident strains. In contrast to other *Candida* species, some of which are associated with birds (*8*,*9*), *C. dubliniensis* has not been found to date in nonhuman environmental sources. This has led to speculation that the species may be restricted to humans, possibly occupying sites deep within the oropharynx or upper respiratory tract (*5*).

The Study

Fungal strains were obtained from *Ixodes uriae* ticks (as part of a National Environment Research Council–funded study of a tickborne virus) at a seabird breeding colony on Great Saltee Island, Ireland (52°07′N, 6°36′W). The ticks were taken from cracks in cliffs used by common guillemots (*Uria aalge*). Tissue cultures of tick homogenates undertaken for virus isolation were occasionally contaminated with yeastlike fungi.

To investigate this, individual adult ticks were homogenized in 1 mL minimum essential medium (MEM). After centrifugation (30 s, 10,000× *g*), 0.2 mL of supernatant was added to 4 mL MEM, 5% fetal bovine serum, and 100 μg/mL penicillin-streptomycin. Cultures incubated at 37°C were examined microscopically daily for up to 6 days. Positive cultures were plated twice on Sabouraud dextrose agar (SAB) with chloramphenicol (bioMérieux, Marcy l'Etoile, France) before phenotypic testing. Isolates were identified by using API identification kits (bioMérieux) and by conventional methods (*10*). Antifungal drug susceptibility was tested according to the Clinical Laboratory Standards Institute guidelines (*11*). The control strains were *C. albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, and *C. dubliniensis* NCPF 3949.

Internal transcribed spacer 1 and 2 regions (ITS1/ITS2) and the 5.8S rRNA gene were amplified with primers ITS1 and ITS4, described by White et al. (*12*). Template DNA was prepared by boiling single SAB-grown colonies in 50 μL ultrapure water for 10 min. After centrifugation (5 min 10,000× *g*), 15 μL supernatant was added to 50 μL PCRs containing 1× reaction buffer, 1 μmol/L ITS primers, 1.5 mmol/L $MgCl_2$, 400 μmol/L deoxynucleoside triphosphates, and 2.5 U Immolase (BiolineLtd, London, England, UK). Cycling parameters were 7 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 58°C, 1 min at 72°C, and a final extension of 5 min at 72°C. Products were purified (QiaQuick kit, QIAGEN Ltd., West Sussex, England, UK) and sequenced (BigDye kit and ABI 377 sequencer; Applied Biosystems, Foster City, CA, USA) by using the ITS primers. Sequences were assembled by using Lasergene 6, Seqman version II, and aligned by using BioEdit software (*13*).

Fungal isolation was undertaken on 2 separate days with samples from 2 distinct sites on the island (Table 1). On both days, Happy Hole West (HHW) ticks were processed immediately after Labour in Vain (LIV) ticks in the same class II microbiologic cabinet. No fungi were detected in HHW ticks, whereas 16.7%–27.6% of ticks sampled from 2 locations within LIV gave positive cultures (Table 1). Twenty-two isolates were obtained (Table 1); SL370–429 were from LIV-1 and SL495–531 from LIV-2 (SL = Saltee).

On SAB the colonies from positive cultures were a creamy white color with a glabrous appearance similar to *C. albicans*. SL375 had a mixed phenotype (large and small colonies, designated SL375–1 and SL375–2). Like *C. albicans*, all SL isolates were germ-tube positive and produced chlamydospores at 37°C on Corn Meal Tween 80

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Table 1. Male and female ticks positive for fungi in culture*

Site	No. positive/no. examined		
	Male	Female	Both sexes
Happy Hole West (HHW)–1	0/26	0/25	0/51
HHW-2	0/17	0/23	0/40
Labour in Vain (LIV)–1	5/30	8/29	13/59†
LIV-2	5/23	4/20	9/43‡

*Adult *Ixodes uriae* ticks were collected from within 2 guillemot-breeding colonies on Great Saltee on August 25, 2004. The ticks were stored frozen and processed by M.A.N on November 11, 2005. (HHW-1 and LIV-1) and 20. 07.06 (HHW-2 and LIV-2).
†Isolates SL370, SL371, SL375, SL387, SL397, SL407, SL410, SL411, SL413, SL414, SL417, SL422, and SL429.
‡Isolates SL495, SL497, SL500, SL501, SL509, SL510, SL522, SL529, and SL531.

agar (Oxoid Ltd, Basingstoke, England, UK) and Czapek Dox (1%) Tween 80 agar (Oxoid) (Figure 1A). None of the SL isolates grew at 43°C on SAB (Figure 1B), which suggested that they might be *C. dubliniensis* (*14*). This was confirmed by carbohydrate assimilation tests (Table 2) and by sequencing the 5.8S rRNA gene (Figure 2). With the API 20C AUX kit, all SL isolates yielded the same profile at 48 h, interpreted as 99.9% *C. dubliniensis* (Table 2). Eleven isolates from LIV-1 and the *C. dubliniensis* (NCPF 3949) reference strain were also tested with API 32C. All had an identical profile (7143100015), interpreted as 81.9% *C. dubliniensis* and 16.9% *C. albicans*.

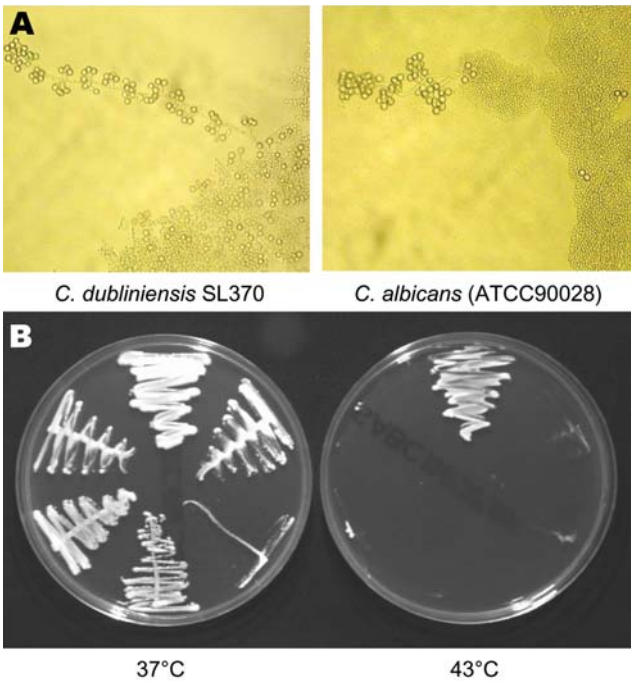

Figure 1. Phenotypic characteristics of environmental *Candida dubliniensis* isolates and reference strain of *C. albicans*. A) Morphology of pseudohyphal terminal chlamydospores of *C. albicans* (ATCC90028) and *C. dubliniensis* SL370 grown at 37°C on Corn Meal Tween 80 agar. Magnification × 50. B) Growth of representative Great Saltee (SL) isolates on Sabouraud agar after 48 h of incubation at 37°C and 43°C. The growth of the following isolates is shown: *C. albicans* (ATCC90028), *C. dubliniensis* (NCPF3949), and *C. dubliniensis* SL370, SL397, SL407, and SL410 (clockwise from the top in each petri dish).

ITS sequences of isolates SL375, SL397, SL407, SL410, SL411, SL417, and SL422 were identical to that of *C. dubliniensis* CD33 genotype 1 (Figure 2). Nevertheless, phenotypic variation among the SL isolate was evident. In addition to variation in trehalose assimilation rates, 3–4 distinct types were apparent in the germ tube test. Three independent inoculations (10^5 CFU/mL, mid log growth phase) of each isolate gave consistent morphologic differ-

Table 2. Substrate assimilation by Great Saltee fungi and *Candida albicans*

Substrate	API 20C AUX assimilation profile code*	
	SL407	*C. albicans* (ATCC90028)
Pentoses		
L-arabinose	–	–
D-xylose	–	+
Hexoses		
D-glucose	+	+
D-galactose	+	+
α-methyl-D-glucoside	–	+
Disaccharides		
D-cellobiose	–	–
D-lactose	–	–
D-maltose	+	+
D-saccharose	+	+
D-trehalose†	–	+
Trisaccharides		
D-melezitose	–	–
D-raffinose	–	–
Alcohols		
Glycerol	+	–
Adonitol	+	+
Xylitol	+	+
Inositol	–	–
D-sorbitol	+	+
Organic acids		
2-keto-gluconate	+	+
Amino acids		
N-acetylglucosamine	+	+
Identification	*C. dubliniensis*	*C. albicans*
API 20C AUX profile code	6172134	2566174
Predictive value	99.9%, excellent	99.2%, very good

*Results for 48 hours are shown. All 22 Great Saltee isolates gave similar profiles.
†By 72 hours all Saltee isolates showed some assimilation of trehalose; the degree of assimilation varied between isolates.

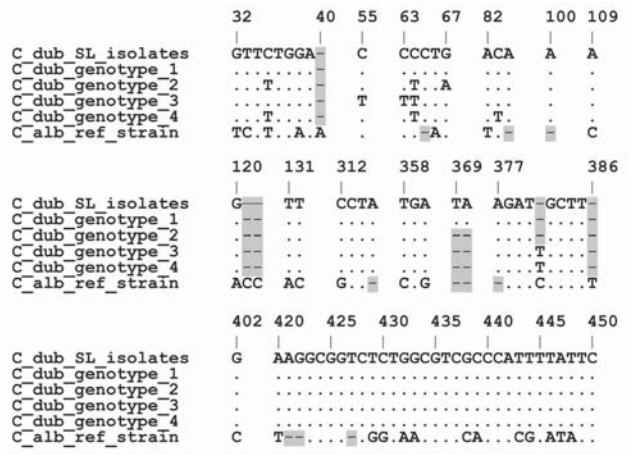

Figure 2. Alignment of the ITS1 5.8S ITS2 DNA region of *Candida dubliniensis* Saltee (SL) isolates and representative human isolates of genotypes 1–4. All polymorphic sites are shown: ITS1 (bp 32–132), 5.8S rRNA exon (bp 312–315), ITS2 (bp 358–450). Dots indicate identity; highlighted dashes indicate gaps in the alignment. GenBank accession nos. are as follows: AJ311895 (CD33 genotype 1), AJ311896 (CD520 genotype 2), AJ311897 (CD519 genotype 3); AJ311898 (p7718 genotype 4), AB049119 (*C. albicans* ATCC90028), and EF032487–EF032495 for SL375–1, SL397, SL375–2, SL387, SL407, SL410, SL411, SL417, SL422, respectively.

ences. One group produced very long germ tubes (SL375–1, SL422, SL417 SL387, SL397, and SL413); another, medium size (SL411, SL407); a third, mainly long germ tubes with cells that aggregate like *C. parapsilosis* (SL375–2 and SL410); and the last, elongated cigarlike cells that clamped together with few germ tubes (SL370 and SL371).

To determine whether *C. dubliniensis* was present on their outer surface LIV-2 and HHW-2 ticks (n = 102) were individually washed in 1 mL MEM before homogenization. *C. dubliniensis* was cultured from the wash supernatants of 8 of 9 ticks that proved positive after homogenization but not from any of the negative ticks. These findings strongly suggest that the fungus is present in particles adhering to the ticks.

The SL isolates (n = 11) were extremely sensitive to antifungals. MIC90s (μg/mL) were as follows: amphotericin B 0.031 (Sigma-Aldrich, Saint Louis, MO, USA), flucytosine 0.031 (Valeant Pharmaceuticals International, Aliso Viejo, CA, USA), fluconazole 0.125 (Pfizer Inc., Sandwhich, Kent, England, UK), itraconazole 0.007 (Ortho Biotech, Bridgewater, NJ, USA), voriconazole 0.007 (Pfizer), posaconazole 0.007 (Schering-Plough, Kenilworth, NJ, USA), ketoconazole <0.007 (Ortho Biotech), and caspofungin <0.007 (Merck & Co., Inc., Rahway, NJ, USA).

Conclusions

Our serendipitous isolation of *C. dubliniensis* from the environment ends speculation (*5*) that the species might be confined to humans. Because ticks were collected from cracks filled with guillemot guano and the fungal isolates were associated with the surface of ticks, the most likely source of the fungus is bird excrement. Thus, like *C. albicans* (*8*), *C. dubliniensis* may inhabit the digestive tract of *marine* birds. Only *C. dubliniensis* was isolated from the Great Saltee ticks; however, we have detected other *Candida* spp. from both soil and tick samples (unpub. data) at other seabird colonies, which suggests that marine as well as terrestrial birds (*9*) carry a variety of yeast species (*15*).

The environmental isolates are members of human C. dubliniensis genotype 1, commonly associated with HIV patients (*6*). Sequencing of the genotype 1 CD36 isolated from an HIV patient is nearly complete (www.sanger.ac.uk/sequencing/Candida/dubliniensis). Phylogenetic comparison of variable loci from environmental and human genotype 1 isolates could be used to estimate when they last shared a common ancestor and thus how often *C. dubliniensis* is transmitted from the environment to humans.

Acknowledgments

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Gulf Coast Ticks (*Amblyomma maculatum*) and *Rickettsia parkeri*, United States

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Geographic distribution of *Rickettsia parkeri* in its US tick vector, *Amblyomma maculatum*, was evaluated by PCR. *R. parkeri* was detected in ticks from Florida, Georgia, Kentucky, Mississippi, Oklahoma, and South Carolina, which suggests that *A. maculatum* may be responsible for additional cases of *R. parkeri* rickettsiosis throughout much of its US range.

The Gulf Coast tick, *Amblyomma maculatum* (Figure), is a Nearctic and Neotropical hard tick found in coastal areas of the southern United States, with inland range extensions in Kansas, Oklahoma, and some other states. It is also found in regions of several Central and South American countries that border the Gulf of Mexico and Caribbean Sea, including Mexico, Guatemala, Belize, Nicaragua, Honduras, Costa Rica, Colombia, Venezuela, and some parts of Ecuador and Peru (*1*).

Rickettsia parkeri, a member of the spotted fever group rickettsiae, was initially identified in Gulf Coast ticks in 1937 (*2*). In 2004, the first confirmed human infection with *R. parkeri* was reported (*3*). Since that report, confirmed cases of *R. parkeri* rickettsiosis have been identified in other persons in Mississippi, Virginia, and possibly other US states (*4*–*6*). Only a few studies, each conducted >50 years ago, document the occurrence of *R. parkeri* in *A. maculatum* ticks (*2*,*7*,*8*). No contemporary surveys have documented the range of *R. parkeri* in the United States or the frequency of *R. parkeri* infection in collections of individual Gulf Coast ticks.

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The Study

A. maculatum ticks collected during 1996–2005 were evaluated by molecular methods for evidence of infection with *R. parkeri*. Ticks were collected from various locations in Florida, Georgia, Kentucky, Mississippi, Oklahoma, and South Carolina. Most were questing adults collected from vegetation by using flannel cloth flags; a few crawling, nonattached, nonengorged adults were obtained (4 from a coyote and 3 from human hosts), and 1 engorged nymph was removed from a cotton rat. Ticks were preserved in 70% ethanol or frozen at –80ºC until evaluation.

Most individual ticks were minced with a sterile scalpel blade, and DNA was extracted by using a QIAamp Mini Kit (QIAGEN, Inc., Valencia, CA, USA). Others were minced or crushed after freezing in liquid nitrogen, and DNA was extracted by using an IsoQuick nucleic acid extraction kit (ORCA Research, Bothell, WA, USA). DNA extracts were evaluated by using nested or heminested PCR assays designed to amplify a segment of the *rompA* gene. For the primary stage of each assay, 5 μL of extract and primers 190–70 and 190–701 (*9*) were used. For the nested reaction, 2 μL of completed primary reaction was used as template with primers 190-FN1 (5′-AAG CAA TAC AAC AAG GTC-3′) and 190-RN1 (5′-TGA CAG TTA TTA TAC CTC-3′); for the heminested reaction, primers 190–FN1 and 190–701 were used. All reactions were prepared by using a High Fidelity PCR Master Kit (Roche Diagnostics, Indianapolis, IN, USA) with final primer concentrations of 300 nmol in a total volume of 50 μL. Thermalcycler parameters for the primary stage consisted of an initial denaturation period of 2 min at 94ºC, followed by 40 cycles of 15 s at 94ºC, 30 s at 60ºC, 45 s at 72ºC, and a 5-min extension period at 72ºC. For the nested and heminested stages, the annealing temperature was changed to 55ºC and the number of cycles was reduced to 30.

PCR products (10 μL) were separated by electrophoresis in 2% agarose gels containing ethidium bromide. For each positive reaction, the remaining 40 μL was subjected

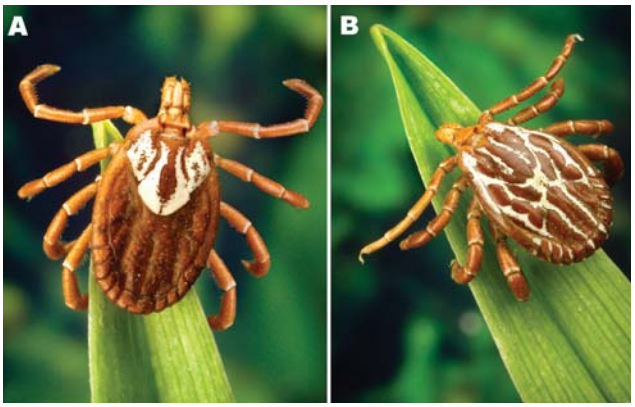

Figure. Adult *Amblyomma maculatum* (the Gulf Coast tick). A) Female; B) Male.

Photographs courtesy of James Gathany, Centers for Disease Control and Prevention

to gel electrophoresis, and products of the appropriate size were excised. DNA was purified from the gel fragments by using the QIAquick Gel Extraction Kit (QIAGEN). Purified PCR products were sequenced using the PCR primers and the GenomeLab DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA). For some products, additional sequencing primers 190-SF3 (5′-GGT ACT ACT CCC GTA GGT C-3′) and 190-SR2 (5′-CCG GCA GTA AKA GTA ACA G-3′) were used to obtain complete sequences for both strands. Sequences were detected by using a Beckman CEQ 8000 automated sequencer. Sequence similarities were determined by using the BLAST program (version 2.0, National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/blast). Sequence-length reaction products (excluding primers) were 590 bp for primary, 559 bp for heminested, and 540 bp for nested.

DNA of *R. parkeri* was amplified from 21 (11.5%) of 182 male and female adult *A. maculatum* ticks collected in Georgia (11/64), Florida (7/89), Kentucky (1/1), Mississippi (1/24), and South Carolina (1/4) and from 1 engorged nymph collected in Oklahoma (Table). A unique *rompA* sequence (GenBank accession no. EF372578) amplified from 5 adult female ticks collected in Florida, Georgia, and Mississippi showed closest homology (≈94%) to several other *rompA* sequences (GenBank accession nos. EF063690, AY093696, AF120021, and DQ365801). PCR amplification of a 208-bp segment of the rickettsial 17-kDa antigen gene (*10*) from these same 5 ticks (GenBank accession no. EF372579) showed 100% homology with the corresponding sequences of *Rickettsia* sp. Arahna (AY360215), *R. montanensis* (DQ402377), *Rickettsia* sp. Hf332 (AB114804), *Rickettsia* sp. Is-1 (DQ344620), and "R. gravesii" (DQ269436).

Precise estimates of infection prevalence could not be assessed from these data because most of the ticks evaluated in this study were collected as relatively small sample sizes or were collected in a discontinuous manner as multiple samples from the same sites over several weeks or months in a given year. However, some collections were flagged synchronously at a single location, including those in Copiah County, Mississippi, during July 2002 (n = 9) and in Franklin County, Florida, during July 2004 (n = 25) and July 2005 (n = 27). Infection prevalence for each of these 3 collections was 11%–12%, which suggests that *R. parkeri* may be a relatively common inhabitant of some populations of Gulf Coast ticks. By comparison, the estimated prevalence of infection of tick vectors with *R. rickettsii*, the etiologic agent of Rocky Mountain spotted fever (RMSF), is typically much lower, as determined by surveys elsewhere, which identified *R. rickettsii* in only 0.05%–1.3% of the collected specimens: 3,705 *Dermacentor andersoni* ticks from canyons bordering the Bitterroot Valley of Montana; 2,123 and 310 *D. variabilis* ticks from RMSF-endemic areas of North Carolina and Ohio, respectively; and 669 *A. aureolatum* ticks from São Paulo, Brazil (*11–14*).

Conclusions

R. parkeri has been isolated in culture from Gulf Coast ticks collected in Alabama, Florida, Georgia, Mississippi, and Texas ([*2,7,8*], C. Paddock, unpub. data). These re-

Table. Gulf Coast ticks (*Amblyomma maculatum*) infected with spotted fever group rickettsiae, United States, 1996–2005

Rickettsia sp. identified	State	County	Total ticks positive/total ticks tested*	Year collected	Stage, sex†	Source
Rickettsia parkeri	Florida	Duval	1/9	1999	Adult, F	Vegetation
		Franklin	3/25	2004	Adults, M, F	Vegetation
			3/38	2005	Adults, M, F	Vegetation
	Georgia	Bulloch	2/20	1999	Adults, M, F	Vegetation
			5/16	2003	Adults, M, F	Vegetation
			3/24	2005	Adults, M, F	Vegetation
		McIntosh	1/4	2005	Adult, F	Vegetation
	Kentucky	Montgomery	1/1	2003	Adult, M	Human
	Mississippi	Copiah	1/9	2002	Adult, M	Vegetation
	Oklahoma	Pittsburgh	1/1	1997	Nymph‡	*Sigmodon hispidis*
	South Carolina	Anderson	1/4	2005	Adult, M	*Canis latrans*
Noncharacterized *Rickettsia* sp.§	Florida	Nassau	1/13	1996	Adult, F	Vegetation
		Franklin	1/38	2005	Adult, F	Vegetation
	Mississippi	Copiah	1/9	2002	Adult, F	Vegetation
	Georgia	Bulloch	1/20	1999	Adult, F	Vegetation
			1/24	2005	Adult, F	Vegetation

*Identified by PCR; some specimens obtained from a given county during a given year were not collected synchronously. Collections without PCR-postive ticks are not listed.
†Nonengorged specimens unless otherwise specified.
‡Engorged specimen.
§Based on a unique *rompA* sequence.

sults, combined with data from the present study, suggest that in the United States *R. parkeri* can be found anywhere that *A. maculatum* ticks are found. In this context, persons exposed to habitats in any region infested by Gulf Coast ticks are potentially vulnerable to infection with *R. parkeri*. A previously undescribed *rompA* sequence, identified in a few ticks collected during this survey, may represent a novel species of spotted fever group rickettsiae associated with the Gulf Coast tick. Attempts to isolate and characterize this species are in progress. Collectively, these findings suggest that the role of *A. maculatum* in the ecology of various spotted fever group rickettsiae deserves further attention. These results and the recent discovery of *R. parkeri* in *A. triste* ticks in Uruguay (*15*) indicate that much remains to be learned about *R. parkeri* and other rickettsiae of human-biting ticks in the Western Hemisphere and their relative contributions to the epidemiology of New World spotted fevers.

Mr Sumner is a molecular biologist at the Centers for Disease Control and Prevention, where he has worked extensively on the molecular detection of various *Rickettsia*, *Ehrlichia*, and *Bartonella* spp. for >15 years. His research interests now focus primarily on PCR-based evaluation of formalin-fixed tissues for bacterial and viral pathogens.

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Outbreak of *Yersinia enterocolitica* Serogroup O:9 Infection and Processed Pork, Norway

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An outbreak involving 11 persons infected with *Yersinia enterocolitica* O:9 was investigated in Norway in February 2006. A case-control study and microbiologic investigation indicated a ready-to-eat pork product as the probable source. Appropriate control measures are needed to address consumer risk associated with this product.

Yersiniosis, which is reportable in Norway, is the third most commonly reported cause of acute enteritis after campylobacteriosis and salmonellosis. In the past 10 years, 80–150 cases of yersiniosis were registered annually; 1–10 of these cases were caused by serotype O:9. Most patients (70%–80%) acquire infection domestically (*1*). During January 2006, the National Reference Laboratory at the Norwegian Institute of Public Health (NIPH) received 6 human isolates of *Yersinia enterocolitica* O:9, which clearly exceeded the expected incidence. All of the infections were diagnosed within a 3-week period (*2*). A multidisciplinary outbreak investigation team was established to find the source and prevent further illness.

The Study

A case-patient was defined as a resident of Norway with *Y. enterocolitica* O:9 isolated from stool or blood and with illness onset between December 15, 2005, to February 15, 2006. Cases were identified through the National Surveillance System at NIPH, which receives reports of laboratory-confirmed cases from laboratories and clinicians nationwide. Eleven cases of *Y. enterocolitica* O:9, biotype 2 infection were identified from December 21, 2005, through February 6, 2006 (Figure). Patients resided in 2 neighboring counties in southern Norway; all were adults except for a 10-year-old child. The median age was 44 years (range 10–88 years); 6 (54%) were female. Reported symptoms included severe abdominal pain, diarrhea, fever, arthralgia, and vomiting. Reactive arthritis developed in 1 patient. Symptoms lasted for a median of 14 days (9 days–6 weeks); 3 patients reported that symptoms had not resolved at the time of interview. Four patients were hospitalized, and 2 patients died; both were elderly with underlying medical conditions.

The first 6 case-patients were interviewed by telephone, with a standard hypothesis-generating questionnaire requesting information on clinical symptoms and demographic data, in addition to food history, animal contacts, and environmental exposures in the 7 days before illness. On the basis of interview results, a case-control study was conducted that included 8 case-patients and 22 controls randomly selected from the national population register (ratio 1:3). Controls and case-patients were matched by age, sex, and municipality of residence. The median interval between illness onset and interview was 33 days (26–49 days), and case-patients and controls were interviewed on the same day. Controls were questioned about exposures in the week before illness of their matched case-patient. Matched odds ratios (mORs) from univariate analysis were calculated by using maximum-likelihood estimates, including 95% exact confidence intervals (CIs) by Fisher exact test (STATA 8.0, Stata Corporation, College Station, TX, USA). The same software was used for multivariate analysis by conditional logistic regression.

All isolates of *Yersinia* spp. from human patients were characterized phenotypically (*3*), biotyped (*4*), and serogrouped against absorbed rabbit antiserum produced at NIPH, representing O-antigen factors 1–34 (*5*). All suspected foods were examined for pathogenic *Y. enterocolitica* at the Norwegian Veterinary Institute by using 2 conventional cultivation methods (*6*,*7*). Isolates identified as *Y. enterocolitica* were forwarded to the National Reference Laboratory for verification and serotyping. Nested PCR targeting of chromosome-located virulence genes was also used (*8*).

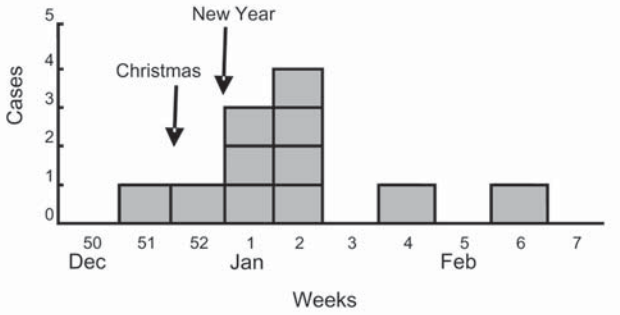

Figure. Distribution of patients with *Yersinia enterocolitica* 0:9 infection (n = 11) by week of onset, Norway, December 2005–February 2006.

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In univariate analysis, eating processed pork product ("julesylte," Christmas brawn) was associated with *Y. enterocolitica* O:9 infection (mOR 6.7, 95% CI 0.7–64.1). Patients were also more likely than controls to have eaten pork chops (mOR 5.9, 95% CI 0.6–61.3) (Table 1). Consumption of brawn and consumption of pork chops were both independently related to increased risk in conditional logistic regression analysis. On February 10, the public health and food safety authorities issued a public warning advising consumers against eating brawn.

The case-control study did not identify specific producers. Consequently, products were sampled on the basis of tracing of product deliveries to implicated shops. Sixty-two samples were examined from major producers and small local butchers; the samples comprised 54 brawn (3 obtained from opened packages from patients' homes, 9 from opened and sliced batches at retailers), 1 cooked ham, and 7 other pork products from patients' homes (Table 2). None yielded pathogenic *Yersinia* organisms by culture methods. However, nonpathogenic *Y. enterocolitica* or *Y. intermedia* strains were isolated from 6 (10%) samples. Positive PCR results were obtained for 20 (32%), including 16 brawn samples from patients' homes and retailers. Eleven of the 20 positive brawn from unopened packages came from 6 different producers. To ascertain whether positive PCR results were from viable or dead target bacteria, colonies from enrichment cultures were retested by nested PCR. Nineteen colonies, including those originating from brawn from patients' homes, were positive. DNA sequence analysis of the PCR product from 1 brawn from a patient's home showed 321 of 322 base pairs matched when compared with the PCR product obtained from 1 random patient isolate.

No other public health measures were introduced because brawn is sold and consumed only at Christmas. Other, long-term measures—including establishing levels of contamination of raw materials and ready-to-eat pork products, assessment of production processes, and establishing reliable microbiologic methods—were suggested.

Conclusions

To our knowledge, this investigation confirmed for the first time both an outbreak of *Y. enterocolitica* O:9 in Norway and a ready-to-eat pork product associated with an outbreak. Both epidemiologic and microbiologic findings suggested brawn as the probable source.

Several studies have found pork consumption to be associated with yersiniosis (*9*–*11*). Risk factors for sporadic *Y. enterocolitica* infections were also assessed in a prospective case-control study in Norway in 1988–1990 (*12*). Results from that study showed that infected persons ate significantly more pork products and sausages than did matched controls. Case-patients were also more likely than controls to prefer raw or rare meat and to drink untreated water. All factors were independently associated with disease. Because of the importance of pork products in yersiniosis epidemiology, attempts were made to identify hazards in swine slaughter so that appropriate preventive measures could be defined (*13*). Measures implemented at farm herd level, in abattoirs, and during meat processing were suggested to minimize contamination of retail pork products (*14*). Introduction of these measures has resulted in considerable reduction in contamination and a decrease of human yersiniosis in Norway (*15*).

Although the case-control study suggested brawn as the outbreak source, it also showed an association with eating pork chops. The power of the case-control study was limited by the small number of cases. However, in-depth interviews with 10 of 11 patients—as well as with patients not included in the case-control study—showed that all had eaten brawn.

Brawn is traditional at Christmas. It is prepared by layering pork meat (precooked head muscles), veal, lard, and spices in a mold. The brawn mold is cooked to an assumed core temperature of 74°C before the temperature is reduced and the meat is maintained at a temperature of 70°C for at least 30 min. After removal from the mold, the brawn is vacuum-packed for sale—usually whole, although some producers package sliced products. At some delicatessen

Table 1. Selected exposures of patients with *Yersinia enterocolitica* O:9 infection and matched controls, univariate analysis, Norway, February 2006*

Food exposure	Case-patients, N = 8 Exposed/total (%)	Controls, N = 22 Exposed/total (%)	Matched OR (95% CI)
Brawn	5/8 (63)	5/22 (23)	6.7 (0.7–64.1)
Pork chops	4/7 (57)	5/20 (25)	5.9 (0.6–61.3)
Wiener sausage	5/7 (71)	13/21 (62)	1.6 (0.2–10.3)
Smoked sausage	3/8 (38)	7/21 (33)	1.2 (0.2–6.0)
Salami	3/8 (38)	6/22 (27)	1.2 (0.1–11.3)
Pork burgers	3/6 (50)	10/20 (50)	1.2 (0.2–8.0)
Roulade	2/8 (25)	4/21 (19)	1.1 (0.2–6.3)
Ribs	4/8 (50)	11/21 (52)	0.9 (0.2–5.0)
Saveloy	2/7 (29)	6/21 (29)	0.9 (0.1–6.0)

*OR, odds ratio; CI, confidence interval.

Table 2. Results from PCR analyses of pork products sampled during investigation of *Yersinia enterocolitica* O:9 outbreak, Norway, 2006

Sample types	No. samples	Positive		Negative	
		Broth culture	Colony swab	Broth culture	Colony swab
Brawn	54	16	16	38	38
Cooked ham	1	1	1	0	0
Other pork products	7	3	2	4	5
Total	62	20	19	42	43

counters, packages might be opened and products sliced. Some people also prepare brawn at home.

Correct cooking should eliminate *Y. enterocolitica,* but microbiologic analyses have shown bacteria can survive in the product core. Lard, with its high fat content, can enhance survival of the bacteria. Furthermore, cross-contamination can occur if good hygiene procedures are not strictly observed when the brawn is removed from the mold before packaging. At that point, contamination is superficial, but slicing would spread microorganisms throughout the product. The ability of *Y. enterocolitica* to grow at refrigeration temperatures gives it an advantage over other microorganisms and may further compound the problem.

Despite existing control measures, this outbreak demonstrated that ready-to-eat processed meats may represent an important risk for consumers to acquire *Yersinia* infection. Producers need to be reminded of their responsibility to ensure safe products, since consumers cannot always take precautions. Research to clarify the role of pork meat in *Yersinia* epidemiology and improved laboratory methods for detecting pathogenic *Y. enterocolitica* in food are needed.

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Enteroaggregative *Escherichia coli* Related to Uropathogenic Clonal Group A

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Enteroaggregative *Escherichia coli* (EAEC) are heterogeneous, diarrheagenic *E. coli.* Of EAEC strains from Nigeria, 10 independent antimicrobial-resistant isolates belonged to the multilocus sequence type 69 clonal complex, to which uropathogenic *E. coli* clonal group A belongs. This finding suggests a recent common ancestor for these distinct groups of pathogenic *E. coli.*

Enteroaggregative *Escherichia coli* (EAEC) is an emerging category of diarrheagenic *E. coli.* EAEC are heterogeneous, and the distribution of known virulence genes rarely correlates with phylogeny based on housekeeping loci (*1*). We recently identified 2 loci, involved in iron acquisition, that are distributed among EAEC in a manner that correlates with multilocus enzyme electrophoresis typing based on 20 housekeeping enzymes (*2*). This finding supports the possibility that EAEC clonal groups with increased pathogenic potential exist.

The Study

To identify overrepresented subgroups of potential clonal origin, we examined 131 EAEC strains isolated from children in Nigeria (*3*), 73 from 187 children with diarrhea and 58 from 144 healthy control participants. All 131 strains had previously been categorized as EAEC by the standard HEp-2 cell adherence assay (*3*,*4*). To determine flagellin types, we used an *Rsa*I-based PCR–restriction fragment length polymorphism (RFLP) protocol. Using primers F-FLIC1 (5′-ATGGCACAAGTCATTAATACCCAAC-3′) and R-FLIC2 (5′-CTAACCCTGCAGCAGAGACA-3′), we obtained an internal *fliC* amplicon from 105 (80.1%) of the 131 isolates. RFLP analysis delineated ≥31 flagellin genotypes among these amplicons (online Appendix Table, available at www.cdc.gov/EID/content/13/5/757-appT.htm). Using 16 control strains, which represented 12 antigenically distinct H-types verified at reference centers, we could associate a specific H antigen with 10 of these RFLP patterns. The most common pattern, which corresponded with the H18 antigen, accounted for 18 (17%) of the genotyped isolates (or 14% of all isolates). Two H7 control strains had different genotypes, which indicates that the products of 2 different alleles are recognized by anti-H7 antiserum. Both genotypes were detected among the test EAEC strains (online Appendix Table) and together accounted for 10 (7.6%) of the isolates. Other predominant *fliC* variants were H2 (3%), H11 (3.8%), H21 (7.6%), and H45 (4.9%). Although some *fliC* variants were somewhat more common among strains from children with diarrhea than from control participants (online Appendix Table), differences were not statistically significant ($p>0.05$, Fisher exact test).

Antimicrobial susceptibility profiles were determined by disk diffusion as specified by the Clinical and Laboratory Standards Institute (*5*). Disks containing ampicillin (10 μg), tetracycline (30 μg), trimethoprim (5 μg), nalidixic acid (30 μg), chloramphenicol (30 μg), sulfonamide (300 μg), streptomycin (10 μg), and ciprofloxacin (5 μg) were used for testing on Mueller-Hinton agar (Oxoid, Lenexa, Kansas, USA). Of the 18 Nigerian H18 strains, 13 had the same resistance pattern: ampicillin, chloramphenicol, streptomycin, sulfonamide, tetracycline, trimethoprim (Table 1). PCR identified 3 EAEC-specific and 2 other virulence genes (*2*,*6*). Markers of well-characterized aggregative adherence plasmids, present in typical EAEC strains, are aggregative adherence regulator gene *aggR*, anti-aggregative protein or dispersin gene *aap,* and empiric plasmid probe (CVD432) that represents part of the *aat* secretion system operon (*6*). Of the 131 isolates, <30% harbor these loci (*7*). However, 17 (89%) of 18 H18-positive EAEC isolates harbored ≥1 of these aggregative adherence plasmid loci, and 15 (83%) of 17 harbored all 3 loci (Table 1). Moreover, 15 (83%) of the 18 isolates contained *iucA* (aerobactin synthesis), and 17 (94%) contained *chuA* (heme transport outer-membrane receptor), significantly more than the other 113 EAEC isolates (and 39.8% and 24.8% for *iucA* and *chuA,* respectively; $p<0.001$ for each).

Multidrug-resistant, *chuA*-positive *E. coli* H18 strains are also frequently recovered from patients with urinary tract infection. Some of these strains derive from the successful and globally disseminated multidrug-resistant clonal group A (CGA) (*8*). Uropathogenic *E. coli* (UPEC) clonal group A strains occur in the United States and Europe; typically exhibit serotypes O11:H18, O17:H18, O73:H18, or O77:H18; and share a common resistance and repetitive element. PCR profile (*8*). Recently, at certain US centers, ≥33% of trimethoprim-resistant *E. coli* isolates from un-

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Table 1. Properties of H18 EAEC isolates and selected reference strains*

Strain	Country of isolation	Clinical condition	Serotype	Motility	CGA (*fumC* SNP)	Resistance pattern	*aggR*	*aap*	CVD432 (*aat*)	*astA*	*chuA*	*iucA*
C08	Nigeria	Diarrhea	O86:H18	+	+	Ap St Su Tc Tp	+	+	+	–	+	+
C14	Nigeria	Diarrhea	N/K	+	+	Ap Cm St Su Tc Tp	+	+	+	+	+	+
E23	Nigeria	Diarrhea	N/K	+	+	Ap Cm St Su Tc Tp	+	+	+	–	+	+
E30	Nigeria	Diarrhea	N/K	+	+	Ap Cm St Su Tc Tp	+	+	+	–	+	+
G10	Nigeria	Diarrhea	N/K	+	+	Ap Cm St Su Tp	+	+	+	–	+	+
G17a	Nigeria	Diarrhea	N/K	–	+	Ap Cm St Su Tc Tp	+	+	+	–	+	+
G59	Nigeria	Diarrhea	N/K	–	+	Ap Cm St Su Tc Tp	+	+	+	–	+	+
G67b	Nigeria	Diarrhea	N/K	+	+	Ap Cm St Su Tc Tp	+	+	+	–	+	+
C16	Nigeria	Diarrhea	N/K	+	–	Ap Cm St Su Tc Tp	+	+	+	–	+	–
G55	Nigeria	Diarrhea	N/K	+	–	Ap Cm St Su Tc Tp	–	–	–	–	–	–
E64	Nigeria	Healthy	N/K	+	+	Ap Cm St Su Tc Tp	+	+	+	+	+	+
G108	Nigeria	Healthy	N/K	+	+	Ap Cm St Su Tc Tp	–	–	+	+	+	+
E56	Nigeria	Healthy	N/K	+	–	Ap Cm St Su Tc	+	+	+	–	+	+
E62	Nigeria	Healthy	N/K	–	–	Tc	+	+	+	–	+	+
E68	Nigeria	Healthy	N/K	+	–	Ap Cm St Su Tc Tp	–	–	+	+	+	+
G103	Nigeria	Healthy	N/K	+	–	Ap Cm St Su Tc Tp	–	–	+	+	+	+
G121a	Nigeria	Healthy	N/K	+	–	Ap Cm St Su Tc Tp	+	+	+	–	+	+
G149	Nigeria	Healthy	N/K	+	–	Ap St Su Tc Tp	+	+	+	–	+	+
O42	Peru	Diarrhea	O44:H18	+	–	Cm St Su Tc Tp	+	+	+	–	+	–
44-1	Thailand	Diarrhea	O36:H18	+	–	Ap Cm St Su Tc	+	+	+	–	+	+
144-1	Thailand	Diarrhea	O77:NM	–	+	Cm	+	+	+	–	+	+
E02	Nigeria	Diarrhea	Ont:H18	+	–	Ap Cm St Su Tc Tp	–	–	–	–	+	–
DH5α†	N/A	N/A	N/A	+	–	–	–	–	–	–	–	–
2P9‡	USA	UTI	O15:K52:H1	N/D	–	St	–	–	–	–	+	+
SEQ102‡	USA	UTI	O11:NT	N/D	+	Ap Cm St Su Tc Tp	–	–	–	–	+	+
UMN026‡	USA	UTI	O17:K52:H18	N/D	+	Ap Cm St Tc Tp	–	–	–	–	+	+
CFT073‡	USA	UTI	O6:K2:H1	N/D	–	–	–	–	–	–	+	+

*EAEC, enteroaggregative *Escherichia coli*; CGA, clonal group A; SNP, single nucleotide polymorphism; Ap, ampicillin; St, streptomycin; Su, sulfonamide; Tc, tetracycline; Tp, trimethoprim; N/K, not known; Cm, chloramphenicol; N/A, not applicable; UTI, urinary tract infection; N/D, not determined. All strains were susceptible to nalidixic acid and ciprofloxacin. All pathotypes are EAEC (H18) unless otherwise noted.
†Pathotype K-12.
‡Pathotype uropathogenic *E. coli*.

complicated cases of pyelonephritis and cystitis have represented CGA (*9*). CGA strains also can infect nonurinary, extraintestinal sites (*10*). Furthermore, CGA-like strains have been recovered from human and animal feces, which implies a commensal reservoir (*11*,*12*). Accordingly, we assessed our H18 EAEC isolates for membership in CGA.

A CGA-specific PCR protocol, which yields a 175-bp PCR product in strains that have 3 single-nucleotide polymorphisms within *fumC* (*13*), was applied to the Nigerian EAEC H18 isolates. Positive controls were 2 reference CGA cystitis isolates with UMN026 and SEQ102 (ATCC BAA-457) (*13*); negative controls were non-CGA cystitis isolate 2P9 (O15:K52:H1), UPEC isolates CFT073 (O6:K2:H1) and 536 (O6:K15:H31), and an H18 enterotoxigenic *E. coli* (ETEC) isolate from the Nigeria study (*14*). Of the 18 EAEC H18 isolates, 10 (including 8 from children with diarrhea) were positive, whereas 8 (and the H18 ETEC isolate) were negative. Lack of obvious familial or temporal clustering of patients from whom these strains were isolated suggests that the isolates are not likely to be directly linked through a single point source. Of 21 other EAEC strains from diverse non-African locales that were similarly screened, including 3 isolates bearing the H18 *fliC* allele (*1*), only Thai isolate 144-1 (H18-positive) (*1*) exhibited the CGA-specific *fumC* single-nucleotide polymorphisms. The 2 other non-Nigerian H18 EAEC (Peruvian O44:H18 isolate 042 and Thai O36:H18 isolate 44-1) (*1*) were negative.

To unequivocally assess clonal relationships, we subjected all H18 Nigerian EAEC isolates and the Thai isolate 144-1 to multilocus sequence typing (MLST) (*15*). Briefly, we sequenced designated internal regions of the *adk, fumC, gyrB, icd, mdh, purA,* and *recA* genes. Allele comparisons and sequence type (ST) assignments were done by using the open-source *E. coli* MLST database (http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli). All 11 putative EAEC CGA isolates carried *fumC* allele 35, which has all 3 targets of the CGA single-nucleotide polymorphism screen (G270A, C271T, and C288T) (*13*). Of these isolates, 10 (9 Nigerian, 1 Thai) belonged to ST394, which shares 5 of 7 alleles with ST69 (the predominant ST of UPEC CGA; [*12*]) and, according to the e-BURST algorithm, is placed with ST69

in the same larger ST69 complex, indicative of a recent common ancestor (*15*). One Nigerian H18 EAEC isolate, strain E23, was assigned a new ST, ST432, because of its novel *purA* allele; however, ST432 shares alleles with ST394 at all 6 other loci and so also belongs with the ST69 complex. Of the 8 H18 isolates that were negative in the *fumC35* single-nucleotide polymorphism assay, 7 belonged to STs not previously described. Only 1 of these, strain E62 (ST471), shared 6 alleles with ST394 and 4 with ST69. Another strain, C16 (ST512), shared 5 alleles with ST69 and only 4 with ST394. Of the 8 *fumC35*-negative H18 isolates, 6 did not share 5 alleles with ST69 or ST394 and are therefore considered to be of a different clonal complex. Six *fumC35*-negative H18 isolates had 6 alleles in common and belonged to 1 of 3 STs: 31, 449, or 474. ST31 includes other EAEC in the MLST database (www.mlst.net). One EAEC H18 isolate shared no allele with any other EAEC isolate from this study (Table 2).

Although iron-utilization genes *chuA* and *iucA* are present in all EAEC and UPEC ST69 complex strains, the EAEC virulence plasmid markers *aggR*, *aap,* and CVD432 (*aat*) were found in all 11 EAEC ST394/432 isolates but not in reference UPEC CGA (ST69) isolates (Table 1). EAEC ST394/432 strains and UPEC ST69 (CGA) strains appear to represent diverging lineages of common ancestry, which are adapting to separate niches. Escobar-Paramo (*14*) proposed that certain *E. coli* backgrounds appear to be more likely to acquire virulence genes. Our findings suggest that the ST69 complex progenitor, from which CGA UPEC and ST394 EAEC are derived, may have had a propensity to acquire virulence genes as well as antimicrobial resistance elements, thereby generating at least 2 clonal groups pathogenic for humans, with several nonoverlapping, horizontally acquired virulence factors.

CGA-like strains recently isolated from animal feces and food samples have been proposed by Ramchandani et al. (*11*) as possible reservoirs for UPEC CGA strains. However, although they found similar resistance patterns and serotypes among animal CGA isolates as among humans, they did not find typical UPEC-associated virulence gene profiles (*11*). Tartof et al. (*12*) have subsequently shown that CGA-like strains from animal or environmental sources do not belong to ST69 (as do most human UPEC CGA isolates) but that they are part of the ST69 complex, particularly the ST394 type, which corresponds to the EAEC H18 clonal group we describe. Nonhuman ST394 isolates could possibly represent CGA-like EAEC and point to potential nonhuman reservoirs of EAEC, which remain to be identified.

Conclusions

ST69 and ST394 appear to represent successful, genetically related lineages; isolates belonging to both are commonly resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, and trimethoprim. Widespread use of trimethoprim-sulfamethoxazole has been proposed as a reason for the emergence and spread of UPEC CGA (*8*). This combination, as well as other drugs to which ST394 strains are typically resistant, is commonly used (and misused) in Nigeria and other developing

Table 2. Multilocus sequence types of CGA-associated *fumC* single-nucleotide polymorphism–positive strains*

Strain	Clinical condition, country	Allele profile							ST	ST complex
		adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*		
C08	Diarrhea, Nigeria	21	35	61	52	5	5	4	394	69
C14	Diarrhea, Nigeria	21	35	61	52	5	5	4	394	69
E23	Diarrhea, Nigeria	21	35	61	52	5	72	4	432	69
E30	Diarrhea, Nigeria	21	35	61	52	5	5	4	394	69
G10	Diarrhea, Nigeria	21	35	61	52	5	5	4	394	69
G17a	Diarrhea, Nigeria	21	35	61	52	5	5	4	394	69
G59	Diarrhea, Nigeria	21	35	61	52	5	5	4	394	69
G67b	Diarrhea, Nigeria	21	35	61	52	5	5	4	394	69
E64	Healthy, Nigeria	21	35	61	52	5	5	4	394	69
G108	Healthy, Nigeria	21	35	61	52	5	5	4	394	69
144-1	Diarrhea, Thailand	21	35	61	52	5	5	4	394	69
SEQ102†	UTI (CGA), USA	21	35	27	6	5	5	4	69	69
C16	Healthy, Nigeria	21	22	2	6	5	5	4	512	Unassigned
G55	Healthy, Nigeria	6	4	33	1	20	12	7	423	Unassigned
E56	Healthy, Nigeria	18	22	17	6	5	5	4	31	31
E62	Healthy, Nigeria	21	125	61	52	5	5	4	471	Unassigned
E68	Healthy, Nigeria	18	22	94	6	5	5	4	449	Unassigned
G103	Healthy, Nigeria	18	22	94	6	5	5	4	449	Unassigned
G121a	Healthy, Nigeria	18	22	1	6	5	5	4	474	Unassigned
G149	Healthy, Nigeria	18	22	1	6	5	5	4	474	Unassigned

*CGA, clonal group A; ST, sequence type; UTI, urinary tract infection.
†Data from reference (*14*). Data for all other isolates are from this study.

countries and could provide selective pressure for EAEC ST394/432. Although our numbers were too small to significantly associate ST394/432 strains with disease, 8 of 10 of these isolates were from children with diarrhea. Our study has unveiled what we believe to be a previously unrecognized EAEC clonal group. The *fumC* single-nucleotide polymorphism method, proposed for identifying UPEC CGA, could be useful for assessing its distribution.

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Antimicrobial Drugs and Community-acquired *Clostridium difficile*–associated Disease, UK

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In a population-based case-control study of community-acquired *Clostridium difficile*–associated disease (CDAD), we matched 1,233 cases to 12,330 controls. CDAD risk increased 3-fold with use of any antimicrobial agent and 6-fold with use of fluoroquinolones. Prior use of antimicrobial agents did not affect risk for CDAD after 6 months.

Recent reports suggest that *Clostridium difficile*–associated disease (CDAD), including community-acquired CDAD, is increasing in occurrence and severity (*1*–*4*). Antimicrobial drug use is widely believed to be a key driver of CDAD infections, with differences in risk depending on class of antimicrobial agent (*2*). Differences in risk are postulated to be caused by differences in properties of the microbial agents, such as the magnitude and the duration of their effects on the fecal flora (*5*), their activity against *C. difficile*, and possibly their drug or metabolite levels in the intestinal lumen (*6*). However, most studies of CDAD and antimicrobial drugs have been hospital based. Because inpatients are often exposed to multiple antimicrobial drugs, these studies may have limited ability to evaluate agents rarely prescribed or rarely prescribed alone (e.g., macrolides).

We expand on previous work by assessing whether and to what extent the risk for community-acquired CDAD varies with the type of antimicrobial drug prescribed. We also evaluate whether and how long this effect takes to dissipate after drug discontinuation, beyond the 90-day period previously explored with this population (*3*,*4*).

The Study

We further analyzed data from a population-based case-control study that was constructed using the United Kingdom's General Practice Research Database (GPRD). GPRD is a well-validated (*7*) clinical database that records information taken from general practice records. The cohort used in this study has been extensively described (*3*).

Briefly, we identified all patients who had had CDAD (based on either clinical diagnosis or a positive toxin test result) from 1993 through 2004 and who were registered for ≥2 years in a general practice anywhere in the United Kingdom. Approximately 90% of tests for *C. difficile* toxin had neither a positive nor negative result in the GPRD (the result was not recorded as a variable but may be included as case notes that we were unable to review), and so a clinical diagnosis often indicates a test result that was not available to the investigators. Case-patients were defined as patients with community-acquired CDAD, that is, patients who had not been not hospitalized during the year before their CDAD diagnosis. Each case-patient was matched by practice and age (±2 years) to 10 control-patients who also had not been hospitalized during the prior year. Control-patients were also registered in the GPRD for at least 2 years. By matching these control-patients to specific case-patients, we could assess the antimicrobial use in the source population from which the case-patients arose. Control-patients had the same index date as the case-patient to which they were individually matched, which enabled us to account for changing drug patterns and disease rates over time in this database. We used the British National Formulary to define the following antimicrobial drug classes: penicillins, cephalosporins and other β-lactams, tetracyclines, macrolides, sulfonamides and trimethoprim, fluoroquinolones, and all others.

A total of 1,233 case-patients were matched to 12,330 control-patients. The results of antimicrobial exposure, by drug class, are presented in Table 1. The adjusted odds ratio (OR) for CDAD with use of any antimicrobial drug in the 90 days before the index date was 3.1 (95% confidence interval [CI]: 2.7–3.6). Adjusted ORs for different classes of antimicrobial drugs were as follows: tetracyclines 0.85, sulfonamides 1.88, penicillins 1.89, macrolides 2.15, cephalosporins 2.21, and fluoroquinolones 6.20. The mean number of antimicrobial classes for those who received the drugs was 1.4 for case-patients and 1.2 for control-patients. With respect to patients who received at least 1 antimicrobial drug, 67% of case-patients and 82% of control-patients received only 1 class of antimicrobial drug (compared with 50% of case-patients and 59% of control-patients among fluoroquinolone users who received only a single class of antimicrobial drug).

Table 2 describes the residual effects after discontinuation of antimicrobial agents, fluoroquinolones in particular, as a function of the time since the last prescription. The risk (OR 3.1, CI 2.7–3.6) with current antimicrobial drug use diminished after 3 months, dropped to OR 1.8 (95% CI 1.4–2.3), and essentially disappeared after 6 months (OR 1.3, 95% CI 1.0–1.6). A similar pattern was observed after discontinuation of fluoroquinolone use. This is much lower than the effect in the 90-day window before diagnosis with CDAD.

To reduce the risk for protopathic bias (*8*), we did a sensitivity analysis in which we considered any patient

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Table 1. Antimicrobial drug exposure of patients with and without *Clostridium difficile*–associated disease, UK, 1993–2004*

Antimicrobial drug received, past 90 d	Case-patients, n = 1,233 (%)	Control-patients, n = 12,330 (%)	Crude OR†	Adjusted OR‡ (95% CI)
Any	456 (37)	1649 (13)	5.0	3.7 (3.1–4.4)
Tetracyclines	17 (1.4)	106 (0.9)	1.0	0.9 (0.5–1.5)
Penicillins	202 (16.4)	790 (6.4)	2.4	1.9 (1.6– 2.4)
Sulfonamides and trimethoprim	71 (5.7)	236 (1.9)	2.3	1.9 (1.5–2.7)
Macrolides	80 (6.5)	219 (1.7)	2.7	2.2 (1.7–3.1)
Cephalosporins and other β-lactams	76 (6.2)	207 (1.7)	2.9	2.2 (1.7–3.2)
Fluoroquinolones	70 (5.7)	84 (0.7)	10.9	6.2 (4.4– 8.8)

*OR, odds ratio; CI, confidence interval.
†Adjusted for other antimicrobial drugs and prior antimicrobial drug use to ensure that all comparisons used the same reference group.
‡Adjusted for inflammatory bowel disease, diverticular disease, peptic ulcer disease and gastroesophageal reflux disease, *Helicobacter pylori*–associated disease, pernicious anemia, cancer including solid tumor and hematologic malignancies, diabetes mellitus, chronic obstructive pulmonary disease, cirrhosis, nonsteroidal anti-inflammatory agents, aspirin, H2 blockers, proton pump inhibitors, and antimicrobial drug use in the past 2 years.

who had a recent (90-day) diagnosis of infectious diarrhea to be unexposed to fluoroquinolones. This analysis reduced the size of the adjusted OR for fluoroquinolone use from 6.2 (95% CI 4.4–8.8) to 5.0 (95% CI 4.4–7.2).

We also conducted sensitivity analyses based on the source of the CDAD diagnosis (test-based or clinical only). These analyses showed a similar effect for exposure to any antimicrobial agents for clinical and test-based diagnoses. Among antimicrobial classes, although fluoroquinolones appear to have higher ORs in the test-based group (OR 6.7; 95% CI 4.5–10.0) than in the group with clinically based diagnoses (OR 5.2; 95% CI 2.6–10.6), this difference is not statistically different. All other antimicrobial agents were stronger risk factors for development of CDAD when the definition of CDAD was determined by clinical diagnosis alone, without a toxin-positive test result.

Conclusions

Almost all antimicrobial drugs were associated with increased risk for community-acquired CDAD. The risk associated with fluoroquinolones was particularly elevated, as has been found in other recent studies on CDAD in hospital settings (*2*,*9*–*11*).

Because broad-spectrum antimicrobial drugs are more commonly prescribed for patients with more severe infections, the underlying indications for fluoroquinolone prescription could also be contributing to the increased risk for CDAD. That is, channeling of antimicrobial drugs toward such patients may result in confounding by indication (*12*). The sharp reduction in residual risk among fluoroquinolone users suggests that chronic health status confounding is likely to be minor. Although the residual effects of antimicrobial prescriptions never drop to zero (which may indicate a minor effect of confounding), they drop significantly and quickly. This does not exclude the possibility of confounding by indication due to the infection for which the fluoroquinolone was prescribed.

In addition, our sensitivity analysis suggested some degree of robustness of this result because of misclassification, from either misdiagnosis of CDAD as another form of diarrhea or from the source of the diagnosis. However, despite the high sensitivity and specificity seen in studies that use database codes to identify CDAD cases (*13*) and the successful use of this approach in other studies (*14*,*15*), more validation work on this endpoint remains to be done to completely describe the process of recording

Table 2. Most recent prescription for any antimicrobial drug and effect of proximity on risk of acquiring *Clostridium difficile*–associated disease, UK, 1993–2004*

Exposure to antimicrobial drug	Case-patients, n = 1,233 (%)	Control-patients, n = 12,330	Crude OR	Adjusted OR (95% CI)†
None (reference)	379 (30)	6,449 (52)	1.0	1.0 (reference)
Most recent prescription‡				
1–90 d (current)	456 (37)	1,649 (13)	5.0	3.7 (3.1–4.4)
91–180 d	128 (10)	1067 (9)	2.2	1.8 (1.4–2.3)
181–365 d	131 (11)	1,498 (12)	1.6	1.3 (1.0–1.6)
1–2 y	139 (11)	1,674 (13)	1.5	1.3 (1.0–1.6)
Most recent fluoroquinolone prescription†				
1 – 90 d (current)	70 (5.7)	84 (0.7)	10.9	6.2 (4.4–8.8)
91–180 d	12 (1.0)	70 (0.6)	1.7	1.2 (0.6–2.3)
181–365 d	27 (2.2)	114 (0.9)	2.4	1.7 (1.1–2.7)
1–2 y	36 (2.9)	198 (1.6)	1.9	1.3 (0.9– 2.0)

*OR, odds ratio; CI, confidence interval.
†Adjusted for inflammatory bowel disease, diverticular disease, peptic ulcer disease and gastroesophageal reflux disease, Helicobacter pylori–associated disease, pernicious anemia, cancer including solid tumor and hematologic malignancies, diabetes mellitus, chronic obstructive pulmonary disease, cirrhosis, nonsteroidal anti-inflammatory agents, aspirin, H2 blockers, proton pump inhibitors, and antimicrobial use in the past 2 years.
‡2 y before the index date.

community-based CDAD diagnoses.

Our results suggest that antimicrobial drugs are a risk factor for CDAD, including community-acquired CDAD. Despite the high risk that appears to be associated with fluoroquinolone use, only 7% of the case-patients in this sample were exposed to a fluoroquinolone, and only 37% were exposed to any class of antimicrobial drug. Therefore, while good prescribing practices for antimicrobial drugs should continue to be encouraged, these drugs are unlikely to be the primary driver of community-acquired CDAD infections in this population.

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Chikungunya Virus in US Travelers Returning from India, 2006

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Chikungunya virus (CHIKV), a mosquitoborne alphavirus, is endemic in Africa and Asia. In 2005–2006, CHIKV epidemics were reported in islands in the Indian Ocean and in southern India. We present data on laboratory-confirmed CHIKV infections among travelers returning from India to the United States during 2006.

Chikungunya virus (CHIKV) is a mosquito-transmitted virus (genus *Alphavirus*, family *Togaviridae*) usually associated with acute epidemic polyarthralgia. The virus is serologically and genetically most closely related to o'nyong-nyong, Igbo Ora, and, to a lesser extent, Mayaro and Ross River viruses, all of which are associated with acute polyarthralgia (*1*).

CHIKV epidemics have been described in Africa, the Middle East, India, and Southeast Asia, and may have caused epidemics in the Caribbean and in the United States during the early 19th century (*2*). CHIKV epidemics can be explosive with large numbers of human cases and rapid virus dissemination. In the Réunion Island epidemic from April 2005 to June 2006, ≈270,000 cases were reported, representing nearly 40% of the population (*3*). *Aedes aegypti* is the principal vector; however, in recent epidemics in Réunion Island and southern India, *Ae*. *albopictus* has been co-implicated (*4*,*5*). In Africa, CHIKV is maintained in an enzootic cycle involving primates, but in Asia and in recent large epidemics, the human-mosquito cycle predominates, possibly including mechanical transmission (*6*). Symptoms are characterized by acute onset of joint pain, followed by myalgia, fever, and rash with recovery usually within weeks.

Laboratory diagnosis of CHIKV infection is accomplished by serologic methods, virus isolation, and reverse transcription–PCR (RT-PCR). A typical serologic algorithm involves testing acute- and convalescent-phase serum specimens for immunoglobulin M (IgM) and IgG antibody, followed by a plaque reduction neutralization test (PRNT). Virus isolation and RT-PCR are normally used with early acute-phase specimens (before day 5 post-onset) because duration of viremia is typically 2–4 days.

Recent CHIKV outbreaks have been reported in several islands in the Indian Ocean as well as in southern India, where >1 million cases were reported in 2006 (*4*,*7*). CHIKV infections have also been documented in travelers returning from these areas (*3*,*7*). We report confirmed CHIKV infections among 35 travelers returning from overseas travel; 33 were returning from India and 2 from Réunion Island (Table 1).

The Study

Serum samples were received by the Centers for Disease Control and Prevention (Fort Collins, CO, USA) from April 2006 to December 2006 as part of routine diagnostic and reference services available to public health laboratories. A total of 106 serum samples were received from persons returning from regions with epidemics or where CHIKV is endemic (79 from India and the Indian Ocean islands and 27 from Africa) with compatible CHIKV illness and submitted by state public health laboratories. Serum samples were tested for antibodies to several viruses known to occur in the region of travel and residence by IgM capture ELISA and a standard IgG ELISA (*8*,*9*). The 35 CHIKV IgM- and IgG-positive specimens were tested by using a PRNT (90% reduction cutoff) with several related alphaviruses (Sindbis, o'nyong-nyong, and Semliki Forest viruses) to confirm specificity of reactivity (*10*). A ≥4-fold neutralizing titer difference between antibody to CHIKV and antibodies to other alphaviruses indicated a CHIKV-specific antibody response. IgM-positive and PRNT specificity–confirmed specimens were classified as recent CHIKV infections (Table 1).

All serum specimens were tested by a quantitative, real-time, fluorescent probed–based RT-PCR assay for CHIKV RNA. Two primer probe sets were designed in unique regions of the viral genome and reacted specifically with CHIKV RNA and not with related or unrelated viruses (Table 2). Both sets showed an analytical sensitivity <1 PFU, and CHIKV was detected in virus-spiked serum samples at a concentration of 10 PFU/mL (75 μL of serum assayed). Eight serum specimens showed positive results by the real-time assay; all were acute-phase specimens with number of days post-onset of illness reported as ≤6. Viral titers of these specimens were estimated by quantitative RT-PCR that used CHIKV quantity standards (determined by plaque assay) to generate a standard curve. Titers of 8 specimens ranged from $10^{3.9}$ PFU/mL to $10^{6.8}$ PFU/mL.

All acute-phase specimens (on or before day 8 poston-set) were also tested for CHIKV by virus isolation in Vero cells. Isolation was performed by using a recently developed protocol in which cells were grown in glass shell vials and centrifuged to enhance viral infectivity (J.O. Velez, unpub.

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Table 1. Diagnostic test results for 35 travelers infected with chikungunya virus (CHIKV), 2006*

Sample	IgM ELISA†	IgG ELISA†	PRNT‡	Virus isolation (Vero cells)	RT-PCR§	Viremia, PFU/mL¶	Days from onset of illness to collection	State of US residence	Return date, 2006
1	17.7	3.2	640	–	–	NA	0	NJ	10/12
2	1.7	1.7	<10	–	+	$10^{4.0}$	1	CA	Before 11/28
3	1.2	1.1	ND	+	+	$10^{4.1}$	1	IL	9/29
4	1.8	NS	ND	+	+	$10^{6.8}$	2	CA	Before 9/16
5	1.2	0.76	ND	+	+	$10^{5.1}$	2	MA	9/10
6	1.8	1.6	<10	+	+	$10^{6.0}$	3	PA	Before 8/20
7	1.6	1.2	ND	+	+	$10^{5.3}$	3	CA	10/2
8	NS	1.4	ND	–	+	$10^{3.9}$	4	WI	10/9
9	22.0	4.3	5,120	–	–	NA	4	CA	Before 10/6
10	1.1	0.95	<10	–	+	$10^{4.5}$	6	CA	Before 8/13
11	7.4	0.96	40	–	–	NA	7	CA	Before 9/23
12	15.0	0.60	320	–	–	NA	8	CT	Before 7/6
13	26.2	1.2	160	ND	–	NA	8	DC	Before 10/16
14	12.9	5.8	1,80	ND	–	NA	10	CA	Before 9/22
15	38.8	2.3	2,560	ND	ND	NA	19	CT	Before 7/6
16	12.7	1.5	640	ND	–	NA	20	IL	8/23
17	16.9	4.8	640	ND	–	NA	30	IL	6/25
18	8.3	3.7	640	ND	ND	NA	31	HI	Before 8/2
19	6.6	1.5	640	ND	–	NA	31	CA	Before 8/13
20	2.6	6.8	320	ND	–	NA	34	MD	Before 3/20
21	5.0	NS	640	ND	–	NA	34	IL	Before 9/22
22	15.1	4.1	1,280	ND	–	NA	38	CA	Before 10/2
23	4.3	5.9	2,560	ND	–	NA	42	PA	Before 8/20
24	5.6	NS	320	ND	–	NA	43	Unknown	Unknown
25	3.1	3.7	640	ND	–	NA	44	IL	Before 9/12
26	26.6	13.4	5,120	ND	ND	NA	48	SC	6/24
27	30.7	6.6	5,120	ND	–	NA	61	CA	Before 10/26
28	6.1	11.4	2,560	ND	–	NA	62	CT	Before 10/3
29	9.4	16.0	640	ND	–	NA	63	CA	8/23
30	7.5	8.4	1,280	ND	–	NA	71	MN	6/8
31	5.3	5.8	640	ND	–	NA	71	MN	6/8
32	3.7	16.1	320	ND	–	NA	75	LA	Before 3/30
33	9.8	10.1	2,560	ND	–	NA	92	IL	7/9
34	7.5	3.1	160	ND	–	NA	101	PA	Before 10/13
35	24.6	11.9	20,480	ND	–	NA	Unknown	IL	Before 11/08

*IgM, immunoglobulin M; PRNT, plaque reduction neutralization test; RT-PCR, reverse transcription–PCR; NA, not applicable; ND, not done (sample depleted); NS, nonspecific reaction in ELISA.
†Values are patient sample optical densities divided by a negative control optical density; values ≥3 are positive.
‡Values are 90% plaque reduction neutralization titers.
§Real-time, fluorescence-based assay for detecting CHIKV RNA; positive samples had crossing threshold values ≤37 with both primer sets.
¶Estimated CHIKV PFU/mL by real-time RT-PCR using a standard curve generated with plaque-titrated/calibrated CHIKV standards.

data). Five serum specimens displayed prominent and characteristic cytopathic effect on day 2 postinfection, and virus was identified as CHIKV by RT-PCR. All virus isolates were obtained from acute-phase specimens that also were positive by RT-PCR. Three serum specimens (samples 2, 8, and 10) showed positive RT-PCR results, but CHIKV was not isolated from these specimens. In these 3 specimens, inability to isolate virus may have been related to viral titers, which were lower than most of the virus isolation–positive samples, or to handling or storage of these samples. All 8 virus-positive specimens (whether positive by RT-PCR, virus isolation, or both) were collected <7 days post-onset and were negative for IgM and IgG antibodies to CHIKV. Nearly all of the specimens collected <7 days post-onset were positive by 1 of the virus-based tests. The 2 exceptions, samples 1 and 9, were positive for IgM and IgG antibodies to CHIKV and had high PRNT titers. These findings indicate that these samples were not true acute-phase specimens; the true onset or collection date had likely been reported incorrectly.

To identify the strain of CHIKV in these specimens, a 2,122-bp fragment from the structural region of the genome (nucleotide positions 9,648–11,770) was amplified from all 8 virus-positive specimens by RT-PCR and subjected to nucleic acid sequencing with previously described primers (*11*). All 8 sequences showed nucleotide identity

Table 2. Sensitivity and specificity of chikungunya virus (CHIKV) oligonucleotide primers used in real-time reverse transcription–PCR assay

Primer	Genome position*	Sequence (5′→3′)	Sensitivity†	Specificity‡
CHIKV 874	874–894	AAAGGGCAAACTCAGCTTCAC		
CHIKV 961	961–942	GCCTGGGCTCATCGTTATTC	0.3	CHIKV
CHIKV 899-FAM§	899–923	CGCTGTGATACAGTGGTTTCGTGTG		
CHIKV 6856	6856–6879	TCACTCCCTGTTGGACTTGATAGA		
CHIKV 6981	6981–6956	TTGACGAACAGAGTTAGGAACATACC	0.9	CHIKV
CHIKV 6919-FAM	6919–6941	AGGTACGCGCTTCAAGTTCGGCG		

*On the basis of CHIKV prototype strain S27, GenBank accession no. NC_004162.
†Absolute no. of PFU detected in triplicate testing.
‡No reactivity was observed with the following viruses: o'nyong-nyong, Ross River, Mayaro, Semliki Forest, Sindbis, western equine encephalitis, eastern equine encephalitis, and Venezuelan equine encephalitis subtypes 1AB, 1C, 1D, and 1E.
§Primer labeled at the 5′ terminus with 5-FAM and 3′ Black Hole Quencher 1 (Operon Biotechnologies Inc., Huntsville, AL, USA).

>99.7% (GenBank accession nos. EF451142–EF451149). BLAST analysis (www.ncbi.nlm.nih.gov/blast) of the 8 sequences showed that the highest percentage identity was to CHIKV strains recently isolated from travelers returning from Indian Ocean islands (Réunion, Mauritius, and Seychelles). Percentage identity matches between the 8 viruses and Indian Ocean CHIKV strains were ≥99.5%, with 5 to 8 mismatches occurring randomly. In comparison, percentage identities of the 8 viruses to CHIKV prototype S27 or to a strain previously isolated from India (Nagpur/653496) were 95.1% and 94.4%, respectively.

Conclusions

The data reported confirm that the widespread CHIKV epidemic in southern India has infected US travelers. CHIKV infections among international travelers are not unexpected; in 2005–2006, ≈800 CHIKV infections were reported in France, primarily in travelers returning from Réunion Island (*3*). The more noteworthy observation of this study with potential public health ramifications is that high levels of infectious virus were detected in returning travelers. Primary vectors for CHIKV are *Ae*. *aegypti* and *Ae*. *albopictus*, which are established in several southeastern coastal states in the United States. Vector competence studies of *Ae*. *aegypti* and *Ae*. *albopictus* strains from the United States, as well as strains from the Caribbean and South America, showed that a titer of $\approx 10^4$ PFU/mL in monkeys resulted in productive infection with virus dissemination in these mosquitoes, with *Ae*. *albopictus* showing higher infection and dissemination rates (*12*). The level of viremia reported in most of these imported CHIKV infections, $>10^4$ PFU/mL, could be sufficient to infect North American vectors, given the appropriate environmental conditions. However, the time of year and place of residence of the returning travelers in this study were not conducive to transmission; only 2 (patients 26 and 32) returned to US regions (South Carolina and Louisiana) known to have populations of *Ae*. *albopictus*.

Nevertheless, returning travelers with high viremia levels, who live in areas with established *Ae*. *aegypti* and *Ae*. *albopictus* populations, could facilitate local transmission in the United States. Clinicians should therefore obtain travel histories from persons with CHIKV-compatible illness and include CHIKV in differential diagnoses when appropriate. Public health laboratories must carefully monitor CHIKV infections of returning travelers and conduct surveillance for CHIKV-infected vectors in high-risk areas to prevent local establishment of a new emerging virus. Diagnostic laboratory personnel involved in virus isolation protocols must be aware of the potential of isolating CHIKV (a biosafety level 3 agent) from patients returning from regions endemic for CHIKV or regions with epidemics and take appropriate safety precautions.

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Chikungunya Virus, Cameroon, 2006

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We report the isolation of chikungunya virus from a patient during an outbreak of a denguelike syndrome in Cameroon in 2006. The virus was phylogenetically grouped in the Democratic Republic of the Congo cluster, indicating a continuous circulation of a genetically similar chikungunya virus population during 6 years in Central Africa.

Chikungunya virus (CHIKV), formerly only an anecdotally described arbovirus, is now a worldwide public health problem (*1*). Recently, numerous cases of CHIKV infection have been reported from a major outbreak of febrile illness around the Indian Ocean, which included Comoros, Mauritius, Réunion Island (*2*,*3*), and southern India (*4*).

CHIKV is widely distributed in tropical Africa (*5*,*6*) and in Asia (*7*). In Africa, until 2000, the virus was described as endemic, perpetuated through a sylvatic cycle involving wild primates, humans, and mosquitoes of the genus *Aedes* (*2*,*8*). During the past 6 years, the urban cycle has also tended to play a role in Central Africa (*6*). Nevertheless, although recent serologic surveys suggest a high prevalence of *Togaviridae*, *Flaviviridae,* and *Bunyaviridae* (*9*,*10*), understanding of the circulation and effects of arboviruses in Cameroon remains imprecise. This lack of understanding may reflect confusion between arboviral infections and hyperendemic *Plasmodium falciparum* infection.

We report the first isolation, to our knowledge, of CHIKV in Cameroon. The virus was identified during an outbreak of a febrile syndrome in French soldiers in Douala and in patients from an urban medical center in Yaoundé. We also found evidence of cocirculation of CHIKV and dengue virus (DENV).

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The Study

In Douala, Cameroon, 2 sporadic cases of a denguelike syndrome were recorded in French soldiers (patients 1 and 2) on April 3 and May 22, 2006, respectively (Table). From the end of May through the end of July 2006, more cases of denguelike syndrome, which included fever, asthenia, maculopapular rashes, and arthralgia, were observed in Yaoundé. The number of patients who sought treatment at the Yaoundé Medical Center peaked in mid-June 2006. Blood samples were collected from 30 of the 40 patients who visited the medical center. The 30 patients' ages ranged from 1 to 54 years. The delay between the onset of symptoms and the sampling ranged from 0 to 39 days with a median of 4 days (Table). All but 1 patient lived in Yaoundé, and none of these patients had a history of travel abroad or from Yaoundé. Nine patients were Cameroonian, and all other patients were from other countries; 15 patients were female. A blood sample from a 53-year-old woman who returned to France from Yaoundé was also received. All patients had negative results for *P. falciparum* according to rapid test (Core Malaria Pf, Core Diagnostics, Birmingham, UK) and thick smear examination.

Serum specimens were tested for immunoglobulin M (IgM) and IgG antibodies specific for DENV, West Nile virus (WNV), Wesselsbron virus, Rift Valley fever virus, Bunyamwera virus, and CHIKV by IgM-antibody capture (MAC-ELISA) and IgG sandwich ELISA, respectively (*11*). A serum sample was consider positive if the optical density (OD) ratio of viral antigen to uninfected cells was >3. The presence of CHIKV, DENV, and WNV genomes was tested for by specific real-time reverse transcription PCR (RT-PCR) (*12*). Virus isolation on C6/36 and Vero cells was attempted on samples that were positive by RT-PCR (*11*).

The serologic follow-up of patient 1 (Table) for a 3-week period detected seroconversion to a virus antigenically related to CHIKV virus (the OD ratios obtained with the second sample were >3) for IgM and IgG. A sample from patient 2 was obtained the day after the onset of symptoms, and no antibodies to all tested arboviruses were detected. However, the specimen was positive by real-time RT-PCR for CHIKV. The patient's sample yielded CHIKV when cultured, and the envelope gene was partially sequenced (position 10,238–11,367, GenBank accession no. EF051584). The 1.2-kb sequence genetic analysis did not show any codon deletion or insertion when compared with other African CHIKV sequences available in the GenBank database (*3*,*6*,*8*). A high degree of identity was observed when the sequence was compared with the Democratic Republic of the Congo (DRC) strains isolated in 2000 (*6*). Paired identity ranged from 97% to 98.1% at the nucleotide level and from 98.7% to 99.3% at the amino acid level. The Cameroon isolate displayed a higher nucleotide diver-

Table. Characteristics of patients with febrile acute denguelike syndrome, Cameroon, 2006*

Patient no.	Sex/age, y	City	Symptom onset	Sampling date	Delay, d	IgM†	IgG†	PCR‡
1	M/35	Douala	3 Apr	7 Apr	4	Neg	Neg	Neg
1	M/35	Douala	3 Apr	11 May	21	Pos CHIKV	Pos CHIKV	Neg
2§	M/36	Douala	22 May	23 May	1	Neg	Neg	Pos CHIKV
3	F/54	Yaoundé	11 Jun	12 Jun	1	Neg	Neg	Neg
4	F/49	Yaoundé	10 Jun	13 Jun	3	Neg	Neg	Neg
5	F/42	Yaoundé	11 Jun	12 Jun	1	Neg	Neg	Neg
6	F/41	Yaoundé	7 Jun	7 Jun	0	Neg	Pos Flavi	Neg
7	M/38	Yaoundé	15 Jun	19 Jun	4	Neg	Neg	Neg
8	M/30	Yaoundé	18 Aug	19 Jun	1	Neg	Neg	Neg
9	M/21	Yaoundé	15 Jun	19 Jun	4	Neg	Neg	Neg
10	F/32	Yaoundé	21 Jun	22 Jun	1	Neg	Neg	Neg
11	F/22	Yaoundé	19 Jun	26 Jun	7	Pos CHIKV	Neg	Neg
12	F/53	Imported	20 Jun	26 Jun	6	Pos CHIKV	Pos CHIKV	Neg
13	M/42	Yaoundé	24 Jun	27 Jun	3	Neg	Pos Flavi	Neg
14	F/42	Yaoundé	18 Jun	28 Jun	10	Neg	Neg	Neg
15	F/27	Yaoundé	18 Jun	29 Jun	11	Neg	Pos Flavi	Neg
16	F/43	Yaoundé	26 Jun	30 Jun	4	Neg	Neg	Neg
17	M/31	Yaoundé	16 Jun	30 Jun	14	Neg	Neg	Neg
18	F/37	Yaoundé	22 May	30 Jun	39	Pos CHIKV	Pos CHIKV	Neg
19	F/45	Yaoundé	10 Jun	30 Jun	20	Neg	Neg	Neg
20	M/45	Yaoundé	22 Jun	30 Jun	8	Neg	Pos Flavi and CHIKV	Neg
21	M/1	Yaoundé	23 Jun	30 Jun	7	Neg	Neg	Neg
22	F/9	Yaoundé	10 Jun	30 Jun	20	Neg	Neg	Neg
23	M/4	Yaoundé	26 Jun	30 Jun	4	Neg	Neg	Neg
24	M/54	Yaoundé	4 Jul	6 Jul	2	Neg	Neg	Neg
25	F/45	Yaoundé	14 Jun	5 Jul	21	Neg	Neg	Neg
26	M/48	Yaoundé	24 Jun	6 Jul	12	Neg	Neg	Neg
27	M/20	Yaoundé	30 Jun	1 Jul	1	Neg	Neg	Neg
28	M/37	Yaoundé	4 Jul	11 Jul	7	Pos CHIKV	Pos CHIKV	Neg
29	M/36	Yaoundé	9 Jul	11 Jul	2	Neg	Neg	Neg
30	M/33	Yaoundé	28 Jun	10 Jul	12	Neg	Neg	Neg
31	M/32	Yaoundé	10 Jul	12 Jul	2	Neg	Neg	Pos DENV
32	F/38	Yaoundé	16 Jul	17 Jul	1	Neg	Neg	Neg
33	M/45	Yaoundé	21 Jul	26 Jul	5	Neg	Neg	Neg

*IgM, immunoglobulin M; Neg, negative; Pos, positive; CHIKV, chikungunya virus; Flavi, flavivirus; DENV, dengue virus.
†DENV, West Nile virus, Wesselsbron virus, Rift Valley fever virus, Bunyamwera virus, and CHIKV antibodies tested.
‡DENV, West Nile virus, CHIKV tested by real-time reverse transcription–PCR.
§CHIKV isolation successful.

gence (paired identity ranging from 95% to 95.5%) when compared with the 2006 Réunion Island strains (*2*,*3*,*13*). However, amino acid sequences were highly conserved (99%–99.5%). The sequence identity among these isolates highlights their common origin and particularly the genetic stability of CHIKV despite the 6 years and the geographic distance from the DRC outbreaks. As shown in the phylogenetic tree (Figure), the CHIKV Cameroon strain clustered with DRC CHIKV strains with a high bootstrap value of 100. This genotype of CHIKV was closely related to strains from the Central African Republic and the 1982 Uganda isolate (*6*,*8*). The close genetic relationship suggests a continuous circulation of a homologous CHIKV population in Central Africa with a high degree of genetic stability. The genetic stability of the Central African CHIKV strains during 24 years, whether associated with epidemic or sporadic cases, highlights the peculiar importance of the few mutations detected in the recent Réunion Island isolates (*3*). This also suggests that the Central African strain CHIKV zone of circulation now includes India (*4*), the Indian Ocean, and Cameroon.

The phylogenetic tree also illustrates the differences between the Cameroon isolates and the Asian subgroup isolates. Moreover, when compared with Asian CHIKV, including the 2006 isolates, the Cameroon strain showed 91%–91.9% and 96.8%–98.8% identity at the nucleotide and amino acid levels, respectively. Despite the similarity, cross-neutralization experiments must be conducted to confirm the protective effect of the Asian CHIKV-based vaccine against Central African strains (*2*).

Among patients from Yaoundé, 1 (patient 11) had only IgM antibodies specific to CHIKV, while patients 18 and

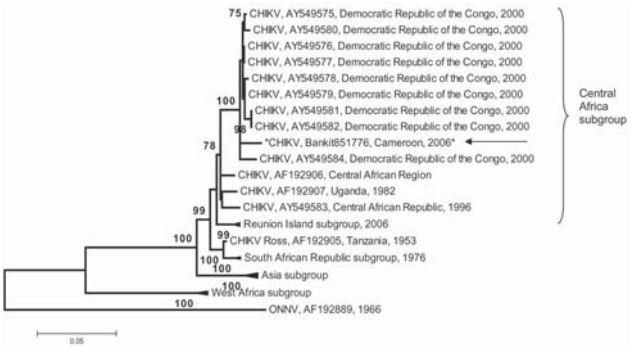

Figure. Phylogenetic tree of chikungunya virus (CHIKV) based on partial nucleotide sequences (3′ extremity of E1/3′-UTR, position 10,238–11,367). Phylogram was constructed with MEGA 2 program and tree drawing used the Jukes-Cantor algorithm for genetic distance determination and the neighbor-joining method. The percentage of successful bootstrap replicates (1,000 bootstrap replications, confidence probability >90%) is indicated at the nodes. The length of branches is proportional to the number of nucleotide changes (% of divergence). Asterisk (*) and arrow indicate the strains isolated in this work. The dark triangle corresponds to viruses clustering together. O'nyong-nyong virus (ONNV) sequence has been introduced for correct rooting of the tree. The GenBank reference no. for the Cameroon CHIKV isolate is EF051584.

28 had both IgM and IgG antibodies specific to CHIKV (Table). One patient from Cameroon (patient 20) had IgG specific to both CHIKV and flavivirus. Three patients (nos. 6, 13, and 15), 2 of whom were Cameroonian, had antibodies specific for flavivirus. All samples were negative for WNV and CHIKV by RT-PCR. One sample (from patient 31) was positive for DENV; however, no virus was detected by cell culture. These results suggested a cocirculation of CHIKV and dengue virus during the same period, which is consistent with the suspected circulation of dengue virus, CHIKV, and yellow fever virus observed in a study from 2000 through 2003 in Cameroon (*9*).

In Cameroon, as in DRC (*6*), patients were likely infected in urban or periurban centers (Yaoundé, the capital of Cameroon; Douala, a major city). These infections occurred in a context where *Aedes albopictus* tends to replace indigenous *Ae. aegypti* in rural and urban Cameroonian environments (*14*). This finding suggests that urban cycles and urban vectors, in addition to the traditional forest-dwelling vectors, may play an important role in the maintenance and amplification of CHIKV in Africa.

Conclusions

Since its first isolation in 1953 (*8*), CHIKV has been isolated in different Central African countries (*8*,*6*). Until now, only 2 alphavirus strains antigenically suspected to be CHIKV had been isolated from human patients in Cameroon (*15*). Recent serosurvey studies suggested a possible CHIKV circulation in Cameroon (*9*,*10*). Our Cameroon CHIKV isolate confirmed its circulation in this country. Our study suggests a 6-year continuous circulation of genetically stable and indigenous strains in Central Africa rather than importation of CHIKV from the recent Indian Ocean or Asian outbreaks. Moreover, the genetic stability of the Central African CHIKV highlights the importance of the unique molecular features that was shown in Réunion Island isolates (*3*).

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Avian Influenza (H5N1) Virus in Waterfowl and Chickens, Central China

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In 2004, 3 and 4 strains of avian influenza virus (subtype H5N1) were isolated from waterfowl and chickens, respectively, in central People's Republic of China. Viral replication and pathogenicity were evaluated in chickens, quails, pigeons, and mice. We analyzed the sequences of the hemagglutinin and neuraminidase genes of the isolates and found broad diversity among them.

Several avian influenza outbreaks occurred in Asia during 2003–2004. We performed virus isolation in central People's Republic of China in early (January–March) 2004 by injecting infected bird tissue homogenates into 10-day-old specific pathogen–free embryonated chicken eggs, according to standard procedures (*1*). Seven strains of avian influenza (H5N1) virus were isolated and named A/widgeon/Hubei/EWHC/2004 (EWHC), A/chicken/Hubei/327/2004 (CKDW), A/chicken/Hubei/JZJ/2004 (CKJZ), A/chicken/Hubei/TMJ/2004 (CKTM), A/chicken/Hubei/XFJ/2004 (CKXF), A/goose/Hubei/ZFE/2004 (GOZF), and A/duck/Hubei/XFY/2004 (DKXF). During the outbreak, CKDW, CKJZ, CKTM, and CKXF were isolated from free-range chickens in villages. GOZF, which was responsible for 50% of the cases and 30% of the related deaths in infected geese, was isolated from a farmed goose in a flock of >1,000. EWHC was isolated from a Eurasian widgeon in a large lake where many widgeons were found dead. DKXF was isolated from an asymptomatic domestic duck on a farm where ducks sporadically died.

The isolates were characterized in Madin-Darby canine kidney (MDCK) cells, embryonated eggs, chickens, and mice. The 50% tissue culture infectious dose ($TCID_{50}$), 50% egg infectious dose (EID_{50}), intravenous pathogenicity index (IVPI), intracerebral pathogenicity index (ICPI), and 50% lethal dose in mice (MLD_{50}) were determined in the respective models. For MLD_{50} determination, mice (n = 5 per dose) were intranasally inoculated with serial dilutions of virus at 10^1–10^6 EID_{50} in a volume of 50 μL. $TCID_{50}$, EID_{50}, and MLD_{50} were calculated by using the Reed-Muench method (*2*). IVPI and ICPI were determined as previously described (*3*). All experiments were performed in a Biosafety Level 3 laboratory. Related data are summarized in Table 1.

Pathogenicity of the 7 isolates was evaluated in birds. Six-week-old White Leghorn (WL) chickens, 4-week-old quails, and 4-week-old pigeons, 64 each, all free from avian influenza (H5N1) virus infection, were divided into 8 groups of 8 birds. For each species, 7 groups were intravenously inoculated with 10^6 EID_{50} of 1 of 7 avian influenza (H5N1) isolates, and 1 group received allantoic fluid as a negative control. Birds were monitored daily, and the number of deaths was recorded until 10 d postinfection, at which point all surviving birds were killed. Viral tissue tropism was analyzed in dead (on the day of death) and surviving birds (10 d postinfection) by a double-antibody sandwich ELISA for the nucleoprotein of influenza A virus (*4*). The mean time to death (MDT) was calculated (Table 2).

All chickens injected with the CKDW or EWHC isolates died within 24 h (MDT<1.0); those injected with GOZF, CKXF, or CKJZ died within 2 d (MDT<1.5). CKTM caused a 75% death rate for chickens, with an MDT of 4.7; all chickens injected with DKXF survived. Quails were also susceptible to infection, but the MDTs in quail (2.8–6.3) were higher than those in chickens. CKDW, EWHC, GOZF, and CKJZ caused a 100% death rate; CKXF and CKTM caused 62.5% and 37.5% death rates, respectively; and DKXF did not cause death. Pigeons were more resistant to these isolates than the other birds; the highest death rate was 37.5% for the EWHC group, with an MDT of 4.3. CKDW and GOZF caused a 25% death rate with an MDT of 5.0; CKJZ caused a 12.5% death rate with an MDT of 6.0. CKTM, CKXF, and DKXF exhibited low pathogenicity in pigeons, with no deaths by day 10.

Most chicken tissue samples had positive ELISA results for avian influenza (H5N1) infection; no particular tissue tropism pattern was found in quail samples. In contrast, all tested pigeon glandular stomach samples from the 7 infected groups had positive results, while most other organs had negative results.

The pathogenicity of the 7 isolates was also evaluated in mice. Forty-eight 6-week-old female BALB/c mice (6 in each of 8 groups) were anesthetized with dry ice and intranasally inoculated with 10^6 EID_{50} of 1 the isolates in a volume of 50 μL. One group was inoculated with normal allantoic fluid as a negative control. The mice were monitored daily for clinical signs and death. The surviving mice were killed at 14 d postinfection for the tissue tropism study. EWHC, CKDW, and GOZF caused lethargy, weight

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Table 1. Characteristics of the 7 avian influenza (H5N1) isolates obtained in central People's Republic of China, 2004*

		Avian				Mammalian		
	HA titers	EID_{50}	IVPI	ICPI	Pathogenicity in chickens†	$TCID_{50}$	MLD_{50}	Pathogenicity in mice‡
EWHC	2^{10}	$10^{7.5}$	3.0	1.91	High	$10^{4.41}$	$10^{1.3}$	High
CKDW	2^{8}	$10^{8.23}$	3.0	1.78	High	$10^{7.8}$	$10^{2.3}$	High
GOZF	2^{8}	$10^{9.8}$	2.98	1.88	High	$10^{7.35}$	$10^{1.5}$	High
CKXF	2^{8}	$10^{6.22}$	2.96	1.88	High	$10^{4.67}$	10^{6}	Medium
CKJZ	2^{8}	$10^{6.33}$	3.0	1.9	High	$10^{5.24}$	$>10^{6.5}$	Low
CKTM	2^{8}	$10^{7.23}$	1.71	1.36	High	$10^{2.0}$	$>10^{6.5}$	Low
DKXF	2^{8}	$10^{6.67}$	0	1.25	Low	$10^{6.57}$	$>10^{6.5}$	None

*HA, hemagglutinin; EID_{50}, 50% egg infectious dose; IVPI, intravenous pathogenicity index; ICPI, intracerebral pathogenicity index; $TCID_{50}$, 50% tissue culture infectious dose; MLD_{50}, 50% lethal dose in mice; EWHC, A/widgeon/Hubei/EWHC/2004; CKDW, A/chicken/Hubei/327/2004; GOZF, A/goose/Hubei/ZFE/2004; CKXF, A/chicken/Hubei/XFJ/2004; CKJZ, A/chicken/Hubei/JZJ/2004; CKTM, A/chicken/Hubei/TMJ/2004; DKXF, A/duck/Hubei/XFY/2004.
†Avian influenza virus isolates are considered to be highly pathogenic if they cause a death rate >75% within 10 days or have an IVPI >1.2 (World Organization for Animal Health criteria).
‡The pathotypes in mice were divided into none, low (MLD_{50}>6.5 $\log_{10}EID_{50}$), medium (3.0 $\log_{10}EID_{50}$<$MLD_{50}\leq$6.5 $\log_{10}EID_{50}$), and high ($MLD_{50}\leq$3.0 $\log_{10}EID_{50}$).

loss, lymphopenia, neurologic disorders, and death within 4–13 d. Mice in the EWHC group showed more rapid and obvious weight loss than those in the CKDW and GOZF groups. CKXF caused moderate clinical signs and a 50% death rate; DKXF, CKTM, and CKJZ caused no obvious clinical signs and no deaths (Figure 1). The virus recovery rates of the 7 isolates showed that the lung had the highest recovery rate (19/42) and the heart had the lowest (3/42); they indicated that the virus could be isolated from the brain (17/42), kidney (13/42), liver (11/42), and spleen (7/42). EWHC showed the highest recovery rate (25/36), and DKXF had the lowest (4/36) among the isolates. Despite no obvious clinical signs in mice injected with DKXF, CKTM, and CKJZ, virus could be reisolated from the tissues; the rates of recovery were 6/36 (CKJZ), 6/36 (CKTM), and 4/36 (DKXF). These results indicated that the isolates DKXF, CKTM, and CKJZ could replicate in mice but at a low efficiency.

To characterize the antibody responses in mice after infection with avian influenza virus, 6-week-old BALB/c mice were intranasally inoculated with nonlethal doses (CKTM, DKXF, and CKJZ, 10^6 EID_{50}; GOZF, CKDW, CKXF, and EWHC, 10^3 EID_{50}). Serum samples were collected at 0, 15, 30, and 60 d postinfection. Antibody analyses showed that GOZF, DKXF, CKDW, CKXF, and EWHC elicited antibody responses in mice at 30 d postinfection and that the antibody levels increased markedly at 60 d postinfection. In the CKTM and CKJZ groups, however, no antibodies were detected at any point.

The hemagglutinin (HA) and neuraminidase (NA) genes from the isolates were amplified by reverse transcription–PCR (*5*) and sequenced. Phylogenetic analysis was performed with Mega 3.1 software (Evolutionary Function Genomics, Tempe, AZ, USA) (*6*). A neighbor-joining tree was based on the nucleotide sequences of HA and NA genes (Figure 2). For the HA gene, the 6 isolates of higher pathogenicity clustered together and were related to the China Guangdong isolate from wild duck (A/wild-duck/Guangdong/314/2004). The HA gene of the less pathogenic DKXF isolate was at a separate position and displayed a close relationship with the Hong Kong goose isolate (A/goose/HongKong/3014.5/2000), which seems to share a common ancestral sequence with the other 6 isolates. For the NA gene, except for CKXF, the other 6 isolates clustered together and were more closely related to several other Chinese isolates obtained in 2004 than to isolates belonging to other strains. The NA gene of CKXF was more closely related to isolates from humans in Indonesia during 2005.

Table 2. Death rates and mean time to death (MDT) from avian influenza virus (H5N1) infections in 3 types of birds inoculated with the 7 isolates obtained in central People's Republic of China, 2004*

	Chicken		Quail		Pigeon	
Isolate	No. deaths/ no. inoculated	MDT (days)	No. deaths/ no. inoculated	MDT (days)	No. deaths/ no. inoculated	MDT (days)
EWHC	8/8	1	8/8	2.8	3/8	4.3
CKDW	8/8	1	8/8	3.4	2/8	5
GOZF	8/8	1.3	8/8	3.3	2/8	5
CKXF	8/8	1.4	5/8	4	0/8	–
CKJZ	8/8	1.1	8/8	3.4	1/8	6
CKTM	6/8	4.7	3/8	6.3	0/8	–
DKXF	0/8	–	0/8	–	0/8	–

*EWHC, A/widgeon/Hubei/EWHC/2004; CKDW, A/chicken/Hubei/327/2004; GOZF, A/goose/Hubei/ZFE/2004; CKXF, A/chicken/Hubei/XFJ/2004; CKJZ, A/chicken/Hubei/JZJ/2004; CKTM, A/chicken/Hubei/TMJ/2004; DKXF, A/duck/Hubei/XFY/2004. Dashes represent no deaths.

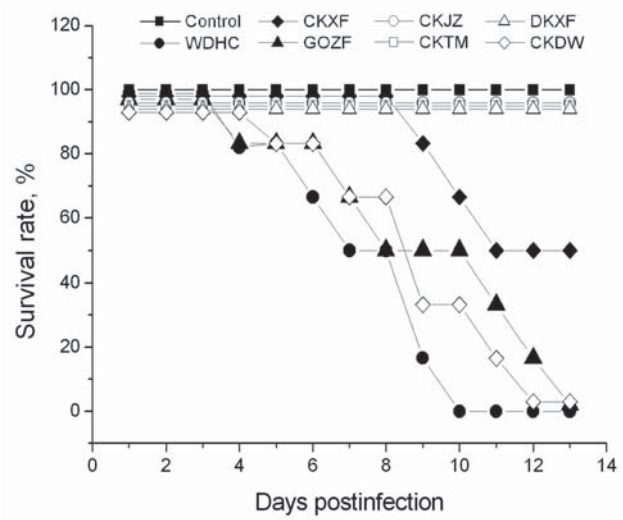

Figure 1. Survival times of mice infected with the 7 avian influenza virus (H5N1) isolates. Mice were intranasally inoculated with 10^6 50% egg infectious dose of viruses in a volume of 50 μL. A/widgeon/Hubei/EWHC/2004 (EWHC), A/chicken/Hubei/327/2004 (CKDW), and A/goose/Hubei/ZFE/2004 (GOZF) induced a 100% death rate within 4–13 days, A/chicken/Hubei/XFJ/2004 (CKXF) induced a 50% death rate, and A/duck/Hubei/XFY/2004 (DKXF), A/chicken/Hubei/TMJ/2004 (CKTM), and A/chicken/Hubei/JZJ/2004 (CKJZ) caused no clinical signs or death.

Conclusions

In this study, influenza virus strains of different pathogenicity were isolated from central People's Republic of China during the same outbreak. Distinctive from most other studies, our work may lead to a more detailed understanding of the complexity of the genetic and biologic variations among avian influenza (H5N1) isolates from outbreaks. All viruses except DKXF were highly pathogenic to avian species, while their pathogenicity in mice was variable (Table 1). The pathogenicity of EWHC in mice was higher than that of CKDW, but the virus titer in MDCK cells was lower than that of CKDW. The HA and NA gene sequences suggested that these viruses were closely related, but they had different clinical and biologic characteristics. DKXF exhibited low pathogenicity in avian species and mice. These data indicate that the isolated strains had diverse biologic characteristics.

DKXF was highly infectious to chicken eggs and MDCK cells (Table 1) and was found to replicate in chickens and mice despite its lack of pathogenicity. In addition, DKXF could induce high antibody titers in mice and chickens. Moreover, a strong cross-neutralizing reaction between DKXF and CKDW has been recently shown (*7*). Future studies should assess the feasibility of using DKXF as a candidate strain for vaccine development.

Early studies showed that pigeons were more resistant to the highly pathogenic avian influenza (H5N1) strain (A/chicken/Hong Kong/220/97; HK/220) than other birds (*8*). In our experiment, we demonstrated that isolates from 2004 could infect pigeons and could be reisolated from pigeon tissues, especially from glandular stomachs. Sequence analysis indicated that the avian influenza isolates used in this study and the HK/220 strain share high homology at the amino acid level for the HA (95%–97%) but not the NA gene (homology 79%–88%). This study indicates that the pigeon may be an asymptomatic carrier of avian influenza virus.

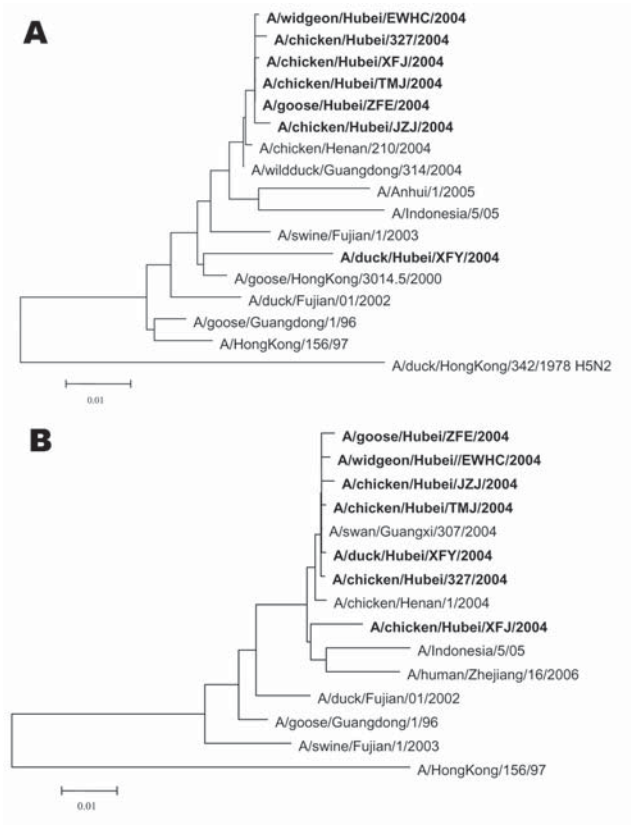

Figure 2. Phylogenetic relationship of various avian influenza virus isolates based on the nucleotide sequences of the A) hemagglutinin and B) neuraminidase genes. All isolates are avian influenza (H5N1) subtype except as indicated. **Boldface** indicates strains isolated in this study.

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Tuberculosis Drug Resistance and HIV Infection, the Netherlands

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In the Netherlands during 1993–2001, multidrug-resistant tuberculosis among newly diagnosed patients was more frequent in those with HIV coinfection (5/308, 1.6%) than in those with no HIV infection (39/646, 0.6%; adjusted odds ratio 3.43, p = 0.015). Four of the 5 patients coinfected with multidrug-resistant tuberculosis and HIV were foreign-born. DNA fingerprint analysis suggested that transmission had occurred outside the Netherlands.

HIV infection strongly increases the risk for tuberculosis (TB) infection: TB disease occurs in 7%–10% of patients with HIV infection each year (*1*). The increase in numbers of patients with both HIV infection and TB has raised the potential for increasing transmission of drug-resistant *Mycobacterium tuberculosis* strains (*2*).

Reports on associations of HIV coinfection and drug resistance among patients with TB have been contradictory. Some studies found strongly increased risks for multidrug-resistant TB (MDR TB) among patients coinfected with TB and HIV (*3*–*7*), whereas other studies found no increased risk (*8*–*11*). Population-based data are limited, however, in particular from low-prevalence countries. We report on a population-based study of anti-tuberculosis drug resistance patterns and associations with HIV infection in the Netherlands during 1993–2001.

The Study

Patient data were obtained from the Netherlands Tuberculosis Register (NTR), which contains data on all TB cases since 1993 reported by TB control departments of municipal health services. Data on drug susceptibility were obtained from the National Tuberculosis Reference Laboratory (National Institute of Public Health and the Environment, Bilthoven, the Netherlands), which performs drug-susceptibility testing (DST) and restriction fragment length polymorphism (RFLP) typing on all *M. tuberculosis* complex strains isolated from patients in the Netherlands. DST is performed according to an absolute concentration method on 7H10 agar, with a proportional cut-off (*12*). RFLP typing is based on the standardized method (*13*). Laboratory records were matched to NTR records by a combination of postal code, date of birth, and sex.

In the NTR, HIV infection is recorded as a response option on the item on impaired immunity. Patients with a record of impaired immunity due to HIV infection were considered HIV positive. If immunity was reported to be impaired because of other causes than HIV infection, or if immune status was unknown, HIV status was considered negative. If the item on impaired immunity was missing (not filled out), the record was excluded from the analysis.

In the analysis, the association was determined between HIV status and resistance to various drugs and combinations of drugs used to treat TB. MDR TB was defined as resistance of *M. tuberculosis* to at least isoniazid and rifampin. DST results before start of treatment were included unless cultures had only been taken during treatment. Patients were categorized as either previously treated or new (i.e., previously untreated). Previous treatment was defined as a history of TB treatment for >4 weeks, a sputum sample obtained during treatment, or both.

For comparison of categorical variables, significance testing was done by χ^2 test with continuity correction or by 2-sided Fisher exact test as appropriate. Multivariate analysis was conducted by logistic regression. A p value <0.05 was considered statistically significant unless stated otherwise. Analyses were conducted with SPSS version 12.0.1 (SPSS Inc., Chicago, IL, USA).

During the study period, 13,943 TB cases were reported to NTR (57% in foreign-born patients), including 8,450 persons with positive *M. tuberculosis* cultures. Of the case-patients with positive cultures, 7,354 were identified in the laboratory database (87.0%). Excluded were 264 (3.6%) of 7,354 case-patients because of missing information on impaired immunity, which left 7,090 case-patients for the analysis. No significant differences between included and excluded case-patients were found in age, sex, nationality, localization of disease, place of residence, risk groups, or year of diagnosis. More included than excluded case-patients had received a TB diagnosis in a hospital, 5,500 (78%) versus 4,310 (62%), respectively (p = 0.034).

HIV infection was reported in 329 (4.6%) of the 7,090 included case-patients. Of these, 232 (71%) were male, 198 (60%) were of foreign origin, 118 (36%) were of Dutch origin, and 13 (4%) were of unknown origin. The 198 case-patients of foreign origin included 128 (41%) patients from Africa and 24 (8%) from industrialized countries; the remaining 49 (14%) patients were from Asia and Central and South America.

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Of the 7,090 case-patients included in the study, 6,775 (95.6%) were new and 315 (4.4%) were previously treated. Among the new cases, drug resistance was reported in 817 (12.1%); isoniazid resistance was reported in 449 (6.6%), rifampin resistance in 51 (0.8%), and multidrug resistance in 44 (0.7%). Multidrug resistance was significantly associated with HIV infection both before (odds ratio [OR] 2.78, p = 0.033) and after adjustment by multivariate analysis for age, sex, and continent of origin (adjusted OR 3.43, p = 0.015). In addition, near-significant associations with HIV infection were observed for resistance to isoniazid (OR 1.50, 95% confidence interval [CI] 0.99–2.26) and resistance to rifampin (OR 2.35, 95% CI 0.82–6.24) (Table 1).

Among the 315 previously treated patients, drug resistance was reported in 68 (21.6%); isoniazid resistance was reported in 52 (16.5%), rifampin resistance in 19 (6.0%), and multidrug resistance in 17 (5.4%). HIV infection was significantly associated with any rifampin resistance (OR 4.12, CI 1.01–15.67, p<0.05) (Table 2). Monoresistance to rifampin was found in 2 previously treated patients; both were HIV infected.

The 5 new HIV-infected MDR TB patients (4 men, 1 woman; age range 22–31 years) originated from the Netherlands, Liberia, Angola, South Africa, and Portugal. Four had diagnoses of pulmonary TB and 1 extrapulmonary TB. None had a known history of intravenous drug use. Two patients completed treatment, 2 died during treatment, and 1 continued treatment at an unknown location.

The 2 previously treated HIV-infected MDR TB patients (both women, ages 28 and 43 years) originated from Europe. Pulmonary TB was diagnosed for both; 1 was an intravenous drug user. One died during treatment, and the other was lost to follow-up.

Each of the 7 patients with both MDR TB and HIV infection had different RFLP patterns. Four of these patients, all new, shared an RFLP pattern with ≥1 other patient in the database. For 1 of these, the cluster included other MDR TB patients. Transmission could have occurred from this patient with MDR TB and HIV infection to 2 other patients without HIV infection whose TB had been diagnosed in the same year. The same patient, whose infecting strain was resistant to isoniazid, rifampin, and streptomycin, could have acquired the TB infection in the Netherlands from an African patient with HIV infection who had received a diagnosis of TB 3 years earlier and harbored a strain resistant to isoniazid and streptomycin. For the other 3 clustered patients, the resistance patterns or date of entry into the country made transmission to or from other patients within the Netherlands impossible.

In the 2 cases of monoresistance of TB to rifampin, the isolates were obtained before treatment from patients with HIV infection originating from Cape Verde and Somalia. Both had combined pulmonary and extrapulmonary TB and had been treated before in the Netherlands. One had completed 6 months of treatment; for the other, treatment completion was not recorded.

Conclusions

Overall, MDR TB occurred in 5 (2.5%) of 198 foreign-born patients with TB and HIV infection, compared with 2 (1.7%) of 118 Dutch-born patients with TB and HIV coinfection (p = 0.730). We found low prevalence of multidrug resistance among patients with TB, in accordance with an earlier study that covered a shorter period (*8*). In the Netherlands, transmission of MDR TB has been rare. During the study period, single secondary MDR TB cases, as confirmed by RFLP typing, were documented in only 2 instances, 1 nosocomial (M. Sebek, pers. comm.). This may be related to the presence of a system of drug resistance surveillance with national coverage, active contact

Table 1. Association between HIV infection and primary drug resistance among new tuberculosis patients, the Netherlands, 1993–2001*

	No. (%) HIV negative (n = 6,467)	No. (%) HIV positive (n = 308)	OR (95% CI) (unadjusted)	p value†
Fully susceptible	5,695 (88.1)	263 (85.4)	1.00	
Resistant to 1 drug	544 (8.4)	29 (9.4)	1.15 (0.76–1.74)	0.542
Resistant to 2 drugs	193 (3.0)	11 (3.6)	1.23 (0.63–2.36)	0.622
Resistant to 3 drugs	24 (0.4)	5 (1.6)	4.51 (1.50–12.57)	0.001
Resistant to 4 drugs	11 (0.2)	0	–	–
Any resistance	772 (11.9)	45 (17.1)	1.26 (0.90–1.77)	0.188
Any resistance to:				
Isoniazid	420 (6.5)	29 (9.4)	1.50 (0.99–2.26)	0.059
Rifampin	46 (0.7)	5 (1.6)	2.35 (0.82–6.24)	0.075
Streptomycin	538 (8.3)	31 (10.1)	1.25 (0.83–1.86)	0.303
Ethambutol	42 (0.6)	1 (0.3)	0.52 (0.03–3.49)	1.000
Multidrug resistance‡	39 (0.6)	5 (1.6)	2.78 (1.09–7.10)	0.033

*New patients are defined as those not previously treated for tuberculosis. OR, odds ratio; CI, confidence interval.
†p value determined by Fisher exact test or χ^2 test (Yates corrected), as appropriate.
‡Resistant to at least isoniazid and rifampin.

Table 2. Prevalence of secondary drug resistance among previously treated tuberculosis patients, the Netherlands, 1993–2001*

	No. (%) HIV negative (n = 294)	No. (%) HIV positive (n = 21)
Fully susceptible	232 (78.9)	15 (71.4)
Resistant to 1 drug	34 (11.9)	4 (19.0)
Isoniazid	20 (6.8)	2 (9.5)
Rifampin	0	2 (9.5)
Streptomycin	14 (4.8)	0
Resistant to 2 drugs	17 (5.8)	0
Resistant to 3 drugs	4 (1.4)	0
Resistant to 4 drugs	7 (2.4)	2 (9.5)
Any resistance	62 (21.1)	6 (28.6)
Any resistance to		
Isoniazid	48 (16.3)	4 (19.0)
Rifampin	15 (5.1)	4 (19.0)*
Streptomycin	36 (12.2)	2 (9.5)
Ethambutol	9 (3.1)	2 (9.5)
Multidrug resistance†	15 (5.1)	2 (9.5)

*Unadjusted odds ratio 4.12 (95% confidence interval 1.01–15.67, p = 0.036).
†Resistant to at least isoniazid and rifampin.

tracing around infectious TB cases, and directly observed treatment of patients with MDR TB in specialized centers under strict respiratory isolation.

Only 7 cases of MDR TB occurred among 329 patients with HIV infection during this 9-year period (2.1%). Despite this small number, MDR TB was significantly more frequent among previously untreated patients with TB and HIV infection than among those without HIV infection. Even though the results were adjusted, at least partially, for origin of the patient, non-Dutch origin appears to play an important role in this association. Of the 5 patients with new TB and HIV infection, 4 were foreign-born, including 3 from sub-Saharan Africa. Because transmission in the Netherlands could be ruled out in 4 of the 5 new cases, most if not all of these infections were acquired abroad. The MDR TB infections in these patients may have been acquired in institutional settings such as hospitals, but data on the pre-immigration history were lacking.

Four (19%) of 21 case-patients with previously treated TB and HIV infection had rifampin-resistant isolates, including 2 (10%) that were rifampin monoresistant. Acquisition of monoresistance to rifampin is associated with HIV infection and may be related to intestinal malabsorption, intermittent treatment with rifabutin, and drug interactions (*14*,*15*). In the patients in our study, the contribution of these factors could not be established.

In conclusion, among new TB patients in the Netherlands, multidrug resistance is associated with HIV infection, predominantly as an imported disease. In patients with HIV infection who have previously been treated for TB, the possibility of rifampin resistance should be considered. Routine surveillance of resistance to anti-TB drugs will improve timely recognition of MDR TB cases and help prevent further transmission.

Dr Haar previously worked in Tanzania in a refugee camp and is now training as a public health specialist in the Netherlands. Her research interests focus on HIV–tuberculosis coinfection.

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Detecting Epidemic Malaria, Uganda

To the Editor: In the field of malaria epidemic early warning, there exists an unfortunate but frequently accurate perception that health systems in many affected countries learn of epidemics by way of the popular press rather than through formal disease surveillance systems. Malaria epidemics are often easily recognized (albeit too late) by laypersons (*1*), but most routine disease surveillance systems lack the ability to provide accurate, timely indications of aberrations in case numbers. The World Health Organization (WHO) has set specific targets for early detection and control of malaria epidemics as part of a wider strategy to cut the global impact of malaria in half by 2010 (*2*). We describe experiences during a recent epidemic in southwest Uganda and examine the performance of a pilot early detection system.

In 2002, the Ugandan Ministry of Health began developing and piloting a new district-level malaria monitoring system in Kabale and Rukungiri (*3*). Located in Uganda's southwestern highlands, these districts have experienced several serious malaria epidemics in recent years, most notably during the El Niño year of 1998 (*4*,*5*). In this new system, data generated from representative health facilities are collated, entered on computer, and analyzed by district teams on a weekly basis. Incoming data on clinical malaria are compared with a baseline of historical illness data from which the effects of long-term temporal trends have been removed, and an objective anomaly measure, or "standardized departure," is used to provide a simple, intuitive index of deviation from expected weekly levels of incidence (*3*). Electronic reports are disseminated by email to the National Malaria Control Programme (NMCP) and others, including WHO and the United Nations Children's Fund.

The monitoring system detected 2 malaria outbreaks in Kabale, 1 each in 2005 and 2006. During the most recent outbreak, the first warnings of abnormally high malaria incidence were communicated from the district team to the NMCP on June 5, 1 month before reports of the outbreak appeared in the press and >2 weeks before case numbers began to peak (Figure, panel A). In the 6 weeks from May 29 to July 9, Kabale's 5 sentinel sites recorded 4,637 clinical malaria cases, 159% more than expected for this period. Although the sentinel network consists of health centers with limited inpatient facilities, available data on admissions showed a similar temporal pattern, with 616 patients admitted during the same 6-week period, 188% more than expected.

Although the ability of the system to generate timely epidemic warnings is encouraging, data from 1 sentinel site highlighted a potential limitation of using routine data from clinical diagnoses of malaria as a basis for epidemic detection. As elsewhere, routine outpatient data for Bufundi Health Centre suggested the occurrence of a malaria outbreak starting in early June and peaking in early July of 2006 (Figure, panel B). The slight delay in the onset of the outbreak at this site was plausible, given its high elevation (2,200 m) and geographic remoteness. Data for patients with parasitologically confirmed malaria, available through an ongoing malaria transmission study at Bufundi Health Centre (*3*), showed a different temporal pattern of incidence, however. As

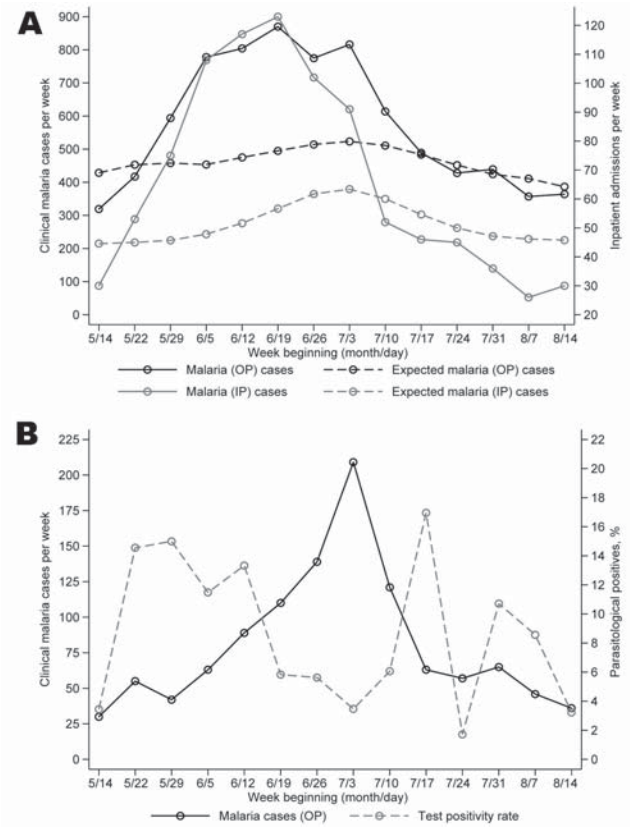

Figure. A) Weekly observed and expected numbers of outpatient (OP) and inpatient (IP) cases of clinically diagnosed malaria from sentinel health centers in Kabale district, southwestern Uganda, May–August 2006. B) Weekly numbers of clinically diagnosed malaria cases and the proportion of case-patients subsequently testing positive for *Plasmodium falciparum* infection by rapid diagnostic test at Bufundi, Kabale district, southwestern Uganda, May–August 2006.

part of this study, all samples from malaria case-patients identified through clinical diagnosis were subject to a Paracheck-Pf immunoassay test (Orchid Biomedical Systems, Verna, Goa, India). Results indicated that, at the peak of the apparent malaria outbreak, the percentage of samples from clinically diagnosed cases that produced a positive diagnostic test was as low as 4% (Figure, panel B). These results are unlikely to reflect poor diagnostic performance of the testing (*6*); febrile illness other than malaria was likely the cause of the outbreak.

Recent experiences in Kabale also highlight the potentially unwieldy nature of indoor residual spraying campaigns in the absence of spatial targeting. In Kabale, a district-wide spraying campaign supported by the US President's Malaria Initiative (*7*) was planned for the 2006 transmission season. However, shortages of trained personnel and other institutional delays meant that spraying could not begin until the third week of June, by which time the epidemic had peaked (and densities of vector mosquitoes had presumably begun to fall). By July 17, <50% of the targeted structures had been sprayed. In the future, careful targeting of spraying to areas of highest epidemic risk might lead to more timely completion of spraying activities. It might also be beneficial to create special spray teams that can respond quickly to specific alerts.

Recent experiences in Kabale have underlined the potential value of simple monitoring tools for early detection of epidemics but have also shown potential barriers to effective epidemic control. Our findings highlight the need to build systems that improve routine collection of data on parasitologically confirmed cases of malaria and allow rapid investigation of anomalies in incoming clinical data. It is equally important to develop procedures that translate early warning information into timely decisions concerning which epidemic control measures to use and how best to target them (*8*). Without these procedures, the value of early detection will be seriously undermined.

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Extensively Drug-resistant Tuberculosis, Italy and Germany

To the Editor: Twenty-three countries have reported ≥1 case of extensively drug-resistant tuberculosis (XDR TB) (*1*); however, information about XDR TB is still incomplete. In particular, the response of XDR TB to treatment in countries with low incidence is not known. We compared mortality rates from XDR TB with those from multidrug-resistant (MDR) TB.

We analyzed data from all culture-confirmed TB cases diagnosed during 2003–2006 by the TB clinical reference centers in Italy (Sondalo, Milan, Rome) and Germany (Borstel, Grosshansdorf, Bad-Lippspringe) and reviewed original clinical records. Drug susceptibility testing for first- and second-line anti-TB drugs was performed according to World Health Organization (WHO) recommendations by quality-assured laboratories and retested at WHO Supranational Reference Laboratories (Rome/Milan; Borstel) (*2–4*).

XDR TB was defined as resistance to at least rifampin and isoniazid (MDR TB definition) in addition to

any fluoroquinolone and ≥1 of 3 injectable anti-TB drugs (capreomycin, kanamycin, amikacin) (*3*). Characteristics of MDR TB and XDR TB cases were compared by χ^2 test (categorical variables), Student *t* test (admission days), and Kaplan-Maier curve (sputum smear, culture conversion), where appropriate.

Of 2,888 culture-positive TB cases analyzed (Italy 2,140, Germany 748), 126 (4.4%) were MDR (Italy 83, Germany 43) and 11 (0.4%) were XDR (Italy 8, Germany 3). We estimate that the TB cases analyzed represent 24% of culture-positive cases reported in Italy (69.7% of MDR) and 4.2% of those reported in Germany (12.6% of MDR). XDR TB was diagnosed in each year of the study. All 11 XDR TB patients were receiving retreatment, and of the 126 MDR TB patients, 74 (58.7%) were receiving retreatment. All XDR TB patients were HIV seronegative; and of 109 MDR TB patients tested for HIV, 10 (9.2%) were HIV seropositive. Details about previous treatment regimens, drug resistance, and duration of treatment of XDR TB patients are summarized in the online Appendix Table (available from www.cdc.gov/EID/content/13/5/780_appT.htm). XDR TB patients were significantly more likely than MDR TB patients to be resistant to all first-line drugs (8/11 vs. 36/126, p<0.005); 2 of these patients were resistant to all tested drugs (online Appendix Table).

In Germany, nonnationals accounted for 95.3% (41/43) of MDR TB cases and 100% (3 of 3) of XDR TB cases (all from the former Soviet Union); in Italy, they accounted for 72.3% (60/83) and 50% (4/8), respectively (p<0.001). Of 126 patients with MDR, 8 (6.3%) died, 45 (35.7%) were treated successfully, 67 (53.2%) were still receiving treatment (after achieving bacteriologic conversion, radiologic and clinical improvement, or both), and 6 defaulted (4.8%). Of 11 patients with XDR, 4 (36.4%) died and 7 (63.6%) were still receiving treatment. Compared with MDR TB patients, XDR TB patients had a 5-fold higher risk for death (relative risk 5.45; 95% confidence interval 1.95–15.27; p<0.01) and required longer hospitalization (mean ± SD 241.2 ± 177.0 vs. 99.1 ± 85.9 days; p<0.001) and longer treatment durations (30.3 ± 29.4 vs. 15.0 ± 23.8 months; p<0.05). Smear and culture conversions were observed for 4 XDR TB patients compared with 102 MDR TB patients (smear median 110 vs. 41 days; culture median 97.5 vs. 58 days, respectively); time to smear and culture conversion significantly differed between the 2 groups (p<0.01). A higher percentage of XDR TB than MDR TB patients had received previous anti-TB treatment (100% [11/11] vs. 59% [74/126], respectively, p<0.01) and were >45 years of age (64% [7/11] and 23% [29/126], respectively, p<0.01). Radiologic patterns of the thorax did not differ between XDR TB and MDR TB patients. In the overall sample, the only variable significantly associated with death (other than XDR TB status) was immigrant status (p<0.01). The association between XDR TB status and risk for death remained significant after stratification by immigrant status (p<0.05).

Our findings suggest that mismanagement of TB cases plays a major role in emergence of the problem in Europe (along with suboptimal infection control in congregate settings) (*5*), while in high HIV-prevalence settings (e.g., South Africa) XDR TB was mainly observed in patients never treated previously (*6*). Mortality rates among MDR TB patients treated in reference centers (6.3%) were lower than the rate observed in a previous study in general hospitals in Italy (8.7%) (*5*), although a proportion of our MDR TB patients are still completing treatment. This difference in rates is probably due to better management of MDR in the reference centers. Because of the high proportion of XDR TB patients still receiving treatment, further follow-up is necessary to assess potential for cure. The clinical relevance of resistance to all first-line drugs or other factors (e.g., delayed or inadequate treatment, suboptimal observation of drug intake) as major determinants of death needs further evaluation. The appearance of XDR TB in western Europe confirms that poor management and poor infection control in congregate settings exist and that new rapid diagnostic tests and new drugs are urgently needed.

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Buruli Ulcer, Nigeria

To the Editor: Buruli ulcer (BU), a neglected tropical disease caused by *Mycobacterium ulcerans*, is characterized by necrosis of subcutaneous tissue, leading to chronic, painless, and progressive ulcers. Without proper treatment, BU results in severe and permanent disability in more than a quarter of patients. Most patients are children <15 years of age. BU has been reported in >30 countries (*1*). The World Health Organization (WHO) has described the epidemiology, clinical features, diagnosis, and treatment of BU (*1*–*3*).

In 1967, Gray et al. described 4 BU cases in the Benue River Valley in Nigeria (*4*). The authors also described unpublished reports of the disease in Banbur, Adamawa State, in the upper part of the Benue River Valley. In 1976, Oluwasanmi et al. described 24 BU cases in and around Ibadan (*5*). Since then, there has been no official report of BU in Nigeria. However, unofficial reports indicate that the disease is still present in the country. For example, between 1998 and 2000, BU cases from the Leprosy and Tuberculosis Hospital in Moniaya-Ogoja, Cross River State, were bacteriologically confirmed at the Institute of Tropical Medicine in Belgium (*6*). More recently, patients from Nigeria have been treated in the neighboring countries of Benin (*7*) and Cameroon (*8*).

To clarify the BU situation in Nigeria, the government, with technical assistance from WHO, carried out a rapid assessment in the southern and southeastern states of the country, where cases had been previously reported. Preassessment sensitization workshops for health workers within the selected states were held in June and July 2006. The assessment took place November 15–19, 2006. The team, which was made up of international experts and national and state health officials, was divided into 2 groups. Group A visited Akwa Ibom and Cross Rivers States, and group B visited Anambra, Ebonyi, and Enugu States.

Based on the WHO case definitions (*1*), 14 of 37 patient examined were considered likely to have BU (9 active and 5 inactive cases); 9 were children ≤15 years of age. Eight patients were female, and 6 were male. One of the patients with active disease had the edematous form, 1 had osteomyelitis and ulcer, and the other 7 had ulcers (Figure). Ten of the patients had lesions on the lower limbs, 3 on the upper limbs, and 1 on the face. All cases were documented by registration on a modified version of the BU 02 form (*1*) and photography. Swab specimens were taken from all active ulcerative lesions. A fine-needle aspiration technique was used to obtain specimens from the edematous patient. In 4 (44%) of the 9 patients with active cases, the clinical diagnosis was confirmed by the IS2404 PCR at the Institute of Tropical Medicine.

The locations and number of cases identified in each are as follows: Ifite Ogwari village, Ayamelum Local Government Area (LGA), Anambra State (4 cases); Ndo Etok village, Ogoja LGA, Cross River State (3 cases); Nkpo Hamida village, Igbo-Eze North LGA, Enugu State (1 case); Iburu village, Ohaozora LGA, Ebonyi State (1 case); Akofu village, Ikwo LGA, Ebonyi State (1 case); Amagunze village, Nkanu East LGA, Enugu State (1 case); Okro Mbokho village, Eastern Obolo, Akwa Ibom State (1 case); Oron village, Oron LGA, Akwai Ibom State (1 case); and Ugwu Tank, Awka South LGA, Anambra State (1 case).

In conclusion, 30 years after the last publication (*5*) of cases in southwestern Nigeria, BU cases have been found in the southern and southeastern parts of the country. A similar phenomenon occurred in Cameroon, where a case search in 2001 in 2 districts where cases had last been reported 24

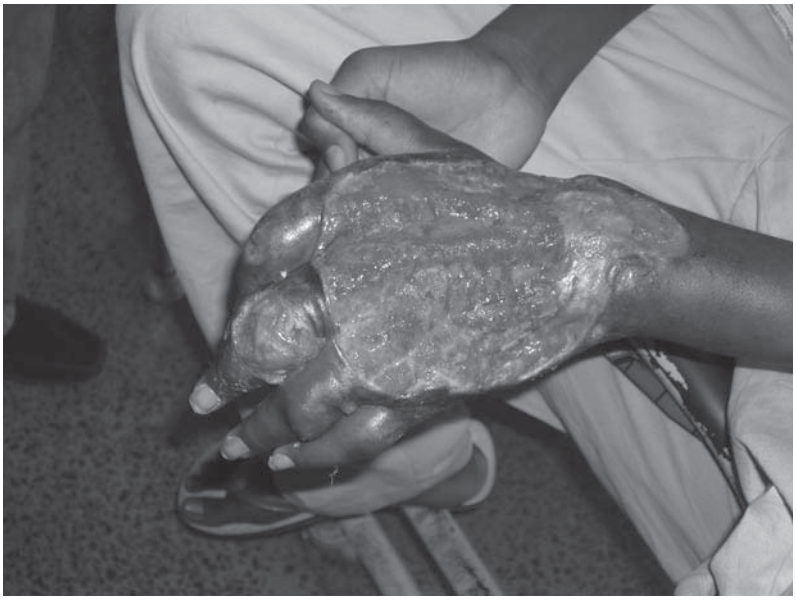

Figure. A typical Buruli ulcer in a 17-year-old boy identified during the assessment.

years earlier found 436 active and inactive cases (*8*). These findings demonstrate that BU has not disappeared from Nigeria and that the absence of any regular reporting should be investigated. Although the assessment team was able to visit only 5 of the 36 states (8 of 774 LGAs and 10 communities), it concluded that BU is still present in Nigeria and may be more prevalent than had been previously thought. The lack of familiarity with the disease by health workers may also have contributed to poor reporting.

The assessment team recommended 5 measures: 1) inclusion of BU treatment and control activities in the Tuberculosis and Leprosy Control Program at federal, state, and LGA levels to enhance surveillance of the disease; 2) training of health workers at all levels; 3) a detailed assessment of the extent of BU in the 5 states visited as well as in other states; 4) approaching partners supporting tuberculosis and leprosy control activities in Nigeria to include BU; and 5) incorporating BU into the national surveillance system to allow better data collection.

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Acetobacter cibinongensis Bacteremia in Human

To the Editor: The genus *Acetobacter* belongs to the group of acetic acid bacteria that oxidize alcohols or sugars incompletely, leading to the accumulation of acetic acid. Acetic acid bacteria are of great industrial interest because of their use to produce vinegar from spirits, wine, beer, and cider in temperate regions of Europe, the Americas, and Japan. Several species seem to be associated with tropical climates. In Southeast Asia, *Acetobacter* spp. have been found in fermented foods such as tea fungus beverage, palm vinegar, palm wine, nata de coco, and pickles (*1*). *A. cibinongensis* is mainly found in tropical fruits and flowers (*2*). We describe a case of human infection with a member of the genus *Acetobacter*.

The patient was an HIV-seronegative, 40-year-old man who for 1 year had been receiving chronic hemodialysis for end-stage renal failure. He had a history of intravenous drug use, and continued use was suspected. In February 2005, when admitted for a routine dialysis session, he had fever (38°C) and bronchitis and was receiving empiric treatment with amoxicillin (2 g/day). His respiratory status improved slightly, but fever persisted after 48 hours. On his right forearm, he had a large inflammatory skin lesion that followed the course of an arteriovenous fistula, suggestive of staphylococcal infection. Treatment was switched to pristinamycin (2 g/day for 4 days). The patient's leukocyte count was within normal limits, but his C-reactive protein level was elevated (50 mg/L). Two blood samples were drawn, 1 through a subclavian catheter implanted in 2004 and the other through the arteriovenous fistula. After 4 days, a gram-variable polymorphic rod, named nîmes373, grew from both aerobic culture vials and yielded very small polymorphic colonies on Columbia sheep blood and blood-chocolate agar plates (Bio-Rad, Marnes-la-Coquette, France). Enhanced growth was observed on *Legionella* agar (AES Chemunex, Bruz, France) and on R2A agar plates (BD-BBL, Le Pont de Claix, France). Biotyping of this catalase-positive, oxidase-negative rod with API 20NE and API 20E strips (bioMérieux, Marcy l'Etiole, France) and with the ID-GNB card system on a VITEK 2 apparatus (bioMérieux) did not identify the bacterium. Positive reactions were obtained for acetoin production and citrate assimilation. Antimicrobial drug–susceptibility pattern could not be validated owing to the lack of interassay reproducibility. Pristinamycin was replaced by broad-spectrum antimicrobial therapy consisting of cefazolin (1 g every 48 hours) and tobramycin (225 mg after each hemodialysis session). After 3 courses of this regimen, treatment was changed to amoxicillin (2 g/day, plus 1 g after each dialysis session) for 4 weeks. The local inflammation and fever subsided, the C-reactive protein level returned to normal, and the patient's clinical status improved with no recurrence of infection.

A 1389-bp 16S rDNA sequence was obtained for strain nîmes373, as described (*3*). The sequence matched those of *A. cibinongensis* deposited in the GenBank database (>99.6% identity). The 16S rDNA-based phylogeny confirmed the affiliation of strain nîmes373 to the species *A. cibinongensis* in the family *Acetobacteraceae* (Figure). The growth of nîmes373 on 0.7% calcium carbonate agar plates with 7% ethanol (pH 3.5) cleared calcium carbonate. This indicated tolerance of the isolate to acidic conditions and ability to produce organic acids from alcohol, thereby proving its affiliation to the acetic acid bacteria group.

Currently, 6 genera of acetic acid bacteria are recognized in the Alpha Proteobacteria lineage (Figure). Only the main genera (*Acetobacter*, *Gluconacetobacter*, *Gluconobacter*) are discriminated by analytic methods, such as high-performance liquid chromatography, which are not used in routine microbiology (*4*). The tree showed that 16S rDNA sequencing was effective for genus and species identification of acetic acid bacteria (Figure). This method is also rapid and convenient for use in a medical microbiology laboratory.

Bacteria have been used in food processing and have thus been ingested, live or dead, throughout human history. The safety of lactic and acetic acid bacteria has been confirmed over the years (*5*). However, in recent years, lactic acid bacteria belonging to the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, or *Bifidobacterium* have been isolated from a variety of clinical samples, generally from patients with diabetes, cancer, or immunosuppression (*6*). As lactic acid bacteria are part of the normal human flora, their clinical relevance is often difficult to establish. In contrast, acetic acid bacteria have never been isolated from human flora; recently, the pathogenicity of an unknown bacterium that probably belonged to acetic acid bacteria, *Granulobacter bethesdensis,* was found in a patient with lymphadenitis (*7*).

Our patient denied travel outside France and had no exotic dietary habits. He was probably infected by direct inoculation, possible during intravenous drug injection or after unnoticed injury when he worked in vineyards. For hemodialysis patients, vascular access infections are a major cause of illness and death (*8*). Also for these patients, unusual bacteria with low inherent pathogenicity are increasingly reported as potential causes of bacteremia (*9*). The decreased phagocytic activity of neutrophils and monocytes associated with chronic renal failure may increase susceptibility to bacteria that would otherwise have low patho-

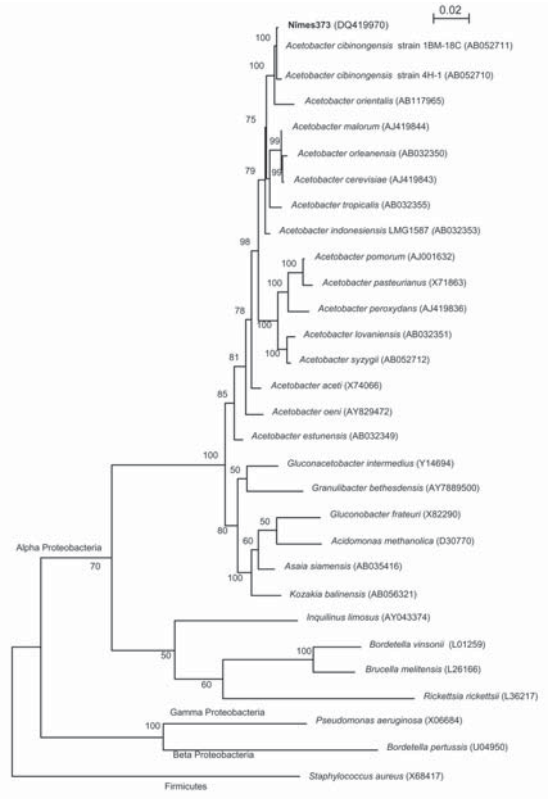

Figure. 16S rDNA maximum-likelihood phylogenetic tree showing the relationships of the isolate nîmes373 with 15 species of the genus *Acetobacter* and 6 strains representative of the 6 other genera of acetic acid bacteria in the Alpha Proteobacteria. Sequences of Alpha, Beta, and Gamma Proteobacteria of clinical relevance were also included in the tree. *Staphylococcus aureus* (*Firmicutes*) 16S rDNA was used as an outgroup. The 16S rDNA sequences used to reconstruct this tree were obtained from the GenBank database, and their accession numbers are indicated in brackets. The tree was reconstructed using DNAML from the PHYLIP package v. 3.6.6, on the basis of the F84 (+ gamma distribution + invariant sites) substitution model. The scale bar indicates 0.02 substitutions per nucleotide position. Numbers given at the nodes represent bootstrap percentages calculated on 100 replicates.

genicity (*10*). This second known report of human infection with acetic acid bacteria should alert clinicians to the risk for opportunistic infections with these bacteria, which are broadly used in food processing.

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Risk for Epidemics after Natural Disasters

To the Editor: Myths that disaster-affected populations are at high risk for outbreaks and that dead bodies contribute to this risk are common (*1*). Conversely, some experts deny high, short-term risk after disasters (*2*).

We agree with Watson et al. (*3*) that the risk for communicable diseases

transmission after natural disasters is low but real and that it is not directly related to the disasters and dead bodies, but primarily associated with the characteristics of the displaced population within the local disease ecology. This belief supports the need for rapid but accurate assessment of health status, risk, and needs, the results of which greatly influence the nature of relief activities (*4*). Key functions of relief teams are communicable diseases surveillance, early warning, and rapid response to epidemic-prone situations or outbreaks.

As an example, on October 26, 2005, after an earthquake in Pakistan, the World Health Organization asked the French military epidemiologic assessment team (1 epidemiologist and 1 veterinarian) to perform a sanitary assessment after cases of acute bloody diarrhea were reported in the camp of Tariqabad (estimated population ≈2,000), near Muzaffarabad. The assessment highlighted a lack of safe water and sanitation facilities, low routine immunization coverage, and disruption of healthcare services.

To prevent further diarrhea, we recommended improving the overall water and sanitation conditions. A medical team from a French nongovernment organization was also provided to help the 1 physician at the camp. Concurrently, we recommended a vaccination campaign as preventive strategy against diseases likely to occur in such conditions: tetanus, diphtheria, and measles. These measures were quickly implemented to reduce the overall risk, and no further unusual increases in disease incidence were noted during the following weeks. As in another outbreak documented in a camp in the Muzaffarabad area (*5*), rapid detection, response, and implementation of control measures are critical for minimizing the illness and death associated with outbreaks in these high-risk populations.

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Recombinant Sapovirus Gastroenteritis, Japan

To the Editor: Sapovirus and norovirus are causative agents of gastroenteritis in children and adults. Norovirus is the most important cause of outbreaks of gastroenteritis, whereas only a few outbreaks of sapovirus have been reported (*1*,*2*). On the basis of complete capsid gene sequences, sapovirus can be divided into 5 genogroups, among which GI, GII, GIV, and GV infect humans, whereas sapovirus GIII infects porcine species.

We report 2 outbreaks of gastroenteritis in Hokkaido, Japan. The first outbreak (A) occurred at a college from May 29 to June 2, 2000. A total of 12 persons (11 students and 1 teacher) reported symptoms of gastroenteritis (nausea, vomiting, stomachache, diarrhea, and fever); 11 stool specimens were collected from days 1 to 7 after onset of illness (Table). These specimens were negative for norovirus (data not shown), but 5 were positive for sapoviruslike viruses by electron microscopy (Table).

The 11 specimens were then examined for sapovirus by using nested reverse transcription–PCR (RT-PCR) as described (*3*). A total of 9 (82%) of 11 specimens were positive for sapovirus. Sequence analysis showed that these 9 viruses had 100% nucleotide identity and likely represented the same sapovirus strain (termed Yak2 strain, GenBank accession no. AB046353). To determine the number of cDNA copies per gram of stool, we performed real-time RT-PCR as described (*4*). The number of sapovirus cDNA copies ranged from 5.36×10^5 to 7.47×10^9/g stool (median 5.49×10^9 copies/g stool) (Table).

The second outbreak (B) occurred at a kindergarten from February 1 to 22, 2005. A total of 23 persons (15 children and 8 adults) reported symp-

Table. Analysis of 18 stool specimens for sapovirus during 2 outbreaks of gastroenteritis, Japan*

Outbreak, specimen	Date of illness onset	EM	Nested RT-PCR	Real-time PCR†
A				
Yak1	Jun 2, 2000	–	+	7.47×10^9
Yak2	May 29, 2000	–	–	–
Yak3	May 30, 2000	–	–	–
Yak4	May 31, 2000	+	+	6.55×10^9
Yak5	Jun 1, 2000	–	+	9.38×10^8
Yak6	Jun 1, 2000	–	+	1.30×10^8
Yak7	May 29, 2000	–	+	1.46×10^9
Yak8	May 29, 2000	+	+	2.78×10^{10}
Yak9	Jun 1, 2000	+	+	3.00×10^9
Yak10	Jun 1, 2000	+	+	2.05×10^9
Yak11	Jun 1, 2000	+	+	5.36×10^5
B				
Nay1	Feb 17, 2005	NT	+	1.65×10^{10}
Nay2	Feb 14, 2005	NT	+	1.82×10^9
Nay3	Feb 18, 2005	NT	+	1.14×10^9
Nay4	Feb 17, 2005	NT	+	5.41×10^{10}
Nay5	Feb 16, 2005	NT	+	5.26×10^{10}
Nay6	Feb 18, 2005	NT	+	2.50×10^{10}
Nay7	Feb 17, 2005	NT	+	2.38×10^{10}

*EM, electron microscopy; RT-PCR, reverse transcription–PCR; NT, not tested.
†cDNA copies/g stool.

toms of gastroenteritis (nausea, vomiting, stomachache, diarrhea, and fever); 7 stool specimens were collected (Table). These specimens were negative for norovirus (data not shown), but all were positive for sapovirus by nested RT-PCR. The 7 sequences from this outbreak had 100% nucleotide identity and likely represented the same sapovirus strain (termed Nay1 strain, GenBank accession no. EF213768). The number of sapovirus cDNA copies ranged from 1.14×10^9 to 5.41×10^{10}/g stool (median 2.50×10^{10} copies/g stool) (Table).

One positive sapovirus specimen from each outbreak was subjected to further sequence analysis in which a single overlapping PCR fragment covering the partial polymerase gene and capsid gene was amplified. The Yak2 and Nay1 sequences shared ≈71% nucleotide identity for this fragment and likely represented different sapovirus strains. The Yak2 sequence closely matched sapovirus GIV Ehime1107 and SW278 sequences (GenBank accession nos. DQ058829 and AY237420, respectively) and had 98% and 97% nucleotide identity for the entire fragment, respectively (*5*). The Nay1 sequence closely matched the sapovirus GII C12 sequence (AY603425) and had 91% nucleotide identity for the entire fragment.

The Nay1 sequence closely matched the C12 sequence, which was detected in Osaka, Japan, in 2001 (*6*), whereas the Yak2 sequence closely matched the Ehime1107 sequence, which was detected in Matsuyama, Japan, in 2002 (*5*), and the SW278 sequence, which was detected in Sweden in 2003 (*1*). We recently described the C12 strain as intragenogroup recombinant sapovirus strain (*6*), whereas the Ehime1107 and SW278 strains were described as intergenogroup recombinant sapovirus strains (*5*). Our results indicate that recombination sites in intragenogroup and intergenogroup recombinant sapovirus strains were at the polymerase and capsid junction (*5*,*6*). Sapovirus Sydney53 (DQ104360) and Sydney3 strains (DQ104357), which were detected in Australia from August 2001 to August 2004 (*7*), closely matched C12 and Ehime1107/SW278 sequences, respectively. These results showed that recombinant sapovirus strains are stable in the environment and may be globally distributed. Our findings also suggest a changing distribution of sapovirus-associated gastroenteritis in Hokkaido because different sapovirus GI strains were predominant in outbreaks of gastroenteritis in Hokkaido (*8*,*9*).

In a recent study, the number of norovirus cDNA copies per gram of stool specimen was analyzed and a discrepancy was found between the different norovirus genogroups (*10*). Chan et al. found that noroviruses GI and GII showed medians of 8.4×10^5 and 3.0×10^8 copies/g of stool specimen, respectively, and speculated that increased viral loads were caused by higher transmissibility of norovirus GII strains (*10*). Our results showed that sapovirus GII Nay1 and GIV Yak2 strains showed higher viral loads than norovirus GII strains. These results suggest that a high degree of shedding of sapovirus GII Nay1 and GIV Yak2 strains may have caused the outbreak of gastroenteritis. However, to elucidate this suggestion, further studies are needed with other sapovirus strains.

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Salmonella Typhimurium in Hihi, New Zealand

To the Editor: The recent finding of a previously unrecorded *Salmonella* strain in an endangered New Zealand passerine (the hihi, *Notiomystis cincta*; [*1*]) offers the rare opportunity to observe the initial arrival and pathology of an epizootic and to determine its population-level effect. Over 8 days in February 2006, 6 freshly dead hihi were discovered in a free-living island population. Pathologic findings were similar: birds were in good body condition with substantial subcutaneous fat reserves and no gross lesions in the crop, indicating death from a highly pathogenic disease. Histopathologic examination showed septicemia and inflammatory necrosis of organs, particularly the liver and spleen, typical of salmonellosis in birds (*2*). Microbiologic examination of liver samples isolated heavy growths of the bacterium *Salmonella enterica* serotype Typhimurium DT195. During the same period, 3 more dead hihi were found, but they were too decomposed for postmortem examination.

Hihi are nectar-feeders that declined to near extinction after European colonization of New Zealand and survived on a single island refuge (Hauturu). Since 1980, 14 attempts have been made to reintroduce the species to 6 other sites, resulting in 3 new populations that persist with management. The *S.* Typhimurium DT195 outbreak occurred within a reintroduced population on Tiritiri Matangi Island. Management includes providing supplementary food (sugar water) diluted with local rain water; feeders are sterilized before each use.

Because disease in hihi is closely monitored, the outbreak indicates that *S.* Typhimurium DT195 is a novel serotype for this species. During December 2005, fecal screening of 18 broods (37 nestlings) from Tiritiri Matangi Island found no evidence of enteric pathogens; screenings in February and May 2005 (40 adult and juvenile birds) from Tiritiri Matangi Island similarly returned negative results. Screening in all hihi populations during 2004 also found no evidence of *Salmonella* infection (32 adults and juveniles at Tiritiri Matangi, 29 at Hauturu, and 27 at Kapiti), and a 15-year pathology database from 230 dead hihi collected across these populations and a captive breeding facility lists no salmonellosis cases (J.G. Ewen and M.R. Alley, unpub. data).

Documentation of the emergent stages of infectious disease in endangered species is rare (*3*,*4*). This bacterium strain is absent from New Zealand's livestock and wildlife (www.surv.esr.cri.nz/enteric_reference/non human_salmonella.php). Nontyphoid *Salmonella* spp. are a major health concern worldwide (*5*), and New Zealand conducts intensive surveillance to maintain food safety. The New Zealand Wildlife Health Centre has not reported *S.* Typhimurium DT195 despite necropsies of >3,000 wild birds during 1996–2006, which suggests this strain is rare in New Zealand, despite its presence in other countries (*6*).

S. Typhimurium DT195 has been detected in 3 human patients in New Zealand (1 each in 2002, 2003, and 2006). The *S.* Typhimurium DT195 isolated from hihi in the February 2006 outbreak were indistinguishable from those isolated from the human case-patient in 2006 (see [*2*] for methods). Tiritiri Matangi is an isolated island nature reserve 3 km off the New Zealand coast, which prevents movement of hihi to other areas. How this strain appeared in a human patient and as an epizootic in an isolated island nature reserve is intriguing. The most recent human case was diagnosed on the North Island of New Zealand, but the person was not living in close proximity to the birds. Tiritiri Matangi receives ≈30,000 human visitors per year, but whether the person with *S.*

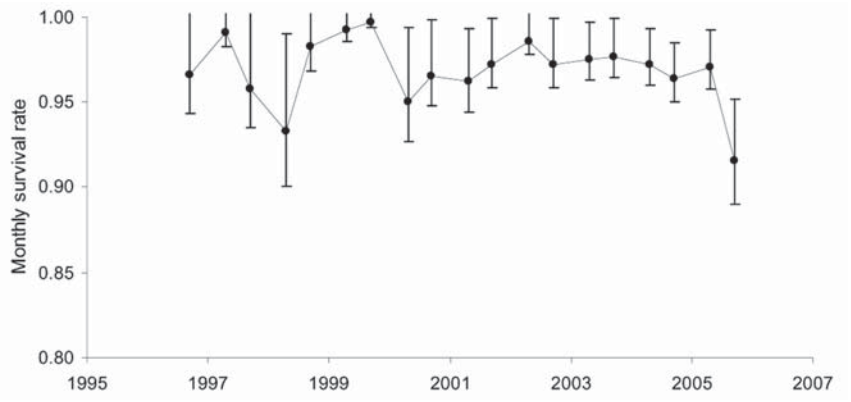

Figure. Survival rates from September–February and February–September among hihi in New Zealand during 1996–2006, estimated by using mark–recapture analysis, show that the transmission of *Salmonella* Typhimurium DT195 to hihi during the February 2006 epidemic caused a substantial drop in population. Bars indicate 95% confidence intervals.

Typhimurium DT195 ever visited is not known. An unidentified infection source may be present in New Zealand that periodically spills over into alternate host species. Given their historic isolation, hihi may have low or no exposure to many diseases, which makes negative reactions more likely (*7*).

The transmission of *S.* Typhimurium DT195 to hihi caused a substantial drop in their population (Figure). The 9 bodies recovered represent a small proportion of the birds that died, given the difficulty of recovering dead birds (*8*). We used mark–recapture analysis (*9*) to estimate that adult survival probability was 0.64 (95% confidence interval [CI] 0.53–0.74) from September 2005 through February 2006, compared with an expected survival of 0.87 (95% CI 0.85–0.89), according to data from the previous 10 years (data not shown). The quotient of these 2 probabilities is 0.74 (95% CI 0.60–0.84); hence, we can infer that ≈26% of birds were killed by the epizootic.

With such high virulence, fade-out may occur as susceptible individuals are rapidly removed from the population (*10*). Subsequent monitoring has failed to detect further evidence of *S.* Typhimurium DT195. This apparent fade-out mirrors classic predictions from epidemiology (*10*). It is unknown whether the pathogen resides in resistant hihi or whether threats from the unknown source remain.

The key issues for endangered species management are identifying the risk of pathogens entering a host population and the probability that this occurrence would result in host extinction (*3*). The 2006 salmonellosis outbreak in hihi could easily have remained undetected, leaving conservation managers unaware of what caused the population decline. How often this occurs in poorly monitored wildlife is unknown. This study shows the need for increased awareness of these processes when considering biodiversity conservation.

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Travel-related *Salmonella* Agama, Gabon

To the Editor: Traveler's diarrhea affects >50% of travelers to regions such as sub-Saharan Africa (*1*). Worldwide, enterotoxigenic *Escherichia coli* is the leading bacterial pathogen that causes traveler's diarrhea, followed by *Campylobacter jejuni* and then *Salmonella* spp., which are the causative pathogens for ≥25% of traveler's diarrhea in Africa (*1*). Nontyphoidal salmonellosis is mostly caused by the *Salmonella* serotypes Enteritidis and Typhimurium (*2*). To our knowledge, only a few cases of salmonellosis due to *S.* Agama have been reported in medical literature, none as a travel-related disease (*3*,*4*).

S. Agama was characterized in 1956 as a new serotype of *Salmonella enterica* from the feces of the agama lizard (*Agama agama*) in Nigeria (*5*). Subsequently, *S.* Agama was isolated from geckos and mammals in Africa (*4*,*6*,*7*) and the United Kindgom (*8*,*9*). Human infections with *S.* Agama were once reported in Nigeria and related to the lizards as possible reservoirs (*4*). Another clinical case of *S.* Agama infection was described in France in a 9-month-old child with fever and diarrhea (*3*); fruits imported from Africa were discussed as potential source of infection. We report what is, to our knowledge, the first travel-related case of salmonellosis due to *Salmonella* Agama experienced by a tourist who had traveled to Gabon in central Africa.

A previously healthy 25-year-old man in Germany sought treatment for 2 episodes of intermittent fever ≤39°C, as well as headache, nausea, abdominal pain, diarrhea, arthralgia, and cough. Symptoms started the day he returned from a 1-month trip to Gabon, a country in central Africa, where he stayed with a friend who lives near the Albert Schweitzer Hospital in Lambaréné and took occasional excursions to other areas.

Before traveling, the patient had been immunized against hepatitis A, hepatitis B, yellow fever, polio, typhoid fever, tetanus, measles, and mumps; he reported taking atovaquone-proguanil for malaria prophylaxis during his first 3 weeks in Gabon. While in Gabon, he frequently drank tap water, ate food sold by street vendors, and had repeated fresh water contact while swimming in the Ogooué River. He exhibited no symptoms during his trip.

His first examination was performed 2 weeks after his return to Germany and the onset of symptoms. Physical examination showed no pathologic findings, malaria was excluded by repeated thick blood smears, and in the absence of abnormal laboratory findings a common cold disease was assumed on clinical grounds. No specific treatment was prescribed, and the patient recovered from symptoms except for intermittent mild diarrhea.

Four weeks after his return to Germany, a second episode with reappearance of all former symptoms led to a new examination. At this time, the patient was afebrile, and physical examination showed no pathologic findings. Laboratory values were within the normal range except for C-reactive protein, which was elevated at 47mg/dL (normal value <5 mg/dL). Pneumonia was excluded by radiography, and a stool sample was obtained for parasitologic examination and bacterial culture. The patient was treated with clarithromycin, 500 mg orally twice a day for 7 days, for a presumed upper respiratory tract infection. The patient's symptoms disappeared.

Stool sample test results were negative for intestinal helminths and other parasites. However, growth of *Salmonella* species was observed in 1 culture. The isolate was characterized as *Salmonella* Agama (*S. enterica* subspecies *enterica* serotype Agama 4,12: i: 1,6). It was sensitive to ampicillin, cefotaxime, cefuroxime, ceftriaxone, imipenem, ciprofloxacin, gentamicin, trimethoprim-sulfamethoxazole, and fosfomycin but resistant to clarithromycin (MIC 96 mg/L). Five weeks after clinical resolution, further stool samples were found to be negative for any enteric pathogen.

In the light of the microbiologic evidence of *S.* Agama infection, we interviewed the patient about any consumption of meat or poultry and contact with animals. The patient reported no contact with animals during and after his trip to Gabon and said he is a vegetarian who abstains from consumption of any meat, including poultry. In Gabon, lizards are plentiful around all habitations, including the terrace of the house where the patient stayed; he reported that he ate sitting on the floor of the terrace. Lizards are also sometimes seen in food displays at street markets, including among foods that are commonly eaten uncooked (Figure).

Given microbiologic results and travel history, *S.* Agama was the most likely cause for the gastroenteritic and unspecific symptoms experienced by our patient. We may speculate about transmission of *S.* Agama by direct or indirect contact with lizards, but other routes of transmission cannot be ruled out.

Gastrointestinal and unspecific symptoms lasted 2 weeks with un-

Figure. Photograph taken at a local street market in Gabon shows a lizard in a basket of onions, which are frequently eaten uncooked. *Salmonella enterica* subspecies *enterica* serotype Agama has been isolated from lizards in Africa.

dulating severity and relapsed after a latent period of another 2 weeks. Although the isolate was highly resistant to clarithromycin in vitro, the patient improved clinically as symptoms disappeared. Results of stool cultures taken 5 weeks after resolution of clinical symptoms were negative. The clinical course of this patient's illness suggests that *S.* Agama may cause self-limiting infections and asymptomatic shedding, as do other nontyphoidal Salmonella infections. The course of disease may be affected by the ingested infective dose, host factors, and virulence of *S.* Agama isolates.

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Small Anellovirus Infections in Korean Children

To the Editor: Recently, Jones et al. (*1*) identified circular DNA sequences, classified as *Anellovirus* genus, in plasma from patients with acute viral infection syndromes. These anelloviruses were then labeled as "small anellovirus (SAV)" because of their smaller genomes when compared with Torque Teno Virus (TTV) and Torque Teno Mini Virus (TTMV), which have small, circular, single-stranded DNA genomes. Although anelloviruses are not associated with any specific disease, TTV has been suggested to play a role in acute respiratory disease (ARD) and in asthma of children (*2*,*3*).

Kawasaki disease and Henoch-Schonlein purpura are important vasculitis disorders in children, possibly triggered by unknown infectious agents. Recently, Gergely et al. (*4*) reported that molecular mimicry involving TTV and the generation of autoantibodies may have a role in the pathogenesis of systemic lupus erythematosus. The purpose of our study was to investigate the prevalence of SAV and its association with various clinical diseases in children.

The study population comprised 81 serum samples from healthy children and 151 serum samples from children hospitalized with hepatitis (81 cases), ARD (40 cases), Kawasaki disease (12 cases), or Henoch-Schonlein purpura (18 cases) during the period January 2002–June 2006. Nasopharyngeal aspirates paired with serum samples were collected from 34 children with ARD, including upper respiratory tract infections, pneumonia, and acute bronchiolitis. Samples were collected after informed consent was obtained at admission from patients' parents.

PCRs for SAV were performed to amplify a 5′ noncoding region of SAV

with specific primers, as described previously (*5*). PCR products were directly sequenced, and nucleotide sequences were registered in GenBank (accession nos. DQ978791–DQ9788810). The χ^2 test with Yates correction and Mann-Whitney U-test were used for statistical comparison by using MedCalc (MedCalc Software, Mariakerke, Belgium). A p value <0.05 was defined as statistically significant.

In our study population, serum SAV DNA was detected in 28 (34.5%) of 81 children in the control group and in 66 (43.7%) of 151 children in the disease group. In the healthy control group, the SAV-positive rate was 7.4% (6/81) in children <12 months of age, 16.0% (13/81) in children 1–4 years of age, and 11.1% (9/81) in children 5–15 years of age. In the disease group, the SAV-positive rate was 35.8% (29/81) in patients with hepatitis, 67.5% (27/40) in ARD, 50% (6/12) in Kawasaki disease, and 22.2% (4/18) in Henoch-Schonlein purpura, respectively (Table). Among 34 nasopharyngeal aspirates collected from children with ARD, SAV DNA was detected in 19 (55.9%). Codetection of SAV and respiratory syncytial virus in nasopharyngeal aspirates was observed in 4 patients.

Percent similarity of nucleotide sequence of PCR products was 99% among SAV isolates. To our knowledge, this is the first report of SAV infections in children. The prevalence and role of SAV in clinical diseases have yet to be determined. Recently, Biagini et al. (*5*) reported that the prevalence of SAV infection was 20% (12/60) in French blood donors. In an Italian study (*6*), the positive rate of SAV DNA was 9.1% (5/55) in patients with hepatitis C compared with 8.6% (3/35) in healthy controls. Thus, the prevalence of SAV in Korean children is much higher than that reported in adults from other countries (*5*,*6*). Further studies are needed to confirm this finding.

In our study, the prevalence of SAV did not differ significantly between the hepatitis group and the healthy control group. Our results indicate that SAV presence does not appear to have a defining role in hepatitis, as do TTV or TTMV infection (*7*). In a previous study, several groups of viruses, including TTV, were ruled out as etiologic agents of Kawasaki disease (*8*), findings similar to those of our study. We found that the prevalence of SAV was significantly higher in patients with ARD and that SAV-positive results from serum were consistent with those of nasopharyngeal aspirates in 76% (26/34). These findings suggest that the respiratory tract may be a transmission route of SAV in children.

In conclusion, we confirmed the presence of SAV in serum samples and nasopharyngeal aspirates from Korean children. A significantly higher detection of SAV DNA was observed in children with ARD compared with healthy children or children with other clinical diseases.

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Table. Prevalence of SAV viremia in the study population, Republic of Korea, January 2002–June 2006*

Group	Sex, M/F	Age range (mean age)	No. tested	No. positive (%)	p value
Control	48/33	1 mo–15 y (47 mo)	81	28 (34.5)	
Hepatitis	44/37	1 mo–15 y (58 mo)	81	29 (35.8)	1
HBV	8/12	1–15 y	20	10 (50)	0.30
HCV	11/0	3–10 y	11	5 (45.4)	0.71
Others	10/9	0–13 y	19	7 (36.8)	0.93
Unknown	15/16	0–13 y	31	7 (22.5)	0.3
ARD	22/18	0–5 y (18 mo)	40	27 (67.5)	0.001†
Vasculitis	22/8	1–10 y (46 mo)	30	10 (33.3)	0.91
KD	9/3	1–9 y	12	6 (50)	0.47
HSP	13/5	2–10 y	18	4 (22.2)	0.46
Total	136/96	1 mo–15 y	232	94 (40.5)	

*SAV, small anellovirus; HBV, hepatitis B virus; HCV, hepatitis C virus; ARD, acute respiratory tract disease; KD, Kawasaki disease; HSP, Henoch-Schonlein purpura.
†p<0.05, statistically significant.

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Antibodies against *Leptospira* spp. in Captive Collared Peccaries, Peru

To the Editor: Leptospirosis is endemic to tropical South America and is a major public health problem for persons living in some regions of the Amazon Basin (*1*–*3*). For local inhabitants, the collared peccary (*Tayassu tajacu)* represents a major source of meat and income and is one of the most hunted species. As a result, several farms are attempting to produce captive collared peccaries (*4*). Although spirochetes have been isolated from bats, marsupials, and rodents in the Peruvian Amazon (*5*), local popular game animals have not been tested.

From May through December 2003, 96 collared peccaries from 4 experimental farms in 2 Amazonian provinces of Peru (Loreto and Ucayali) were surveyed for antibodies against *Leptospira* spp. Although the initial stock of each farm came from the wild, most animals had been born in captivity, remained on their respective farms, and had no contact with animals from the different farms. Blood samples were taken from animals that were born or maintained on the farm for ≥6 months, were in good physical condition, and showed no signs of disease. Samples that had been hemolyzed or otherwise contaminated were discarded, leaving optimal samples from 96 animals (sex ratio 1:1, 71% ≥1 year of age).

The microscopic agglutination test was performed with a panel of 24 antigens belonging to 17 serogroups of *Leptospira* spp. used for screening surveys at the National Leptospirosis Reference Laboratory. An additional distinct strain, obtained from a febrile human patient in the Peruvian Amazon and provisionally designated as Var10, was added (*2*). Serum samples were considered positive if they had 50% agglutination and titers >100 (*6*). Chi-square tests were used for statistical comparisons of sex and age; significance was set at $p<0.05$.

Among the screened samples, 64.6% reacted to 15 serovars (strains) that belong to 11 serogroups (Table). Seroprevalence did not differ significantly in relation to sex or age. Var10 was the most prevalent (56.2%) strain. This strain was isolated from a human patient in Iquitos (Loreto, Peru) and involved in recent outbreaks in the northern Peruvian Amazon (*2*); its taxonomic classification is pending. In terms of its distribution, 32 peccaries had positive results for Var10 only; 12 serum samples were reactive to >1 known serogroup. *Leptospira* sp. Var10 reacted mainly with serogroups Australis and Hebdomadis. Maximum titers were 6,400 for serogroup Tarassovi and 3,200 for Icterohaemorraghiae. High seroprevalence (15.6%) against serogroup Australis (serovar bratislava) has been reported in collared peccaries and in feral and domestic pigs (*7*,*8*)

Seroprevalence on the farm in Loreto (n = 27) was 100%. At this farm, peccaries are kept near aquatic species and numerous ponds of stagnant water, which provide an ideal environment for the development of *Leptospira* spp. Because of recent human leptospirosis outbreaks in the area (*2*), 3 of the peccary caretakers were tested for antibodies against *Leptospira* spp.; their results were negative.

Although similar to animals described in previous reports (*7*,*9*), none of the sampled animals showed evidence of disease at the time of sampling; however, absence of clinical disease does not exclude the possibility of subclinical or past infections. Furthermore, the high prevalence of antibodies to multiple serotypes suggests a wide exposure to *Leptospira* spp. Despite reports that suggest the collared peccary could act as a reservoir for *Leptospira* spp. (*7*,*9*), the finding of high antibody titers in some individual animals could indicate that collared peccaries are incidental rather than reservoir hosts. However, the prevalences found at 4 distant farms also indicate that this species could play some role in the maintenance and spread of leptospirosis in the Amazon Basin.

Multiple titers to different serovars or serogroups in the same serum sample are common with serologic testing and difficult to interpret. Multiple titers can result from cross-reactions between different serovars or from true multiple infections (*10*). Regardless, serologic tests are only indicative of exposure to leptospires. Further efforts are necessary to isolate lepstospires from the urine or renal tissue of collared peccaries to confirm the presence of spirochetes and their potential dissemination into the environment.

Our findings indicate that persons who have contact with collared peccaries and their products, particularly animal caretakers, researchers, hunters, and game traders, are at risk for

Table. Prevalence of antileptospiral agglutinins per positive serogroup in captive collared peccaries, Peruvian Amazon, May 2003–Dec 2003*

			Loreto area		Ucayali area					
			BIOAM (n = 27)		Pucallpa Natural Park (n = 6)		San Juan (n = 52)		Club Divina Montaña (n = 11)	
Serogroup	Positive reactions no. (%)	Max titer	Positive reactions no. (%)	Max titer	Positive reactions no. (%)	Max titer	Positive reactions no. (%)	Max titer	Positive reactions no. (%)	Max titer
Total	62 (64.6)	6,400	27 (100)	6,400	1 (16.6)	100	27 (51.9)	3,200	7 (63.6)	100
Var10†	54 (56.2)	1,600	27 (100)	1,600	1 (16.6)	100	19 (36.5)	1,600	7 (63.6)	100
Australis	15 (15.6)	800	5 (18.5)	100	0	–	9 (17.3)	800	1 (9.1)	100
Hebdomadis	7 (7.3)	100	5 (18.5)	100	0	–	2 (3.8)	100	0	–
Icterohaemorrhagiae	4 (4.2)	3,200	2 (7.4)	100	0	–	2 (3.8)	3,200	0	–
Autumnalis	4 (4.2)	100	3 (11.1)	100	0	–	1 (1.9)	100	0	–
Bataviae	4 (4.2)	400	2 (7.4)	200	0	–	2 (3.8)	400	0	–
Tarassovi	3 (3.1)	6,400	3 (11.1)	6,400	0	–	0	–	0	–
Djasiman	2 (2.1)	800	0	–	0	–	2 (3.8)	800	0	–
Grippothyphosa	2 (2.1)	800	0	–	0	–	2 (3.8)	800	0	–
Ballum	2 (2.1)	100	2 (7.4)	100	0	–	0	–	0	–
Canicola	1 (1.0)	100	0	–	0	–	1 (1.9)	100	0	–
Mini	1 (1.0)	100	0	–	0	–	1 (1.9)	100	0	–

*BIOAM, Biodiversidad Amazónica; Max, maximum; –, titer not applicable.
†Classification pending.

zoonotic disease (*3*). Because further wildlife production in the Peruvian Amazon is expected, movement of animals and high animal densities could increase the chances of spirochete transmission within and between the farms. Therefore, precautions should be taken to limit the potential risks for leptospirosis transmission to domestic animals and humans.

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High Tuberculosis and HIV Coinfection Rate, Johannesburg

To the Editor: Tuberculosis (TB) is the leading cause of illness and death among HIV-1–infected patients in sub-Saharan Africa (*1*–*3*), but valid data on the population-level interaction between the TB and HIV epidemics are scarce (*4*). Our objective was to determine the extent of this dual epidemic in our setting, a hospital in Johannesburg, South Africa. We did this by introducing bedside TB and HIV counseling. We also intended to increase the use of voluntary counseling and testing for our TB patients and facilitate referral to our antiretroviral clinic.

From February to April 2006, 2 volunteers from Community AIDS Response (CARE) counseled patients admitted to the medical wards of the Helen Joseph Hospital. This regional hospital serves a catchment population of >500,000 people, predominantly low-income black Africans. Counselors provided TB and HIV wellness and adherence information, HIV pretest counseling, and referral to the Themba Lethu Clinic for rapid testing that used standard CARE modules.

Basic demographic, TB, and HIV data from patient records were documented on standard data collection forms. Missing data were extracted from the hospital database and Therapy Edge-HIV, the data management system used by the HIV clinic. HIV testing was conducted with a fourth-generation ELISA or rapid finger prick antibody test, according to World Health Organization guidelines.

Most admissions were for pulmonary TB. A total of 467 patients receiving TB treatment were counseled; 8 of these patients refused the TB counseling service, and 2 refused voluntary counseling and testing for HIV. These 467 patients constituted 13% of medical admissions and excluded the 1,075 patients seen at the hospital's outpatient clinic with suspected TB for this 3-month period. Our impression is that this figure constitutes an underrepresentation of the total TB admissions because TB counselors were not able to see every patient with TB.

Laboratory data were retrievable for 373 inpatients. For 301 (81%) of the 373 patients, TB blood culture, smear, or culture results could be traced. Hence, 72 (19%) of 373 patients who were receiving TB treatment had no record of a diagnostic effort to confirm TB. A total of 284 (76%) HIV test results could be traced; 270 (95%) of the 284 accessible TB patients had concurrent HIV infection (Table).

Most (123 [89%]) documented HIV results were from ELISAs performed during admission. Rapid testing performed in the ward was unacceptable to patients because confidentiality was compromised in large, busy wards and patients were often too ill to move to a side room. The system of making an appointment with the HIV clinic at the time of discharge failed because few patients (5%) actually had the rapid test after admission or began antiretroviral therapy. Those who began such therapy would have been captured on our database.

The level of concurrent TB and HIV coinfection at the hospital was 95%. To the best of our knowledge, this is the highest level ever described in the peer-reviewed English-language literature (*5*). This finding may reflect the selection bias for our inpatients, who generally would have more coexisting conditions than outpatients do. Also, HIV data were missing for 24% of the 373 patients, a fact that may also influence this finding.

The peak age incidence of TB in our population corresponds with previously published data and is similar to the peak age incidence of the HIV epidemic in South Africa (*6*). In one third of the admitted patients, no TB investigations were undertaken. This may be because patients provided a history of TB diagnosed elsewhere, or it may reflect the high rate of sputum smear negativity in the HIV-infected population, which lowers the clinician's threshold for empiric TB treatment.

Mycobacteremia appeared to be less common (14%) than reported in other African studies (*7*). However, we did not have a complete dataset—only 195 (52%) of the 373 patients could be evaluated.

TB and HIV have reached unprecedented levels in our urban inpatient population. TB and HIV must be viewed as different sides of the same coin, and services and staff must change accordingly. We need to use the opportunity of hospital admission to educate patients on the interaction between these 2 epidemics and facilitate patient referral for long-term management. Such management would include voluntary counseling and testing, as well as antiretroviral medication. The latter is a recognized strategy of TB control because it reduces the risk for TB by 70%–90% (*8*).

In addition, all inpatient procedures in our TB/HIV control programs need to be strengthened. Infection control interventions to limit the high rates of nosocomial transmission of TB to other vulnerable patients and staff need to be instituted. At our hospital, we are committed to these approaches. To this end, we have secured

Table. Results of HIV testing by method of HIV diagnosis among accessible patients with tuberculosis, Johannesburg, South Africa

Method	No. positive/ no. patients (%)	No. negative/ no. patients (%)
ELISA	110/123 (89)	13/123 (11)
Rapid test	32/32 (100)	0/32 (0)
Clinical diagnosis only	61/61 (100)	0/61 (0)
HIV status known to patient	67/68 (99)	1/68 (1)
Total	270/284 (95)	14/284 (5)

a Presidents Emergency Plan for AIDS Relief Grant via the nongovernmental organization Right to Care, which shares our vision. Urgent and extraordinary measures are indeed required in our combined control programs to achieve the Millennium Development Goals for TB/HIV.

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Tuberculosis Trends, Vietnam

To the Editor: The aims of the global strategy for tuberculosis (TB) control, the directly observed treatment short-course (DOTS) strategy, of the World Health Organization (WHO) are to detect ≥70% of new smear-positive pulmonary TB cases and cure ≥85% of these detected cases (*1*). If these aims are met in a setting of low prevalence of multidrug-resistant TB and HIV infection, TB incidence is predicted to decrease by >7% annually (*2*). Vietnam has a low prevalence of multidrug-resistant TB (2.3% in 1996–1997 [*3*]) and a low level of HIV infection in the adult population (0.4% in 2003, range 0.2%–0.8% [*4*]). It is the only country of 22 countries with the highest number of TB cases worldwide that has reached and exceeded WHO targets for TB control since 1997 (*5*,*6*). However, this country has not shown any decrease in TB reporting (*5*).

Reports may not reflect TB incidence if the proportion of cases detected and treated by the National Tuberculosis Program (NTP) varies over time. This incidence can be captured by assessing diagnostic efforts. We assessed whether TB case reporting rates in Vietnam are not decreasing because of increased diagnostic efforts in urban, rural, and remote (mountainous) settings. Characteristics of the NTP in Vietnam have been reported (*6*,*7*). The research board of the National Hospital for Tuberculosis and Respiratory Diseases in Hanoi provided scientific and ethical clearance for this study.

Reporting and laboratory register data were collected from 66 randomly selected districts; sampling was stratified to include 20 urban, 30 rural, and 20 remote districts. The NTP defines a suspected TB case-patient as a person with a cough for >3 weeks. A suspected case-patient was a person with a diagnostic sputum smear examination result for acid-fast bacilli by direct microscopy. A total of 20% of suspected case-patients were randomly selected and their data were used. Diagnostic effort was the number of suspect cases per 10,000 persons. A case-patient was a person with new smear-positive pulmonary TB. The reporting rate was the number of cases per 100,000 persons. Population sizes were derived from the national population census of 1999 and projected populations (*8*).

We calculated trends in reporting rates for 1997–2004 by age, sex, and setting (urban, rural, and remote) before and after adjustment for diagnostic effort by using Poisson regression and expressed the average annual percentage change with 95% confidence intervals. Observed trends were adjusted for variation in diagnostic effort over time by standardizing the number of notified cases to the rate of suspected cases in 1997 for that particular setting, age, and sex category.

Total number of cases and suspected cases during 1997–2004 were 28,470 and 138,130 in urban districts, 20,328 and 157,296 in rural districts, and 6,879 and 62,227 in remote districts, respectively. The overall reporting rate per 100,000 persons in 2000 was 78 in urban districts, 64 in rural districts, and 42 in remote districts. The annual change in overall reporting rates was 0.0% in urban districts, 0.4% higher in rural districts, and 0.2% lower in remote districts (Figure). Reporting rates decreased annually in elderly persons

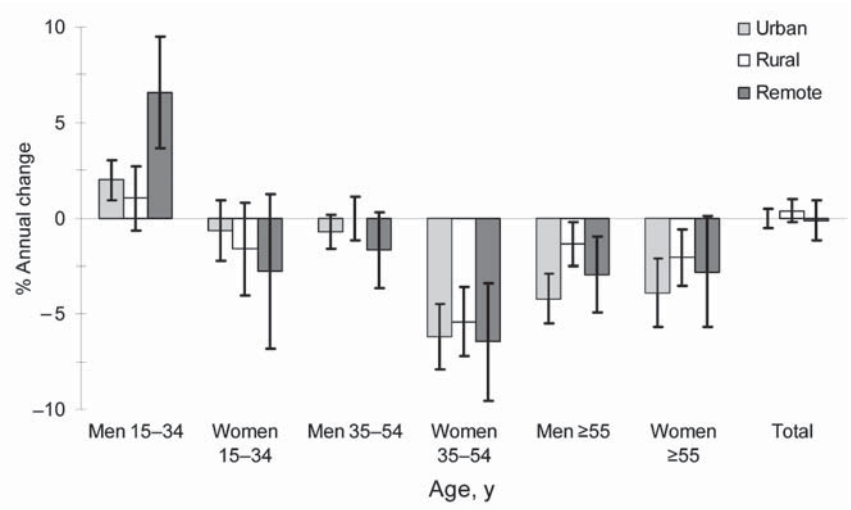

Figure. Sex- and age-specific trends in tuberculosis case reporting rates in urban, rural, and remote (mountainous) districts, Vietnam, 1997–2004. Error bars show 95% confidence intervals.

(≥55 years of age), and most notably in middle-age women (35–54 years of age, range 5.4%–6.5% in different settings). This was offset by an annual increase among young men (15–34 years of age), notably in urban (2.0%) and mountainous (6.6%) districts. The annual change in overall rates of suspected cases was a 6.2% decrease in urban districts, a 0.5% increase in rural districts, and a 0.1% increase in remote districts. Adjustment for rates of suspected cases did not result in a significant decrease in overall reporting rates and did not fundamentally change our conclusions about the age–sex pattern of the trends. Therefore, increased diagnostic effort did not explain the lack of decreasing reporting rates. The observed trends in reporting rates probably reflect underlying trends in incidence.

If aims for case detection and cure were met, TB incidence for 1997 through 2004 was predicted to decrease by 44% (*2*). However, no decrease was observed in overall reporting rates in urban, rural, and remote districts in this period in Vietnam. This is explained by an emerging TB epidemic among young adults, particularly in men in urban and remote districts, which causes concern because TB at younger ages tends to reflect recent transmission (*9*).

There are 3 possible explanations for the lack of effect of the DOTS strategy. First, the true case detection rate is lower than estimated. Second, the true cure rate is lower than reported. Third, a mathematical model insufficiently captures the dynamics of TB epidemiology in Vietnam (e.g., because of HIV infection, emergence of the more virulent Beijing TB genotype or risk factors associated with internal migration are underestimated).

Investigation of factors hampering TB control in Vietnam is urgently needed. Efforts are being undertaken to evaluate the effect of HIV on TB trends and to assess case detection in a nationwide TB prevalence survey. The limited effect of the DOTS strategy in Vietnam may be relevant for predicting the effect of this strategy in other countries.

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Vancomycin-resistant Enterococci, Mexico City

To the Editor: Vancomycin-resistant *Enterococcus* (VRE) has become an important nosocomial pathogen because of its rapid spread, limited therapy options, mortality, and the possibility of transfer of vancomycin resistance to other pathogens such as *Staphylococcus aureus*. Vancomycin-resistant *E. faecium* (VREF) and *E. faecalis* were first described in 1988 (*1*,*2*).They have become major nosocomial pathogens, but their prevalence in Latin America has remained <2% (*3*). In Mexico, VRE has rarely been reported (*4*,*5*). In a recent study in Mexico City, 100% (n = 60) of the isolates of *E. faecium* and *E. faecalis* were susceptible to vancomycin (*6*).

From May 2004 to April 2005, the rate of vancomycin resistance among all *Enterococcus* isolates was 0.27%. However, in May 2005 the first fully VREF was isolated at our hospital, and the rate of vancomycin resistance was 6.23% (a 23-fold increase) during the following 12-month period.

We performed a retrospective study to describe the isolates and the characteristics of patients with VREF. All VREF isolates from May 2005 through April 2006 were included. We collected demographic and clinical data. For the final identification of the isolates, the VITEK system (bioMérieux, Lyon, France) with VITEK GPI cards (bioMérieux, Inc., Durham NC, USA) were used. Antimicrobial drug susceptibility was tested by using the VITEK GPS-111 card and confirmed by MIC determination that used broth microdilution. Resistance to vancomycin and teicoplanin was confirmed by E-test (AB Biodisk, Solna, Sweden). An isolate was considered vancomycin resistant when the MIC was ≥32 μg/mL and was considered to have high-level resistance when the MIC was ≥256 μg/mL. A PCR for detection of the *vanA* or *vanB* genotype was used (*7*). Isolates were characterized by pulsed-field gel electrophoresis (PFGE) (*8*,*9*); a dendrogram was constructed with the GelCompare II 4.0 software (Applied Maths, Kortrijk, Belgium), and the similarity was compared with the Dice coefficient.

In the study period, VREF was isolated from 27 patients. The median age was 40 years (range 22–84 years). VREF was isolated from the abdomen in 14 patients (51.9%); 11 isolates were from an abscess, 2 from infected surgical sites, and 1 from ascites. An additional 8 isolates were from the urinary tract (29.6%), 2 from the bloodstream (7.4%), 2 from soft-tissue (7.4%), and 1 (3.7%) from bone. Residence in the general medical wards during the isolation of VREF was most common, 17 (63%) cases, followed by 6 (22.2%) in the intensive care unit. The remaining 4 (14.8%) were distributed in other areas. Median time of hospitalization before the isolation was 21 days (range 1–84 days). Twenty-five patients (92.6%) had a central line, 12 (44.4%) had mechanical ventilation, and 20 (74.1%) previous surgery. Of the last group, 17 (85%) of 20 had abdominal surgery. Twenty-four patients (88.8%) received an antimicrobial drug before the isolation of VREF: third- or fourth-generation cephalosporins (89%), metronidazole (70.4%), aminoglycosides (70.4%), vancomycin (66.7%), carbapenems (66.7%), amoxicillin or ampicillin (48.1%), antifungal agents (48.1%); and <20% received quinolones, trimethoprim-sulfamethoxazole, colistin, macrolides, and antimycobacterial or antiviral agents. The median time of antimicrobial drug use was 11 days (range 1–84 days). During hospitalization, 7 patients died (crude death rate, 25.9%), 5 of them from sepsis with at least another microorganism isolated; the remaining 2 died of gastrointestinal hemorrhage.

All isolates of *E. faecium* had a vancomycin MIC ≥256 μg/mL and a vanA phenotype (teicoplanin resistance); 26 (96.3%) had *vanA* genotype. Only 1 isolate of *E. faecium* was classified as non–*vanA,* non *vanB,* even though it demonstrated high-level resistance to vancomycin and teicoplanin. Resistance to other antimicrobial agents was as follows: ampicillin and ciprofloxacin, 100%; high-level gentamicin, 48.2%; quinupristin/dalfopristin, 7.4%; and linezolid, 0%.

PFGE analysis showed several genotypes of *E. faecium*; however, 18 of 26 of the isolates had ≤3 band differences from the predominant strain classified as type A. One isolate of *E. faecium* could not be typed (Figure).

As in most tertiary-care centers, our PFGE data suggest that a heterogenous population of VREF exists, but a particular clone established itself as the dominant strain. Although infection control measures are well established in our hospital, in disseminated outbreaks caused by several different clones, infection control measures and control of vancomycin use have shown only limited efficacy. This suggests selection pressure by antimicrobial drugs other than vancomycin (*10*). Early detection of VREF is of extreme importance because of the possibility that the *vanA* gene may be transferred to a variety of gram-positive microorganisms, including *S. aureus.*

The rate of isolation of VREF at our hospital increased considerably during the last year. Even though the number of patients is small, we consider this finding to be of utmost importance, since VREF seems to be emerging in Mexico. To our knowledge, this is the first well-documented outbreak of high-level resistance to vancomycin in enterococci in Mexico. Further research is needed to determine if the problem is limited to our hospital or if it is a nationwide trend.

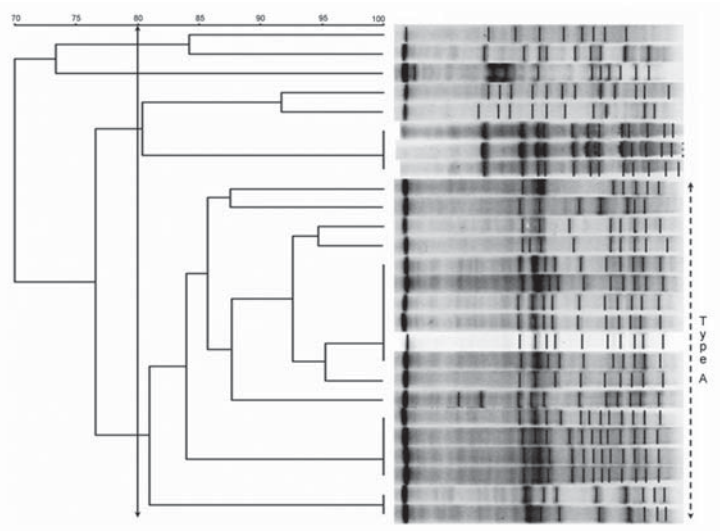

Figure. Pulsed-field gel electrophoresis (PFGE) banding patterns of chromosomal DNA of 26 isolates of vancomycin-resistant enterococci. There is a clear predominant type, classified as type A (≥80% similarity), composed of 18 isolates of *Enterococcus faecium.* There are at least 3 subtypes that display a 100% similarity.

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Disseminated Bacillus Calmette-Guérin Infection and Immunodeficiency

To the Editor: Disseminated bacillus Calmette-Guérin (BCG) infection has been noted in patients with primary immunodeficiency. Incidence rates have ranged from 0.06 to 1.56 cases per million vaccinated, and mortality rates have remained at ≈60% (*1–7*). Of 946 patients with primary immunodeficiency, including 29 with severe combined immunodeficiencies, diagnosed from 1980 through 2006 at the Children's Memorial Health Institute in Warsaw, adverse events after BCG vaccination were observed in 16 (*8*,*9*). All 16 were children who had been vaccinated at birth with BCG, Brazilian strain (Biomed, Lublin, Poland).

Four patients with severe combined immunodeficiency showed adverse reactions to BCG. Patient M.K. had mild inflammation at the site of the BCG injection and was successfully treated with rifampin. The patient subsequently received a bone marrow transplant, and 2 months later poor appetite, failure to thrive, and subfebrile condition were noted. Disseminated skin changes (with pus formation in the subcutaneous layer), osteomyelitis, and multiple lesions in the liver were found. A skin biopsy showed tuberculoma formations, which were PCR-positive for *Mycobacterium tuberculosis* complex (Amplified Myco-

bacterium Tuberculosis Direct [MTD] Test, Gen-Probe, Inc., San Diego, CA, USA) but had negative culture results. Complete recovery, including full immunologic reconstitution, was reached after 12 months of treatment with triple antituberculosis (TB) therapy (rifampin, isoniazid, and ciprofloxacin). Patient M.C., a 6-month-old boy, was admitted to an intensive care unit because of respiratory insufficiency. An unhealed BCG vaccination site was noted. Bronchopulmonary lavage samples were tested for *M. bovis;* positive PCR and culture results led to the diagnosis of disseminated BCG infection. Despite intensive anti-TB therapy, the child died of multiple organ failure. Autopsy showed typical granuloma formations and a hypoplastic thymus, typical for severe combined immunodeficiency. Male patients S.D. and C.G. were admitted to intensive care units at 6 and 8 months of age, respectively, with lymphadenopathy and multiple organ insufficiency. Each boy died of multiple organ failure; postmortem examination found granuloma formation and a hypoplastic thymus in each (*8*).

Eight patients with severe combined immunodeficiency had local adverse events after vaccination with BCG. Inflammation at the vaccination site was observed for all 8. For all except 1, dual anti-TB therapy (rifampin, isoniazid) or monotherapy was successful. For 1 of these patients, anti-TB treatment was stopped 3 months after bone marrow transplant, but increasing inflammation and lymphadenitis appeared 1 month later, with positive PCR and negative culture results for *Mycobacterium* spp. After 12 months of triple anti-TB therapy, this patient fully recovered.

In 2-month-old female patient, W.M., who had interferon-γ–receptor deficiency, axillary lymphadenopathy with normal healing of the vaccination site was noted 7 weeks after BCG vaccination**.** Tuberculous lymphadenitis was diagnosed by histopathologic methods. Despite dual anti-TB therapy and streptomycin administration, the girl died. At autopsy, multiple tuberculous granulomas were found (*5*).

In 4-month-old female patient M.K., who had interleukin-12–receptor deficiency, axillary lymphadenopathy with positive results from *Mycobacterium* typing was noted. Dual anti-TB therapy for 12 months produced good results.

In 7-month-old female patient B.B., who also had interleukin-12–receptor deficiency, axillary lymphadenopathy was noted. Mycobacteria PCR-positive for the *M. tuberculosis* complex were found in the purulent secretion. Despite dual anti-TB therapy, the patient experienced 2 episodes of relapse. After another 2 years of anti-TB therapy, disseminated BCG infection, with pulmonary consequences, developed.

In patient R.C., a 6-month-old boy, osteomyelitis was diagnosed, and delayed healing of the BCG vaccination scar was noted. Investigation of his immunologic status showed no abnormalities. However, because granulomatous inflammation was present in a bone biopsy sample and staining for BCG produced a positive result, triple anti-TB therapy was provided for 12 months, with good results.

The literature describes >200 cases of disseminated BCG infection in patients with primary immunodeficiency (*1–7*). The diagnostic difficulties described for 8 of our patients with primary immunodeficiency have been noted by others (*1–6,8–10*). In only 2 cases was the *Mycobacterium* species successfully isolated and identified as the *M. bovis* BCG strain. We propose novel criteria for the diagnosis of disseminated BCG infection in persons with primary immunodeficiency (Table). These criteria have recently been submitted to the European Society for Immunodeficiencies.

Table. Suggested diagnostic criteria for disseminated bacillus Calmette-Guérin (BCG) infection in persons with primary immunodeficiency*

Diagnosis	Clinical	Laboratory
Definitive	Systemic symptoms such as fever or subfebrile status, weight loss, or stunted growth, and ≥2 areas of involvement beyond the site of BCG vaccination†	Identification of *Mycobacterium bovis* BCG substrain from the patient's organs by culture and/or standard PCR, as well as typical histopathologic changes with granulomatous inflammation
Probable	Systemic symptoms such as fever or subfebrile status, weight loss or stunted growth, and ≥2 areas of involvement beyond the site of BCG vaccination†	Identification of *M. tuberculosis* complex from the organs by PCR, without differentiation of *M. bovis* BCG substrain or other members of the *M. tuberculosis* complex and negative mycobacterial cultures, with the presence of typical histopathologic changes with granulomatous inflammation
Possible	Systemic symptoms such as fever or subfebrile condition, weight loss or stunted growth, and ≥2 areas of involvement beyond the site of BCG vaccination†	No identification of mycobacteria by PCR and culture, with presence of typical histopathologic changes with granulomatous inflammation
Exclusion criteria	Any inflammation without typical histopathologic changes, with no isolation of *M. tuberculosis* complex by PCR analysis in patient with primary immunodeficiency	
Differential diagnosis	Severe, long-term inflammation with granuloma formation in patient with primary immunodeficiency	

*Male or female patient with or without genetic confirmation of severe combined immunodeficiency, interferon-γ–receptor deficiency, interleukin-12–receptor deficiency, or other primary immunodeficiency.
†Areas of involvement may include lymph nodes, skin, soft tissues, lungs, spleen, liver, bones.

We believe that patients with severe combined immunodeficiency and any form of mild local changes at the BCG injection site should be given single or double anti-TB therapy, which should be continued until complete immunologic reconstitution occurs after bone marrow transplant. Severe local BCG infection with regional lymph node involvement needs at least triple anti-TB therapy followed by long-term prophylaxis. Disseminated BCG infection needs anti-TB therapy, including ≥4 anti-TB drugs, until the patient fully recovers.

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Clindamycin-resistant *Streptococcus pneumoniae*

To the Editor: Antimicrobial medications classified as macrolides (e.g., erythromycin) and lincosamides (e.g., clindamycin) show strong activity against streptococci and are commonly used to treat community-acquired infections caused by *Streptococcus pneumoniae*. Moreover, these drugs are the recommended alternatives for patients who cannot tolerate β-lactams.

Two main macrolide-resistant *S. pneumoniae* phenotypes have been reported (*1*). The first has a high level of resistance to all macrolides, lincosamides, ketolides, and streptogramins B due to ribosomal dimethylation, 23S rRNA mutations, or ribosomal protein mutations (MLS_B, MS_B, ML, MKS_B, and K phenotypes). The second is characterized by a low-level resistance (e.g., MIC 2–4 mg/L) to only 14- and 15-member ring macrolides (M phenotype) because of *mef* gene–mediated active drug efflux mechanism.

In January 2005, an erythromycin-susceptible but clindamycin-resistant pneumococcal strain was obtained from a conjunctival swab of a 10-month-old female outpatient attending the daycare center of the Clinic and Laboratory of Infectious Diseases, Siena University, Siena, Italy. To our knowledge, such a phenotype has not been reported in the international literature for *S. pneumoniae*, although a similar phenotype of *S. agalactiae* was described by Malbruny et al. (*2*).

The *S. pneumoniae* isolate was identified by standard procedures (*3*) and confirmed by PCR for the common capsule gene *cpsA* (*4*). Serotyping, performed by Quellung reaction, showed a 35F serotype. Susceptibility testing was carried out by disk diffusion and confirmed with E-test according to Clinical and Laboratory

Standards Institute standards (*5*,*6*) for penicillin, ceftriaxone, ciprofloxacin, erythromycin, clindamycin, linezolid, and quinupristin-dalfopristin. For telithromycin, because an E-test strip was unavailable, a microbroth dilution method was used.

The strain was susceptible to ceftriaxone (MIC 0.125 mg/L), ciprofloxacin (MIC 0.125 mg/L), erythromycin (MIC 0.125 mg/L), linezolid (MIC 1.5 mg/L), quinupristin/dalfopristin (MIC 0.5 mg/L), and telithromycin (MIC <0.0035 mg/L); it was not susceptible to penicillin (MIC 0.125 mg/L) and was resistant to clindamycin (MIC 1 mg/L). A triple disk–diffusion test with erythromycin, clindamycin, and josamycin was performed to test resistance inducibility. No inducible pattern was shown.

To understand the possible resistance mechanism, MICs for 2 lincosamides (clindamycin and lincomycin) were determined by using a microbroth dilution method in the presence and absence of 10 mg/L of the efflux pump inhibitor reserpine (Sigma Chemicals, St Louis, MO, USA), as described (*7*); *S. pneumoniae* ATCC 49619 and *S. mitis* 21A29 (*mefE*$^{+}$) were used as controls (*8*). The MICs remained unchanged in the presence of reserpine: 1 mg/L for clindamycin and 4 mg/L for lincomycin.

The strain was screened for *erm*TR, *erm*B or *mef*A, and *mef*E determinants as described (*8*,*9*). All PCR controls gave the expected results. No PCR product was obtained for the studied isolate.

Preliminary data did not show classic macrolide resistance determinants for *S. pneumoniae*. Low-level lincosamide resistance suggests the presence of some efflux mechanism, even if no inhibition by reserpine was observed. Moreover, no mutations of ribosomal proteins and of known binding sites for lincosamides in rRNA (*1*) were shown by sequencing of L22, L4, and 23S rRNA domain II and V genes with primers described by Canu et al. (*10*). Although these findings are preliminary and the molecular basis for resistance is the subject of ongoing investigation, the identification of this *S. pneumoniae* phenotype may affect clinical management of pneumococcal infections, especially in the treatment of patients intolerant of β-lactams.

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Strain serotyping was performed at Streptococcal Reference Unit of Respiratory and Systemic Infection Laboratory, Centre for Infections, Health Protection Agency, London, UK.

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Expanded-spectrum β-Lactamase and Plasmid-mediated Quinolone Resistance

To the Editor: The emergence of plasmid-mediated, and thus transferable, quinolone resistance determinants has been recently discovered (*1*) and shown to involve the pentapeptide repeat protein Qnr, which interacts with DNA gyrase and topoisomerase IV to prevent quinolone inhibition (*2*,*3*). Qnr determinants confer resistance to nalidixic acid and reduced susceptibility to fluoroquinolones (*3*). They have been identified worldwide in a variety of enterobacterial species and were often associated to expanded-spectrum β-lactamases (ESBLs) (*2*). The association between the ESBL VEB-1 and the QnrA1 determinants was reported (*4*). Because plasmid co-localization of QnrA and VEB-1 encoding genes has been reported repeatedly from scattered clonally-unrelated enterobacterial isolates, our objective was to use replicon typing to trace a possible dissemination of a common plasmid worldwide.

The bla_{VEB-1}- and/or *qnrA*-positive plasmids that have been included in the study were from 17 isolates previously described in detail (*3*–*8*) (Table). *Escherichia coli* transconjugants (Tc) were obtained for 14 of 17 clinical isolates, allowing an accurate replicon typing since original clinical isolates might harbor several plasmids. They were collected from 1999 to 2005, from patients hospitalized in different parts of the world (Table). The 13 bla_{VEB-1}-positive isolates were from 5 countries (France, Turkey, Algeria, Thailand, and Canada), scattered on 4 continents. Among them, the *Providencia stuartii* and *Proteus mirabilis* isolates from Algeria were negative for *qnrA1*. In addition, 4 bla_{VEB-1}-negative but *qnrA1*-positive isolates recovered from France and Australia were also included in the study.

PCR-based replicon typing (PBRT), which recognizes FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, and FII replicons (*9*), was applied to type the resistance plasmids from all the strains.

Table. Features of the VEB-1– or QnrA-positive isolates used in this study*

Strain† (Ref.)	Sp. of origin	Country	Year of isolation	Plasmid size (kb)	ESBL	QnrA1	Replicon	Resistance markers‡
Escherichia coli TcE1 (*5*)	*E. coli*	Thailand	1999	160	VEB-1	+	A/C_2	NAL, K, SSS, C, RA
E. coli TcE4 (*5*)	*E. coli*	Thailand	1999	150	VEB-1	+	A/C_2	NAL, K, TM, SSS, C, RA
E. coli TcE5 (*5*)	*E. coli*	Thailand	1999	150	VEB-1	+	A/C_2	NAL, K, TM, SSS, SXT
E. coli TcE7 (*5*)	*E. coli*	Thailand	1999	150	VEB-1	+	A/C_2	NAL, K, TM, SSS, C, RA
E. coli TcE8 (*5*)	*E. coli*	Thailand	1999	150	VEB-1	+	A/C_2	NAL, K, TM, SSS, TE
E. coli TcE16 (*5*)	*E. coli*	Thailand	1999	140	VEB-1	+	A/C_2	NAL, K, TM, SSS, RA
E. coli TcE18 (*5*)	*E. coli*	Thailand	1999	180	VEB-1	+	A/C_2	NAL, K, SSS, C, RA
E. coli Tc(p1) (*5*)	*E. coli*	Canada	2000	180	VEB-1	+	A/C_2	NAL, K, SSS, C, RA
E. coli Tc(pQR1) (*4*)	*E. coli*	France	2003	180	VEB-1	+	A/C_2	NAL, K, SSS, C, RA, SXT
E. coli Tc(GOC) (*4*)	*Enterobacter cloacae*	France	2003	190	VEB-1	+	A/C_2, FIB	NAL, K, TM, SSS, C
Citrobacter freundii LUT (*3*)	*C. freundii*	Turkey	2004	ND	VEB-1	+	A/C_2, FIB, K	NA
Providencia stuartii 15 (this study)	*P. stuartii*	Algeria	2004	ND	VEB-1	–	A/C_2	NA
E. coli TcMAA (this study)	*Proteus mirabilis*	Algeria	2004	190	VEB-1	–	A/C_2	K, TM, SSS, C, SXT
E. coli TcK147 (*7*)	*Klebsiella pneumoniae*	Australia	2002	160	SHV-12	+	HI2, A/C_1, P	NAL, K, TM, C, TE, SXT
E. cloacae A1 (*8*)	*E. cloacae*	France	2004	75	SHV-12	+	HI2	NA
E. coli TcA2 (*8*)	*Enterobacter aerogenes*	France	2005	150	SHV-12	+	FII	NAL, K, TM, TE
E. coli TcA3 (*8*)	*K. pneumoniae*	France	2005	40	–	+	I1, K	NAL, K, TM, C, TE

*Ref., reference; ESBL, expanded-spectrum β-lactamase; NAL, nalidixic acid; K, kanamycin; SSS, sulfonamides; C, chloramphenicol; RA, rifampin; TM, tobramycin; SXT, trimethoprim-sulfamethoxazole; TE, tetracycline; ND, not determinable; NA, not applicable.
†Tc indicates that this is a transconjugant or a transformant.
‡Non–β-lactam–associated markers.

Amplicons were confirmed by DNA sequencing and used as probes in hybridization experiments on purified plasmids (data not shown).

PBRT results showed that the 13 bla_{VEB-1}-positive plasmids (including 11 *qnrA1*-positive) belonged to the IncA/C incompatibility group. DNA sequencing identified the A/C_2 replicon variant (European Molecular Biology Laboratory no. AM087198) in all these plasmids (Table). Plasmids of this type were recently identified in the United States and in Italy carrying the AmpC-type cephalosporinase CMY-2–encoding gene (*10*). In 2 strains (*E. coli* TcGOC and *Citrobacter freundii* LUT), the IncA/C_2 plasmids were associated with additional replicons, which suggests the presence of multiple plasmids or fusions between plasmids of different backbones. By contrast, all the 4 bla_{VEB-1}-negative isolates but *qnrA1*-positive were negative for the A/C replicon, except transconjugant TcK147; however, sequencing identified an A/C_1-type replicon in that strain. These results indicated that the genes encoding QnrA1 and VEB-1, when identified concomitantly in a given isolate, were always located on plasmids belonging to the same IncA/C_2-incompatibility group that may vary in size and digestion pattern (Table; unpub. data). In addition, we showed that plasmids carrying the bla_{VEB-1} gene but lacking *qnrA1* were also of the IncA/C_2 type (Table). Plasmids that were bla_{VEB-1}-negative but *qnrA1*-positive were of distinct replicon types, thus suggesting independent acquisition of the *qnrA1* gene on different plasmids. It is remarkable that since VEB-1 is apparently always encoded by IncA/C_2 plasmids, when genes for QnrA1 and VEB-1 are found together, they also occur on IncA/C_2 plasmids.

Thus, evidence here shows that the IncA/C_2 plasmid is the main vehicle of the bla_{VEB-1} gene worldwide, on which the *qnrA1* gene may be added. The possibility that both bla_{VEB-1} and *qnrA1* genes may be identified on a single genetic structure in several isolates has been recently shown with their identification within the same *sul1*-type integron (*6*).

Because results of these experiments provided a good marker for tracing bla_{VEB-1}-positive plasmids, and taking in account the property of A/C-type plasmids to have a broad range of hosts (note: this has not been demonstrated for the specific A/C_2 subgroup), we tried to amplify the A/C_2 replicon in a collection of 15 bla_{VEB-1}-positive and clonally unrelated *Pseudomonas aeruginosa* isolates from France, Thailand, India, and Kuwait. The bla_{VEB-1} gene was supposed to be chromosome-encoded in those isolates. PCR failed to give any positive results, confirming the absence of an IncA/C-type plasmid and also ruling out the hypothesis of IncA/C_2-type plasmid co-integration at the origin of bla_{VEB-1} acquisition in *P. aeruginosa*.

The spread of plasmids carrying a large array of resistance genes among *Enterobacteriaceae* is of concern since this provides a convenient genetic mechanism for a given strain to become panresistant to antimicrobial drugs. In particular, the recent identification of the Qnr determinants has shown that plasmids may provide resistance (or at least reduced susceptibility) to quinolones and fluoroquinolones, whereas they are already known to carry resistance to β-lactams, aminoglycosides, chloramphenicol, tetracycline, rifampin, sulfonamides, and disinfectants. pQR1 (*4*) or p1 (*6*) are examples of well-characterized plasmids that mediate multidrug resistance by carrying bla_{VEB-1} and *qnrA1*, together with aminoglycoside resistance genes *aadB*, *aacA1*, and *aadA1*, chloramphenicol resistance gene *cmlA*, rifampin resistance gene *arr2*, disinfectant resistance gene *qacI*, and sulfonamides resistance gene *sul1*.

Our study showed that the IncA/C_2-type plasmids may be the source of such worldwide dissemination. It means that 1 plasmid scaffold has brought the same (or at least very similar) multidrug resistance to multiple enterobacterial species in different continents.

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Viral Load and Crimean-Congo Hemorrhagic Fever

To the Editor: Crimean-Congo hemorrhagic fever (CCHF) is a severe viral disease transmitted to humans by tick bite or contact with blood, excreta, or tissues of infected patients or livestock. The disease is endemic in many African, Asian, and European countries. Sporadic cases or outbreaks have been observed in the Balkan Peninsula (*1*–*5*). Prompt diagnosis of the disease is essential for preventing human-to-human transmission. Reverse transcription–PCR (RT-PCR) is the detection method of choice in first days of illness and in severe cases with no antibody production. In recent years, real-time RT-PCR approaches have been described for detection and quantification of CCHF virus (*6*–*8*). However, no information is available on viral RNA concentration in patients. We describe a real-time RT-PCR for detection and quantification of CCHF virus, present the results of its use with clinical samples, and report the relationship between viral load and severity and outcome of CCHF.

We tested 29 serum samples from Albanian patients with suspected CCHF or their contacts who were living in a CCHF-endemic area of Albania. Serum samples were collected during 2003–2006 and categorized into 3 groups. Group A contained samples from 11 patients with CCHF confirmed by a conventional RT-nested PCR (*9*). Group B contained samples from 5 patients who had negative RT-nested PCR results and positive serologic results. Group C contained samples from 15 persons who were from the same region as the CCHF patients but who did not have any clinical symptoms of CCHF and had negative PCR or serologic results.

One set of primers and 1 probe were designed to amplify an 84-bp genome region of the S RNA segment of CCHF virus on the basis of European sequences (Balkan and Russian strains available in GenBank): primers CCEuS 5′-TGACAGCATTTCTTTAACAGACATCA-3′ and CCEuAs 5′-AAACACGGCAGCCTTAAGCA-3′, and probe 5′-TCGCCAGGGACTTTATATTCTGCAAGG-3′. A 25-μL reaction was conducted in a LightCycler (Roche, Indianapolis, IN, USA) with 10 mmol/L of each deoxynucleotide triphosphate, 600 nmol/L of each primer, 200 nmol/L of probe, and 3 μL of RNA. Cycling conditions were 50°C for 30 min and 95°C for 15 min, followed by 45 cycles at 95°C for 15 s and 58°C for 30 s. A quantification curve was constructed with 10-fold serial dilutions of in vitro–transcribed CCHF virus RNA. Positive results were obtained up to a dilution of 10^{-12}, which corresponds to ≈45 virus genome equivalents (geqs) per reaction.

Twelve samples had positive results: all 11 samples in group A and 1 in group B (Table). Results for the remaining samples in groups B and C were negative. Levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-10, and a 60-kDa soluble receptor of TNF were previously measured in most of the samples in this study (*10*), and their values are shown in the Table.

Viral loads ranged from 14×10^6 to 28.99×10^6 geqs/reaction. The highest level was observed in the patient who died (23/03). High loads were observed in all primary case-patients (23/03, 82/03, 178/04, 252/06) except for patient 154/04, from whom a sample was obtained 18 days after onset of disease. All primary case-patients had severe disease with high fever and clinically apparent hemorrhage. All secondary case-patients, except patient 34/03, were contacts of the patient who died (24b/03, husband; 25b/03, brother-in-law; 50/03 and 52/03, cousins; 56/03, sister-in-law; 40/03, son of sister) and had symptoms of disease ≈1 week after the death of patient 23/03.

Viral load of secondary case-patients was <250 geqs/reaction, which was much lower than that of primary case-patients. This finding suggests that the disease is more severe in primary case-patients and becomes a milder form in secondary case-patients. Samples of secondary case-patients 24b/03 and 25b/03 were obtained on day 9 of illness, and patient 24b/03 had a 4× higher viral load than patient 25b/03. A possible explanation might be that because patient 24b/03 had closer contact with the person who died, he received a higher dose of virus, which might affect severity of the disease. Other secondary case-patients had milder symptoms with no clinically apparent hemorrhage and were not hospitalized. All hospitalized patients had leukopenia, except for the patient whose sample was taken 18 days after the onset of disease. No correlation was observed between viral load and cytokine levels or platelet counts, which suggests that other factors are involved in pathogenicity and immune response.

Table. Epidemiologic, molecular, and clinical data for 12 Albanian patients with suspected Crimean-Congo hemorrhagic fever, 2003–2006*

Patient	Day of illness	In hospital	Outcome	Real-time RT-PCR, geqs/reaction	IFA	TNF-α, pg/mL	sTNF-R, ng/mL	IL-6, pg/mL	IL-10, pg/mL	Leukocytes, × 10^9/L	Platelets, × 10^9/L
Group A primary											
23/03	6	Yes	D	28,990,000	+	68.5	14.0	109.7	388.3	1,700	36,200
82/03	5	Yes	R	450	+	N	N	17.0	23.9	2,300	96,830
154/04	18	Yes	R	33	+	ND	ND	ND	ND	15,000	71,400
178/04	4	Yes	R	7,271,000	+		ND	ND	ND	4,100	62,550
252/06	2	Yes	R	4049	–	ND	ND	ND	ND	3,700	117,900
Group A secondary											
24b/03	9	Yes	R	166	+	1,444.8	N	114.2	N	4,800	63,800
25b/03	9	Yes	R	40	+	N	N	10.3	N	3,800	63,000
50/03	3	No	R	240	–	N	N	N	43.4	ND	ND
52/03	3	No	R	18	–	N	N	N	9.9	ND	ND
56/03	5	No	R	62	+	N	N	26.1	N	ND	ND
34/03	5	Yes	R	46	–	N	8.9	N	N	8,000	102,000
Group B secondary											
40/03	7	No	R	14	+	N	N	N	23.1	ND	ND

*RT-PCR, reverse transcription–PCR; geqs, genome equivalents; IFA, immunofluorescent assay; TNF-α, tumor necrosis factor-α; sTNF-R, soluble TNF-α receptor; IL-6, interleukin-6; D, died; +, positive; R, recovered; N, normal value; ND, not done; –, negative.

The real-time RT-PCR was rapid and more sensitive than the RT-nested PCR because 1 additional positive sample was detected. Samples with positive results from the first round of the conventional RT-nested PCR (23/03, 178/04, 252/06) had the highest viral loads when tested by real-time RT-PCR.

In conclusion, a 1-step real-time RT-PCR for detection and quantification of CCHF virus was developed, used with clinical samples, and provided informative data on the severity, course, and outcome of CCHF. Further studies, preferably in serial samples of patients, should provide insights into the pathology of CCHF and the effectiveness of antiviral drugs.

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Inactivated Whole Virus Influenza A (H5N1) Vaccine[1]

To the Editor: Avian influenza viruses of the H5N1 subtype represent a potential source of the next pandemic (*1*,*2*). Our goal was to determine the safety and immunogenicity of a newly developed vaccine in humans.

The vaccine was produced by the same method as the interpandemic influenza vaccine "FluvalAB" used in Hungary for the past 11 years (*3*,*4*). The method has been validated by meeting the requirements of the European Agency for the Evaluation of Medicinal Products (EMEA) related to interpandemic influenza vaccines each year since 1995, and by having been administered in humans in a total of >15 million cases (*5*).

The virus strain (NIBRG-14), a reverse genetics–derived 2:6 reassortant between A/VietNam/1194/2004 (H5N1) and PR8, was obtained from the National Institute for Biologic Standards and Control, London. It is one of the reference viruses indicated as suitable for use in a mock-up vaccine by the Committee for Medicinal Products for Human Use (*6*).

Hens' egg–grown, formaldehyde-inactivated, whole virus vaccine, developed and produced by the Omninvest Ltd. (Budapest, Hungary), was used. The vaccine contained 6 μg hemagglutinin per dose (as determined by single radial immundiffusion test) in 0.5-mL ampules. Purity was assessed by endotoxin content (determined by chromogenic endotoxin assay, using a modified limulus amoebocyte lysate and a synthetic color-producing substrate), which was considered acceptable in concentrations <0.1 IU/mL. The amount of ovalbumin was determined by ELISA, which was considered satisfactory in concentrations <10 ng/mL. Aluminum phosphate was used as adjuvant, in the amount of 0.31 mg Al per ampule; 0.1 mg/mL merthiolate was added as preservative.

A total of 146 healthy volunteers >18 years of age (mean ± SD 42.07 ± 12.62 years). were enrolled in the study. Sixty-five male and 81 female volunteers participated. The sample size was chosen to exceed the requirement of 50 patients per group set by the European guidelines for yearly influenza vaccine trials (*5*). The sponsor was the National Public Health and Medical Officer Service, Budapest, Hungary.

The injection administered 0.5 mL of vaccine intramuscularly. The injection was not repeated. Serum antibody titers were measured by hemagglutination inhibition (HI) by using chicken erythrocytes, following standard procedures (*7*). Because the protective titer for influenza virus A (H5N1) infections is unknown, immunogenicity was assessed according to the European Medicines Agency criteria related to interpandemic influenza vaccines (Table) (*5*).

None of the study participants displayed measurable levels of HI antibodies before vaccination. According to EMEA requirements, both male and female groups met 2 independent criteria for immunogenicity 21 and 90 days after vaccination (Table).

In 15.7% of the participants, adverse reactions in the form of local pain at the injection site occurred within the first 48 hours; these reactions disappeared within 1 day. No other local reactions, such as injection site induration, erythema, swelling, warmth, or ecchymosis, were noted. No systemic reaction (fever, malaise, headache, shivering) was detected. No serious adverse events were observed. These results are in line with the 11-year experience using the interpandemic vaccine produced by Omninvest Ltd.

[1]The study design has been presented as an oral presentation at the World Health Organization Meeting on Evaluation of Pandemic Influenza Vaccines in Clinical Trials, May 4–5, 2006, Geneva, Switzerland.

Table. Immunogenicity findings of whole-virus influenza vaccine trial, Hungary*†

	CHMP requirement	Total study population	Male	Female
Day 21				
GMT	NA	27.9	31.0	25.6
Post- to prevaccination GMT ratio (increase)	>2.5	5.6‡	6.2‡	5.1‡
% of participants seropositive (titer >1:40)	>70	63.7‡	70.8‡	58.0
% of participants with seroconversion (4-fold titer increase or titer >1:40)	>40	63.7*	70.8‡	58‡
Day 90				
GMT	NA	29.4	31.9	27.4
Post- to prevaccination GMT ratio (increase)	>2.5	5.9‡	6.4‡	5.5‡
% of participants seropositive (titer >1:40)	>70	67.3	73.9‡	61.8
% of participants with seroconversion (4-fold titer increase or titer >1:40)	>40	67.3‡	73.9‡	61.8‡

*CHMP, Committee for Medicinal Products for Human Use, European Medicines Agency; GMT, geometric mean titer; NA, not applicable.
†Hemagglutination-inhibition (HI) titers below the limit of detection were given an arbitrary value of 1:5. GMTs of antibody and their confidence intervals were computed by transforming the results to a logarithmic scale, assuming asymptotic normality conditions were satisfied on the scale and converting back to the original scale. HI endpoints were the GMT at each timepoint and the variables required for interpandemic influenza vaccines: postvaccination seropositivity rate (% of participants with titers ≥40), the post- to prevaccination GMT ratio, and the proportion of persons seroconverting or displaying a 4-fold titer increase postvaccination.
‡Met CHMP standards.

by the same method, where a similar safety profile has been seen after >15 million vaccinations in humans.

This is the first study that reports that an inactivated whole virus vaccine with an aluminum phosphate adjuvant system against influenza A (H5N1) was safe and immunogenic in humans after only 1 injection. This study reports the lowest effective dose used to cause immune response. Other trials used much higher maximum doses and required 2 injections 21 or 28 days apart (*8–10*). Using the lowest possible amount of the antigen and fewer injections is essential for increasing the production capacity of vaccine manufacturers in a pandemic (*2*).

Using 1, instead of 2, injections will shorten the time needed to develop immune response by 3–4 weeks. Unlike previous studies on influenza A (H5N1) vaccines that reported only data from 21, 28, or 56 days after the final vaccination (*8–10*), we report data up to 90 days. The lower dose and fewer injections required to trigger an immune response can be at least partially explained by using a whole virus vaccine and an aluminum phosphate adjuvant system. The use of a different adjuvant system than ours may have influenced the results of other trials (*9*,*10*). Other investigators used a modified HI method with horse erythrocytes, which are known to be more sensitive for influenza A (H5N1) subtype than the conventionally used turkey or chicken erythrocytes (*8*,*9*). Thus, if horse erythrocytes had been used in our study, the vaccine would likely have been even more immunogenic.

This study found fewer, less frequent, and milder side effects than did other trials of influenza A (H5N1) vaccines published so far (*8–10*). This could possibly be explained by the smaller dose used. Also, the endotoxin content of 0.1 IU/mL in our vaccine was much smaller then the allowed amount of 100 IU/mL by standards (*5*).

We report an inactivated whole virus vaccine that is safe and immunogenic in healthy adults and that requires a low dose and only 1 injection to trigger an immune response. We are conducting trials in elderly persons and children.

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Resistance to Dihydroartemisinin

To the Editor: The title of the letter by Cojean et al. (*1*) is misleading. The data presented essentially point to an absence of in vitro resistance to dihydroartemisinin (dhART) in the panel of African isolates studied, with 1 of 397 isolates having an elevated 50% inhibitory concentration (IC_{50}) for dhART. The S769N *PfATPase6* mutation associated with in vitro resistance to artemether (*2*) was observed in 1 isolate. This mutant isolate had a low IC_{50} for dhART, but its IC_{50} for artemether has not been tested. Since the relationship between in vitro susceptibility to artemether and dhART is still uncertain (*3*), these data do not disprove the association of a *PfATPase6* S769N polymorphism with elevated IC_{50} for artemether that was observed in isolates from French Guiana (*2*).

Worth noting is that the association of the S769N *PfATPase6* polymorphism with elevated IC_{50} for artemether was confirmed in an isolate collected in French Guiana in 2005; that isolate had an IC_{50} for artemether of 127 nmol/L. Molecular typing identified 2 clonal types, 1 with a wild-type *PfATPase6* allele and 1 with a S769N

single mutant. After 3 weeks of in vitro cultivation without drug, the mutant allele was no longer detected and the IC_{50} for artemether was 8.2 nmol/L. This finding suggests poor fitness of the mutant allele under standard culture conditions.

The observation of an additional case of in vitro resistance to artemether in French Guiana 3 years after the first cases is of concern. Reinforcement of surveillance is needed as is clarification of the relationship of in vitro susceptibility to artemether and artesunate, the derivatives currently included in artemisinin-based combination therapies (ACTs). Surveillance and clarification would be particularly timely since emerging clinical or parasitologic failures to some ACTs have been reported (*4*,*5*).

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Resistance to Dihydroartemisinin

In Response: The original title of our article was "Lack of *Plasmodium falciparum* in Vitro and Genomic Resistance to Dihydroartemisinin in Travelers Returning to France from Africa." EID's shortening of the title (*1*) led to the perception that the letter title was misleading, but it was not on purpose. We have recently tested the 50% inhibitory concentration for artemether of the S769N *PfATPase6* isolate that we had kept in liquid nitrogen, and it showed susceptibility.

We underline that the previously reported clinical or parasitologic failures to some artemisinin-based combination therapies (*2*,*3*) were not synonymous with the emergence of resistance to artemisinin compounds. In the study by Grandesso et al., a combination of artesunate plus amodiaquine was given to children <5 years of age who lived in an area in which amodiaquine alone was ineffective to adequately treat uncomplicated falciparum malaria in 1 of 3 cases at day 28 (*2*). Such a combination (artesunate plus amodiaquine) was nearly equivalent in 1 of 3 cases to a 3-day artesunate monotherapy, which may fail to completely cure children because of the short half-life of artesunate. In the study by Bukirwa et al., no recrudescence occurred in patients treated with artesunate plus amodiaquine and only 2 of 199 patients treated with artemether plus lumefantrine experienced recrudescence at day 28 (*3*). As Birkiwa et al. themselves acknowledged, artemether plus lumefantrine was not administered with food, and it is known that lumefantrine is absorbed better when it is taken with a small amount of fat. Thus, the clinical failures observed did not necessarily reflect *P. falciparum* resistance to artemisinin compounds.

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Exposure: A Guide to Sources of Infection

Dieter A. Sturchler

ASM Press, Herndon, Virginia, USA, 2006
ISBN: 978-1-55581-376-5
Pages: 910; Price: US $129.95

Exposure: A Guide to Sources of Infection is a dense reference book suited for health professionals and public health officials working within the realm of infectious diseases. This book does not use the typical format of solely detailing microbes in succession. Instead, it takes the novel approach of organizing microbes by sources of exposure, as stated in the title. Sections include animals, the environment, foods, humans, travel, and nosocomial infections. A listing of microbes is provided in the last section, followed by an exposure checklist in the appendix.

By compiling >250 pages of references, the author has tried to give a detailed and current review of the literature. Although some readers may not find this level of detail useful, it is interesting and does propel the reader to think beyond the usual microbes and their common routes of exposure. The author is particularly sensitive to the international nature of infectious diseases and has worked hard to thoroughly discuss cases and outbreaks that have occurred throughout the world. This is particularly evident in the last section of the book, which employs the more familiar format of listing microbes alphabetically. The author cites the effects of many infectious agents by detailing where the microbe is usually found, its prevalence, virulence, and mode of spread.

As the author states, the scope of the book is not clinical but rather epidemiologic. Therefore, this publication does not specifically provide suggestions for treatment and management decisions. However, this book stresses the need to be more conscientious of the many modes of infections, which may prompt a diagnosis that otherwise may have been missed.

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Several Worlds: Reminiscences and Reflections of a Chinese-American Physician

Monto Ho

World Scientific Publishing Company, Hackensack, New Jersey, 2005
ISBN-10: 9812564098
ISBN-13: 978-9812564092
Pages: 348; Price: US $48.00

Dr. Monto Ho is a well-known infectious disease specialist whose major achievements are in interferon research and the control of antimicrobial drug resistance. The son of a Chinese diplomat, he received his early education in Ankara, Berlin, Vienna, and Brooklyn, New York. When he was 14 years old, his father took him back to the People's Republic of China and impressed on him the importance of Chinese culture.

In the first 2 parts of this book, Dr. Ho takes readers through his experiences in childhood and adulthood. During World War II, he was deeply immersed in Chinese culture. He then studied philosophy as an undergraduate and medicine at Harvard University and received his M.D. degree in 1954. After that important period, Dr. Ho began his pioneering work in interferon research and viral infections in transplant patients at the University of Pittsburgh School of Medicine in 1959. He then changed the direction of his research to help Taiwanese doctors face and solve the critical problem of antimicrobial drug resistance in Taiwan.

The 2 most interesting chapters in this book are "Academic Medicine" and "The Ups and Downs of a Department," in which Dr. Ho offers a behind-the-scenes perspective and looks at the role of basic disciplinary sciences at schools of public health. He describes himself as a lifelong learner and problem solver. His insights into Chinese and American cultures will put readers in a thoughtful mood.

Since 1998, Dr. Ho has organized the Taiwan Surveillance of Antimicrobial Resistance Program, a nationwide surveillance project indigenous to Taiwan that is supported by the National Health Research Institutes. He is also involved in the periodic surveillance of antimicrobial drug resistance through the centralized collection and testing of representative isolates from major hospital laboratories.

Dr. Ho uses his personal charm and intelligence in a philosophical and scientific autobiography to provide information that enables readers to ponder their own thinking about scientific research. His concept of education is that it is a lifelong experience and process. He suggests that 1 way to be a successful person is to keep on learning, reflecting, and striving for insights and answers to questions.

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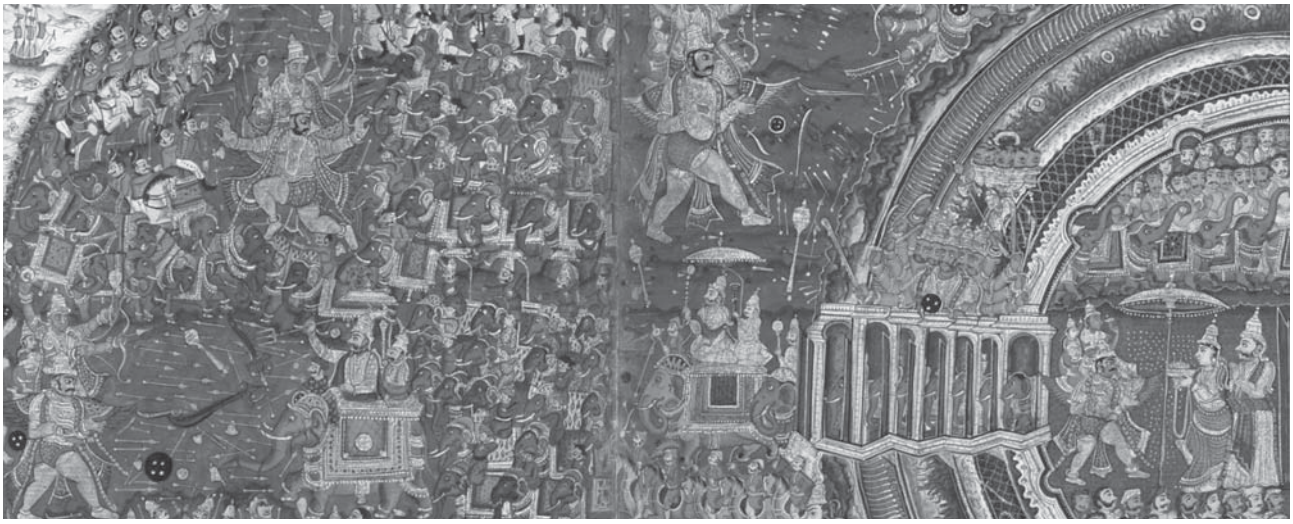

Krishna Storms the Citadel of Naraka (from a Bhagavata Purana). India, Karnataka, Mysore (ca. 1840). Opaque watercolor and gold on paper (25.1 cm × 36.8 cm). San Diego Museum of Art (Edwin Binney 3rd Collection)

Protect Me, Lord, from Oil, from Water, from Fire, and from Ants and Save Me from Falling into the Hands of Fools

– Prayer "uttered by a manuscript." Found at the end of medieval Indian texts

Polyxeni Potter*

Indian paintings on paper, known as "miniatures," can be found in books from as far back as the 11th century, most from the 14th through the 19th century. They vary from postage stamp size to more than a yard in height and are called miniatures partly to distinguish them from murals, which they followed as a genre (*1*). Like the good books they inhabited, they were portable and intimate, meant to be appreciated from close up and, duly treasured, they were tucked away to be handled only from time to time, with care.

Miniature paintings were collaborative, created by groups of artists specialized in drawing, portraiture, background, or border illustration and were exclusively commissioned by patrons—princes, merchants, religious leaders. The importance attached to patronage can be traced in the colophons of surviving books. We know virtually nothing about the anonymous artist who created the painting but can often trace at whose "lotus feet" it was placed when completed.

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Though "All the blessings of heaven" were bestowed on the patron of a manuscript or series, great patrons did not emerge until the late 15th century. Soon after, during the Mughal Empire, interest in art peaked, along with patronage, and schools of painting developed and flourished.

Miniatures were often painted on a wash: sheets of paper glued together and laminated. Ground white chalk or lead formed the foundation for layers of transparent watercolor in vivid, exotic pigments, from gum arabic or crushed seeds of the tamarind. Indian yellow was made of dried urine from cows fed on mango leaves. Gold, in leaf or liquid, embellished clothing and jewelry. Detail was created laboriously with fine brushes of hair from live squirrels, luster achieved from burnishing the surface, which also bonded pigment layers to the support (*2*). Book pages were intricately illustrated, some double-sided, with calligraphic elements on the verso.

The folio on this month's cover comes from the Bhagavata Purana, a celebrated text in Hindu sacred literature recited daily by millions. Though favored and revered by painters and patrons, the Purana, with its collection of "an-

cient and wondrous tales of the Lord" Krishna, has rarely been illustrated with such exuberance (*1*). The embroidered cover of this manuscript, which contains 217 paintings, identifies it as volume 6 in a series. It was written on European paper. A seal on the flyleaf reads, "His Highness, Rajah of Mysore."

Eyes are naturally drawn to Krishna. His name literally means "black" or "dark" or "all-attractive," and he has a very distinct iconography. In his countless avatars, from Vishnu to simple human, his beauty is irresistible, his complexion "tinged with the hue of blue clouds" (*3*). Clad in golden silk, he rides the sun-bird Garuda. The philosophy of this God/cowherd is captured in the epic of the Hindu faith, the Bhagavad-Gita (*4*).

Krishna Storms the Citadel of Naraka recounts the God's exploits against a demon king, a menace who commits atrocities, even against his own mother, the Earth Goddess. Aboard Garuda with his consort Satyabhama, Krishna wings his way to the demon's citadel, "Which heart would not quail at the loud blast …from the Lord's conch?" (*1*). The enemy is barricaded in his impregnable island city, inaccessible by "hilly fortifications and mounted missiles and weaponry" and unapproachable with "moats of water and fire and belts of stormy winds" (*1*).

Krishna, in true form, is Vishnu, four-armed and impervious to "thousands of fearful and strong snares" (*1*). He faces Mura, the five-headed demon (upper right), who soon falls, "like a mountain summit struck by a thunderbolt." Mura's seven sons move in, advancing, "discharging volleys of shafts, swords, maces, darts, double-edged swords and javelins" to perish too, along with their armies (*1*). Naraka joins in and succumbs to Krishna, who appears everywhere, "like a cloud emblazoned in a streak of lightning" (*1*). The citadel is penetrated. Inside, the Earth Goddess, bowing, offers Krishna "a pair of earrings resplendent with jewels and chased in the purest gold…a garland of forest flowers, the umbrella of Varuna…" (*1*).

The unfolding spectacle encompasses the heavens, engaging with ease gods, humans, animals, and mythologic beasts. Tiny figures move about purposefully, elephants carry on with dignity, seas are alive with fish. The monumental story is painted with assurance, as if it could have happened only in this orderly and brilliant way. And flying arrows and severed heads notwithstanding, the event seems a pageant, the celebration of a shift in the balance of power, an interaction whose outcome was never in doubt.

The citadel of Naraka with its formidable fortifications and hordes of defenders begs an equivalent in the microbial world. And not only because vermin threaten everything, even books. In the eternal, complicated interactions between microbes and hosts, supremacy and survival are closely knit. Host defenses are inevitably overcome by adaptation and change, until more sophisticated, specialized defenses can be built. Microbes develop resistance. Hosts mount additional defense. Microbes regroup and reappear in manifestations and avatars rivaling those of Krishna himself.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the June issue for the following topics:

Economics of Catheter-related Bloodstream Infections

Transmission and Surveillance of Chronic Wasting Disease in Deer and Elk

Extent and Costs of Severe Rotavirus Disease, Denmark

Person-to-Person Transmission during *Escherichia coli* O157:H7 Outbreak

West Nile Virus Transmission from Eastern Chipmunk to Mosquito Species

Bovine Spongiform Encephalitis Infection in Meat and Bonemeal After Feed Ban, France

Meningococcal Carriage, Burkina Faso, 2003

Minority Variant pfcrt K76T Mutations and Chloroquine Resistance, Malawi

Alveolar Echinococcosis and Increasing Fox Population Density, Switzerland

Characteristics and Risk Factors of Imported Fatal Falciparum Malaria, France, 1996–2003

Drug-resistant *Escherichia coli* in Humans and Poultry

Isolation and Characterization of Human Parechovirus

Melioidosis Outbreak, Southern Taiwan

Norovirus Infection in Children with Acute Gastroenteritis, Madagascar

Reemergence of Oropouche Fever, Northern Brazil

Complete list of articles in the June issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

May 31–June 1, 2007
2nd International Conference on Avian Influenza in Humans: Recent Developments and Perspectives
Institut Pasteur
Paris, France
http://www.isanh.com/avian-influenza

June 17–23, 2007
Options for the Control of Influenza VI
Toronto, Ontario, Canada
http://www.optionsviconference.com

June 24–28, 2007
Association for Professionals in Infection Control and Epidemiology
34th Annual Conference and International Meeting
San Jose, CA, USA
Contact: 202-454-2638
http://www.apic.org

June 25–27, 2007
National Foundation for Infectious Diseases (NFID) 2007 Annual Conference on Antimicrobial Resistance
Hyatt Regency Bethesda
Bethesda, MD, USA
http://www.nfid.org/conferences/resistance07

June 27, 2007
The Interagency Task Force on Antimicrobial Resistance presents: The Annual Report of Progress on A Public Health Action Plan to Combat Antimicrobial Resistance
Hyatt Regency Bethesda
Bethesda, MD, USA
http://www.nfid.org/conferences/resistance07

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@ cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

EMERGING
INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje. org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by “et al.” Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word’s table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/ EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 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.