EMERGING INFECTIOUS DISEASES

Tracking trends and analyzing new and reemerging infectious disease issues around the world

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Update from Brazil

Measles Elimination

Drug-Resistant Enterococci

Malaria Developments

Hantavirus in Children

Dengue Reemergence in Cuba

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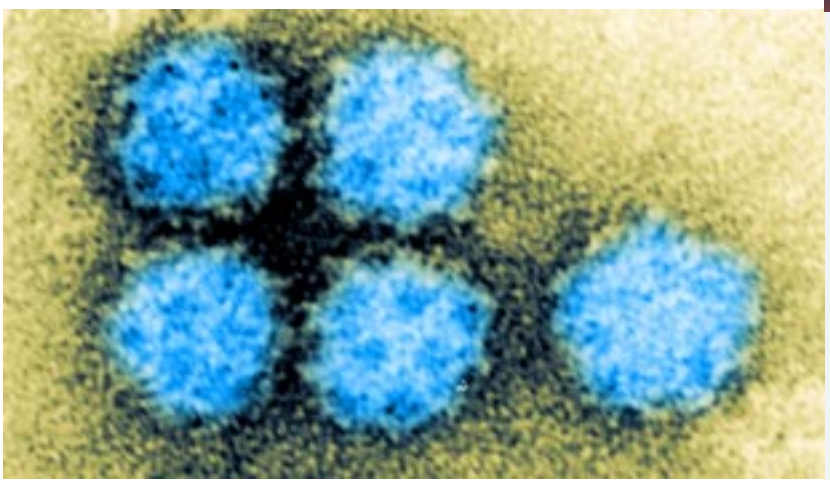

Above: La Voz de la Conciencia (1977) by Perez Celis (Argentina) Used with permission from Brenda and Stephen Morse, Atlanta, Georgia (Photo by Troy Hall).

Left: Electron photomicrograph of cetacean calicivirus Tursiops-1. Photo courtesy of A.L. Smith.

Cover: Landscape with Metallic Interventions (1991) by Antonio Henrique Amaral (Brazil, b. 1935). Used with permission from Brenda and Stephen Morse, Atlanta, Georgia (Photo by Troy Hall).

Antonio Amaral is known for successfully combining elements of Pop Art with Brazilian themes. His paintings are viewed by some as the revival of the tropicalismo of the Tarsila do Amaral generation.

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Above: Macaque grabbing a snack from a passerby in the downtown area of Lop Buri, Thailand. Photo by Cyril Ruoso, reprinted with permission of BIOS.

International Editors

update

In this new section, we welcome commentary and updates from our newly formed Board of International Editors. (See inside front cover for a list of names.)

Emerging Infectious Diseases—Brazil

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Dr. Momen is a senior research scientist; head of the Department of Biochemistry and Molecular Biology, Oswaldo Cruz Institute, FIOCRUZ; and Editor of Memorias do Instituto Oswaldo Cruz. His research interests include biochemical systematics of pathogenic microorganisms. Dr. Momen is temporary adviser to both WHO and PAHO at several technical consultations and scientific working groups.

Brazil's large size (more than 8.5 million km^2 and 150 million population) and inadequate public health infrastructure pose a considerable challenge in assessing the status of emerging infectious diseases. Under the current system, most infectious diseases are not notifiable. In diseases for which notification is required, underreporting is common and varies widely by region and disease, and notification is often delayed, which causes the data to be revised frequently. Moreover, in hospital and clinical settings, the etiologic agent of an infectious disease is often not identified. For example, more than a million hospital admissions are recorded per year by the public health system under parasitic and infectious diseases (the category excludes AIDS and respiratory illnesses); of these diseases, more than 70% are diagnosed as ill-defined intestinal infections and a further 10% as food poisoning and septicemia, both without identification of the etiologic agents. For these reasons, the numbers reported here (1996 data, unless otherwise stated) may not reflect the true numbers of cases, and only diseases whose prevalence has changed markedly in recent years will be included in the review.

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Parasitic Diseases

Malaria

Among parasitic diseases, malaria causes the most illness, approximately half a million cases annually, nearly all from the northern (Amazon) region. The disease has been controlled in the remainder of the country for years. Where systematic and sustained efforts have been made in the Amazon, control has also been successful. Due to the different epidemiologic situations in the Amazon, no single strategy is effective. Worrying developments in this region include the establishment of the disease in the periphery of the principal cities and the increase in drug-resistant parasites.

American Trypanosomiasis

American trypanosomiasis (Chagas disease) is the most lethal parasitic disease in Brazil, causing more than 5,000 deaths per year. Several million people are chronically infected carriers of this disease, for which there is still no effective treatment. A very effective control program against the principal insect vector, *Triatoma infestans,* and improved control of the blood supply have reduced the incidence of new cases to a very low level. Factors that continue to make the disease a re-emerging threat include secondary vectors, forest clearance, and congenital transmission.

Leishmaniasis

Leishmaniasis (cutaneous and visceral) has increased in incidence in recent years. This increase is modified by apparent cyclic variations of undetermined cause (cyclic duration for the visceral form has been estimated at 10 years). The disease has increased not only in the usual areas of forest and recent clearance, but also in areas of traditional colonization as well as in the urban areas of the northeast. One factor involved in the spread of this disease, uncontrolled urban growth due principally to rural migration, has led to pockets (normally on the periphery of the

cities) of extreme poverty that favor the spread of many diseases.

Viral Diseases

Dengue

Among the notifiable viral diseases, dengue causes the most illness. Its mosquito vector, *Aedes aegypti,* was reintroduced into Brazil in the 1970s; large outbreaks followed in the next decade. Since the vector's reintroduction, more than 700,000 cases have been reported (180,000 cases in 1996). The presence of dengue-1 and -2 indicates that cases of hemorrhagic dengue are also occurring. *Ae. aegypti* is now present in all states, and a plan has been formulated by the federal ministry of health to eradicate the vector, although plan implementation has met with operational difficulties. The widespread distribution of *Ae. aegypti,* also the vector of yellow fever, in areas where this disease was considered endemic poses a serious risk for the reemergence of the epidemic form of yellow fever. Vaccination and sanitation campaigns earlier in the century considerably reduced the incidence of the disease (fewer than 100 cases per year—the last urban cases were reported in 1942).

Measles

Measles—a viral disease that was controlled and nearly eradicated—reemerged with a vengeance in 1997. The outbreak began in the state of São Paulo, where only 15 cases of measles were confirmed in 1996, and has spread to other states. As of October 1997, 61,000 cases (48,000 in São Paulo) have been reported, and 17,000 (13,000 in São Paulo) have been confirmed. Most cases have occurred in young adults under 30 years of age. The vaccination campaign has been reformulated in response to the outbreak, and a national campaign has been launched. Given Brazil's history of success in organizing such campaigns, it is likely that the outbreak will be rapidly contained and that the effort for measles eradication will be resumed.

Other Viral Diseases

Other viral diseases include AIDS, with more than 17,000 new cases registered in the last year. The number of deaths due to AIDS was 30% lower in the first half of 1997 than in the first half of 1996. This decrease is attributed to the introduction of new antiviral drug combinations. The spread of the AIDS epidemic, particularly into rural areas, has resulted in coinfections with different infectious agents, producing a variety of novel pathologies. Several subtypes of HIV, as well as novel recombinations, have been reported.

Enteric transmission of hepatitis continues; hepatitis A and D comprise most of the cases, although the peak incidence is now in young adults rather than in young children. The incidence of hepatitis B is declining as a result of vaccination, while that of hepatitis C is increasing. A high prevalence of hepatitis D exists in some regions of the Amazon, where in conjunction with hepatitis B, it is believed to be the principal cause of Labrea black fever.

Influenza is a major concern. Imported vaccine is expensive and not generally available, and the proportion of elderly people (and the threat for a large-scale epidemic) is increasing.

The vast forests that still cover large areas of Brazil are home to many known and unknown viruses. The Evandro Chagas Institute in Belem alone has isolated more than 183 new arboviruses. The increasing exploitation of sylvatic resources put humans in direct contact with these viruses, but because of inadequate or nonexistent medical facilities (entire municipalities still lack a single medical doctor), fevers and even deaths often are not diagnosed or are misattributed. Among new viruses causing fatal infections recently discovered in Brazil are Rocio virus, which causes encephalitis, and Sabiá virus, which causes hemorrhagic fever. Several cases of hantavirus infection have also been reported, but on these and other occasions, the lack of a biosafety level 4 laboratory in the country impeded further work.

Bacterial Diseases

Enteric bacterial infections are an important cause of illness in Brazil. National figures on prevalence are not available, except for cholera. However, an alarming increase has been reported in antibiotic-resistant strains.

Cholera

The cholera epidemic in Brazil started with the reemergence of the disease in Latin America in 1991, reached a peak of more than 60,000 confirmed cases and 670 deaths in 1993, and then declined to 1,000 cases in 1996. In 1997, the disease resurged with more than 5,000 reported cases and 2,600 confirmed cases. The reawakening

of epidemiologic research in cholera caused by its resurgence led to the discovery of a new biotype of *Vibrio cholerae* in the Amazon region. This biotype is of the O1 serotype; it has distinct multilocus enzyme electrophoresis and RAPD profiles from other pathogenic O1 *V. cholerae*. About 50 isolates have been made from cases of diarrhea in the upper Amazon (Solimoes) River. The microbe apparently lacks the principal known virulence factors (e.g., the toxin gene cassette and the major colonization factor, TCP); however, some isolates present a cytotoxic effect for Y-1 cells.

Mycotic Diseases

Fungal infections, including histoplasmosis, paracoccidioidomycosis, and cryptococcosis, occur; however, their cause is often unknown and even if a diagnosis is made, the diseases are not reported.

Public Health Infrastructure

The emerging infectious disease picture in Brazil will not change markedly without a sustained and determined effort to improve the country's public health infrastructure. The existing, generally passive epidemiologic surveillance system produces information that arrives too late to be effective; however, a number of measures, if implemented immediately, can mitigate the impact of any future epidemic: a containment laboratory (biosafety level 4) that can handle known and unknown microbes of high virulence; at least one infirmary, properly designed and fully equipped, to treat highly contagious and virulent diseases. The current lack of this facility poses a great danger to the population should an outbreak of such a disease occur.

The financial, technical, and human resources needed to activate these measures already exist. In infectious diseases, Brazil has a long tradition of fruitful international collaboration that can be tapped for additional support. The success of national vaccination and sanitation campaigns in the eradication and control of some infectious diseases at the beginning of this century and in more recent times demonstrates that much can be accomplished. Brazil can become a successful model for other developing countries.

Acknowledgments

I thank the Fundação Nacional de Saude for providing data on different diseases; Drs. H. Schatzmayr, J.R. Coura, and P. Mason for interesting discussions and criticisms of the manuscript; and Drs. Ana Gaspar, Clara Yoshida, Ana Carolina Vicente, and Marilda Siqueira for useful information.

Risk for Transfusion-Transmitted Infectious Diseases in Central and South America

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We report the potential risk for an infectious disease through tainted transfusion in 10 countries of South and Central America in 1993 and in two countries of South America in 1994, as well as the cost of reagents as partial estimation of screening costs. Of the 12 countries included in the study, nine screened all donors for HIV; three screened all donors for hepatitis B virus (HBV); two screened all donors for *Trypanosoma cruzi;* none screened all donors for hepatitis C virus (HCV); and six screened some donors for syphilis. Estimates of the risk of acquiring HIV through blood transfusion were much lower than for acquiring HBV, HCV, or *T. cruzi* because of significantly higher screening and lower prevalence rates for HIV. An index of infectious disease spread through blood transfusion was calculated for each country. The highest value was obtained for Bolivia (233 infections per 10,000 transfusions); in five other countries, it was 68 to 103 infections per 10,000. The risks were lower in Honduras (nine per 10,000), Ecuador (16 per 10,000), and Paraguay (19 per 10,000). While the real number of potentially infected units or infected persons is probably lower than our estimates because of false positives and already infected recipients, the data reinforce the need for an information system to assess the level of screening for infectious diseases in the blood supply. Since this information was collected, Chile, Colombia, Costa Rica, and Venezuela have made HCV screening mandatory; serologic testing for HCV has increased in those countries, as well as in El Salvador and Honduras. *T. cruzi* screening is now mandatory in Colombia, and the percentage of screened donors increased not only in Colombia, but also in Ecuador, El Salvador, and Paraguay. Laws to regulate blood transfusion practices have been enacted in Bolivia, Guatemala, and Peru. However, donor screening still needs to improve for one or more diseases in most countries.

Preventing the transmission of infectious diseases through blood transfusion in developing countries is difficult given that the resources needed are not always available, even when policies and strategies are in place (1). Avoiding paid donors, selecting blood donors through questionnaires, and limiting the number of transfusions can prevent the transmission of infections. Testing for specific antibodies is the final measure for eliminating unsafe blood.

The risk for transfusion-transmitted infectious diseases can be estimated on the basis of screening level for each infectious agent and the prevalence rate of the infection in the donor population. Estimates may also take into account the sensitivity, specificity, and window period of the testing assays. We report here an estimate of such a risk in 12 Central and South American countries and the cost of reagents required for the screening of these infectious diseases as a proxy of resources needed to reduce the risk.

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Source of Information

This report analyzes data from 1993 on screening of blood donors from five countries (Costa Rica, El Salvador, Guatemala, Honduras, and Nicaragua) of Central America. Data were also analyzed for 1993 from five countries

(Bolivia, Chile, Colombia, Peru, and Venezuela) and for 1994 from two countries (Ecuador and Paraguay) of South America. Information was obtained from Ministry of Health reports during technical meetings in which the situation of each country was reviewed (2-5) or from an official report (6).

In addition, data are presented on the least expensive reagents for detecting antibodies for HIV, hepatitis C virus (HCV), *Trypanosoma cruzi*, and *Treponema pallidum*, and for detecting hepatitis B virus (HBV) antigen (HBsAg) (2,3). All data are national except for Peru, where the information was for the city of Lima only (3). Population data were from the Pan American Health Organization's publication Health Conditions in the Americas (7). Estimates are based on reported results of donor screening activities (2-6).

Assumptions

For the best possible scenario, the following assumptions were made: 1) Because the laboratory procedures and brands of reagents used in the 12 countries may differ in sensitivity and specificity, comparisons between them are not straightforward. In addition, results of the screening are influenced by the existence of an organized system of quality control and proficiency testing for the serology and for the evaluation of the diagnostic kits, which most countries lacked from 1993 to 1994. Most countries reported the use of different brands of second, third, and fourth generation immunologic assays for screenings of HCV, HIV, and HBV, respectively. Therefore, we assumed that the specificity of the tests for viral diagnosis was 100%, but the sensitivity was 90.00% for HCV, 99.99% for HIV, and 99.90% for HBV. These specificity and sensitivity estimates fit well with those reported for second, third, and fourth generations of assays for HCV, HIV, and HBV, respectively, as mentioned in the package insert by two of the manufacturers of reagents used in the countries. Average window periods for those assays were 20 to 25 days (8,9), 82 to 84 days (9,10), and 51 days (9) for HIV, HBV, and HCV, respectively. In the case of *T. cruzi* serology, we selected the upper range of reported sensitivity and specificity (90% and 95%, respectively) (11,12). For *T. cruzi*, the probability that a person may become a donor during the window period is low because infection is usually acquired in childhood and in rural areas. 2) We assumed that prevalence of infection in unscreened donors was the same as the national average prevalence for each infectious disease. 3) Chile (6) and Peru (3) were the only countries that reported a fractionation index, 1.85 and 1.5, respectively. As no other country provided data on the fractionation index or data allowing one to be calculated, to put the countries in the same category, it was assumed that every blood donation corresponded to a single transfusion to one recipient.

Screening Coverage and Prevalence Rates

Table 1 shows coverage of screening and prevalence rates of seropositive tests for specific infectious agents among blood donors reported by each of the 12 countries. For HIV, 100% of the donors were screened in all countries, except Bolivia (36.20%), Ecuador (89.50%), and Colombia (98.80%). Prevalence rates for HIV varied from 3.90 per 1,000 in Honduras to 0.04 per 1,000 in Nicaragua. For HBV, only Costa Rica, Peru, and Venezuela screened 100% of donors. The highest values of HBV prevalence estimated were 14.40 per 1,000 for Venezuela and 13.00 per 1,000 for Paraguay. Bolivia, Costa Rica, and Paraguay did not screen for HCV at all, and all other countries screened fewer than 58% of donors; prevalence rates varied from 0.50 to 9.40 per 1,000. Screening for syphilis was not complete in Bolivia, Chile, Colombia, Ecuador, Nicaragua, and Paraguay; prevalence rates were 5.00 to 28.00 per 1,000. For *T. cruzi* infection, only Venezuela and Honduras screened 100% of donors; prevalence rates were 2.00 per 1,000 in Ecuador to 147.90 per 1,000 in Bolivia. In 1993, Peru and Costa Rica had not yet introduced screening for *T. cruzi*.

Estimating Potential Infectivity of the Blood Supply

The probability of receiving an infected transfusion unit *P(R)* in each country was estimated by multiplying the prevalence of a specific infection by 1-level of screening (Table 1). For those estimates, the sensitivity and specificity of the different tests were taken into account. As the overall assumed sensitivity of HIV screening was 99.99%, the adjustment of prevalence rates makes no material difference to the precision of the figures in Table 1. The probability of getting a transfusion-transmitted infection *P(I)* was calculated as the result of the probability of receiving an infected transfusion

Table 1. Coverage of screening[a] of blood donors and seroprevalence rates (per 1,000) of infectious diseases, by country[b]

Country	HIV		HBV[c]		HCV		Syphilis		*T. cruzi*	
	Cov.[d] (%)	Prev.[e] (/10³)	Cov. (%)	Prev. (/10³)	Cov. (%)	Prev. (/10³)	Cov. (%)	Prev. (/10³)	Cov. (%)	Prev. (/10³)
Bolivia	36.2	0.10	14.5	2.00	0	?[f]	37.9	18.10	29.4	147.90
Chile	100	3.40	98.7	2.00	34.0	6.40	95.2	11.40	76.7	12.00
Colombia	98.8	2.00	98.3	7.00	24.7	9.00	87.3	13.00	1.4	12.00
Costa Rica	100	0.34	100	4.50	0	?	100	5.00	0	?
Ecuador	89.5	1.00	88.2	3.80	32.9	1.40	86.7	11.50	51.0	2.00
El Salvador	100	1.30	96.0	8.00	31.4	2.50	100	19.00	42.5	14.70
Guatemala	100	3.00	79.8	7.00	37.2	8.00	100	19.00	75.0	14.00
Honduras	100	3.90	83.5	2.70	27.8	0.50	100	7.00	100	12.40
Nicaragua	100	0.04	53.1	4.00	53.1	4.40	88.4	16.00	58.4	2.40
Paraguay	100	0.70	92.9	13.00	0	?	66.9	28.00	86.8	45.00
Peru	100	2.80	100	8.60	57.4	4.40	100	9.60	0	23.60[g]
Venezuela	100	2.10	100	14.40	31.0	9.40	100	10.70	100	13.20

[a]Coverage of screening = (number of screened donors ÷ total number of donors) x 100.
[b]Data as reported by the countries from 1993, except for Ecuador and Paraguay, which were for 1994.
[c]HBsAg only.
[d]Coverage.
[e]Prevalence.
[f]Screening not performed and prevalence not known.
[g]Data from a survey of 2,237 samples.

P(R) multiplied by the infectivity risk. For countries reporting 100% of screening coverage for a specific disease, a residual *P(R)* was estimated as prevalence x 1-screening sensitivity (Table 2). Infectivity risk (defined as the likelihood of being infected when receiving an infected transfusion unit) was assumed to be 90% for HIV (13), 75% for HBV (14), 90% for HCV (15), and 20% for *T. cruzi* (16) (Table 2). Estimates for transfusion-acquired syphilis are not presented because the infectivity risk depends on length of refrigeration (17).

Considering the low prevalence rates and the incompleteness of HIV screening, only Bolivia, Colombia, and Ecuador could have missed detecting an HIV-infected transfusion unit; the probability of getting an infection in these countries was estimated at 0.57, 0.22, and 0.95 per 10,000 transfusions, respectively. For HBV and HCV this risk is higher. Up to 14.21 HBV infections (Nicaragua) and 67.09 HCV infections (Colombia) per 10,000 transfusions may have occurred. The highest risk for transfusion-transmitted infection was estimated for *T. cruzi:* 219.28 per 10,000 and 49.56 per 10,000 for Bolivia and Peru, respectively, and approximately 2 to 24 per 10,000 for the other seven countries (Table 2).

Table 3 shows estimates of the absolute number of infections that may have been induced

Table 2. Probability of receiving an infected transfusion *P(R)*[a] and probability of getting a transfusion-transmitted infection *P(I)*[b], by country[c]

Country	HIV (x10⁴)		HBV (x10⁴)		HCV (x10⁴)		*T. cruzi* (x10⁴)	
	P(R)	*P(I)*	*P(R)*	*P(I)*	*P(R)*	*P(I)*	*P(R)*	*P(I)*
Bolivia	0.64	0.57	17.27	12.95	NSP[d]	NSP	1096.38	219.28
Chile	0.00	0.00	0.26	0.20	46.46	41.82	29.36	5.87
Colombia	0.24	0.22	1.20	0.90	74.55	67.09	124.24	24.85
Costa Rica	0.00	0.00	0.45[x]	0.34	NSP	NSP	NSP	NSP
Ecuador	1.05	0.95	4.52	3.39	10.33	9.38	10.29	2.06
El Salvador	0.00	0.00	3.23	2.42	18.87	16.97	88.75	17.75
Guatemala	0.00	0.00	14.28	10.71	55.26	49.74	36.75	7.35
Honduras	0.00	0.00	4.49	3.37	3.97	3.57	13.02[x]	2.60
Nicaragua	0.00	0.00	18.95	14.21	22.70	20.43	10.48	2.10
Paraguay	0.00	0.00	9.32	6.99	NSP	NSP	62.37	12.47
Peru	0.00	0.00	0.87[x]	0.65	20.62	18.56	247.80	49.56
Venezuela	0.00	0.00	1.45[x]	1.09	71.35	64.21	13.86[x]	2.77

[a]*P(R)* = probability of receiving an infected transfusion = prevalence of infection x 1- level of screening; [x]for countries in which reported screening level was 100%, a residual *P(R)* was estimated as prevalence x 1- screening sensitivity rate x 10,000.
[b]*P(I)* = probability of getting a transfusion-transmitted infection = *P(R)* x infectivity index (infectivity indexes used were HIV=90%; HBV=75%; HCV=90%; *T.cruzi*=20%). For calculations of *P(R)* and *P(I)* the prevalence was corrected taking into account the sensitivity of the screening.
[c]Data from 1993, except for Ecuador and Paraguay, which were for 1994.
[d]No Screening performed, so *P(R)* and *P(I)* not known.

by transfusion, calculated as [no. of donors x *P(I)*], for each country. Because Chile (6) and Peru (3) reported fractionation of blood units by 1.85 and 1.5, respectively, the estimated number of infected units transfused in those countries was multiplied by these factors. For the remaining countries, it was assumed that each donated unit was given to only one recipient. An index of infectious disease spread through blood transfusion was calculated by dividing the estimated total number of transfusion-related infections (for any one of the infectious agents considered) by the total number of donors. This index indicates the health risks associated with blood transfusion and can be used as an outcome indicator to assess the cost-effectiveness of screening programs.

The highest value for the infection spreading index was obtained for Bolivia, where 233 transfusion-related infections may have occurred per 10,000 donations. This was a result of a very high prevalence rate of antibodies to *T. cruzi* and a lower level of screening. For most other countries considered, the index was 68 to 103 infections per 10,000 donations. Due to low seroprevalence rates and good screening levels in some cases, the risk for transfusion-related infections was relatively low in Honduras (nine per 10,000), Ecuador (16 per 10,000), and Paraguay (19 per 10,000).

Table 3 also shows the ratio of number of infections per donation by country. One infection (HIV, HBV, HCV, or *T. cruzi*) might have been transmitted in every 43 (Bolivia) to 1,072 (Honduras) donated units.

Screening Costs

The unitary cost for serologic screening, estimated solely from expenditures on the least expensive laboratory reagents in each country and considering the prevalence rates reported by the countries was US$0.9 to US$2.4 for an HIV enzyme-linked immunosorbent assay (ELISA), US$0.5 to US$3.5 for HBV screening (enzyme immunoassay, radioimmunoassay, or passive reverse hemagglutination), US$3.5 to US$10.0 for HCV ELISA, US$0.25 to US$1.0 for a *T. cruzi* test (ELISA, radioimmunoassay, or indirect hemagglutination) (Table 4), and US$0.09 to US$0.60 for syphilis serology (RPR or VDRL). Using other tests might have increased the costs significantly for some of the infections. For example, the rapid agglutination test for HIV is usually more expensive than ELISA.

The cost of preventing the transfusion of one infected unit was estimated as [(no. of donors x cost of each test)/total number of positive donors] as reported by each country. This value represents the cost of detecting one unit positive for any one of the infections studied in each country by using one diagnostic test for each infectious disease. For example, using two tests, one for antibody detection and one for antigen detection of HIV, increases costs. Detection of *T. cruzi* was the least expensive (US$11-$209 per positive unit), followed by HBV (US$90-US$599 per unit), HCV (US$438-$7,136 per unit), and HIV (US$232-$23,000 per unit) (Table 4). The wide variation of cost primarily reflects differences in the prevalence of each infection and in the cost of each test in the countries.

The costs per capita to carry out a complete screening of blood donors in each country was US$0.008 to US$0.04 for HIV, US$0.008 to US$0.02 for

Table 3. Estimates of transfusion-transmitted infectious diseases, by country[a]

Country	No. of donors	Absolute no. of transfusion-transmitted infectious diseases[b]					Infection spreading index[c] /10[4]	Ratio of infections: donations
		HIV	HBV	HCV	*T. cruzi*	Total		
Bolivia	37,948	2	49	NA[e]	832	883	233	1:43
Chile[d]	217,312	0	8	1681	236	1925	88	1:113
Colombia	352,316	8	32	2364	875	3279	93	1:107
Costa Rica	50,692	0	2[f]	NA	NA	NA	NA	NA
Ecuador	98,473	9	33	92	20	154	16	1:639
El Salvador	48,048	0	12	82	85	179	37	1:268
Guatemala	45,426	0	49	226	33	308	68	1:147
Honduras	27,885	0	9	10	7[f]	26	9	1:1072
Nicaragua	46,001	0	65	94	10	169	37	1:272
Paraguay	32,893	0	23	NA	41	64	19	1:514
Peru[g]	52,909	0	4[f]	147	393	544	103	1:97
Venezuela	204,316	0	22[f]	1312	57[f]	1391	68	1:147

[a]Data from 1993 except for Ecuador and Paraguay, which were for 1994.
[b]Number of cases transmitted by blood transfusion = [number of donors x *P(I)*]. For calculations of number of infections, the prevalence was corrected taking into account the sensitivity of the screening.
[c]Infection spreading index = (total number of infections transmitted ÷ number of donors) x 10,000.
[d]Fractionation index = 1.85.
[e]Data not available.
[f]Residual infectivity considering that sensitivity of diagnostic tests is not 100%.
[g]Fractionation index = 1.5.

Table 4. Estimated unitary cost of preventing transfusion-transmitted infections, by country, 1993[a]

	Cost (US$)							
	HIV		HBV[b]		HCV		*T. cruzi*	
Country	Single test	Preventing one infected unit	Single test	Preventing one infected unit	Single test	Preventing one infected unit	Single test	Preventing one infected unit
Chile	2.3	676	1.2[c]	599	3.5[d]	547	NA[e]	NA
Costa Rica	1.1	3,280	0.5[c]	111	NA	NA	NA	NA
Ecuador[f]	1.7	1,708	1.0[c]	263	10.0	7,136	0.35[g]	175
El Salvador	2.0	1,550	1.9[c]	238	4.5	1,802	1.0[g,h]	68
							1.0	
Guatemala	1.8	601	1.7[c]	243	3.5	438	0.9[g]	65
Honduras	0.9	232	0.9[c]	334	3.5	6,971	0.45[h]	36
			0.5[h]	186				
Nicaragua	1.0	23,000	0.5[h]	125	3.5	797	0.5[h]	209
Peru (Lima)	2.4	858	3.5[c]	407	8.2	1,862	0.25[g]	11
			2.4[i]	279				
Venezuela	1.3	619	1.3[c]	90	4.5	479	0.5	38
							0.3[g]	23

[a]Cost of preventing (=detecting) one infected unit was calculated as [(number of donors x test cost) ÷ (total number of positive donors detected)]. All costs refer to enzyme-linked immunosorbent assay, unless otherwise indicated.
[b]HBsAg only
[c]Enzyme immunoassay.
[d]Estimated cost based on cost of test in other countries.
[e]Data not available.
[f]Donors and prevalence for 1994, costs for 1993.
[g]Indirect hemagglutination.
[h]Radioimmunoassay.
[i]Passive reverse hemagglutination.

HBV, US$0.01 to US$0.08 for HCV, US$0.0008 to US$0.003 for syphilis, and US$0.0025 to US$0.009 for *T. cruzi*.

Condition of the Blood Supply

These estimates indicate that the condition of the blood supply in Central and South America is far from ideal. Roughly, one case of transfusion-related infection occurs every 43 to 1,072 donations, varying with the infectious agent and the country.

In three of the 12 countries, transfusion recipients might become infected with HIV; in nine countries, with HCV; in all countries, with HBV; and in ten countries, with *T. cruzi*. However, it was not possible to establish the potential number of tainted units/infections from countries in which there was no information on donor prevalence for HCV (e.g., Bolivia, Costa Rica, and Paraguay).

No serologic tests for *T. cruzi* were done in Costa Rica and Peru. Data on the prevalence of *T. cruzi* serology in blood donors from Costa Rica from 1983 to 1985 (18,19) suggest a risk. Data from a recent report of a survey among donors in Lima indicate a prevalence of 2.36% (3). If this is the real prevalence in the city, the number of tainted units transfused would have been 1,872 in 1993, while the number of persons infected through blood transfusion could have been 393.

On the other hand, considering the number of donors and the prevalence of the infection in the 12 countries, if blood had not been screened at all, more than 35,000 infected units would have been transfused. However, infections have different patterns of evolution. HIV-infected persons are expected to get AIDS at some time during their lives (20), while only 50% and 38% of persons, respectively, will get posttransfusion hepatitis after infection with HBV or HCV (21). On the other hand, 20% to 30% of those infected with *T. cruzi* will get Chagas disease (16,18,19).

Limitations of the Data

Difficulties and limitations of the use of public health data for policy decisions, even in industralized countries, are well recognized. Figures presented here were generated to establish an approximation of the problem by providing an overview of the risk of receiving

tainted blood in different countries in Central and South America. One potential cause of underestimation of viral infections transmitted by blood is the residual risk because of the window period, even when 100% of donors are screened by serology (8,9). However, this residual risk would be difficult to ascertain in most countries of Central and South America. Investigation of clinically identified cases after a transfusion, follow-up of recipients for seroconversion, and special laboratory studies detecting seronegative donors for missed infections are laborious and expensive and could seldom be undertaken in those countries. Another possibility would be studies that combine estimates of incidence rates of infection among repeated and first-time donors (who seroconvert) with estimates for the duration of the preseroconversion period for a specific infectious agent (22). Excellent results were obtained by this method in the United States, where more than 80% of donations come from repeat donors. Those studies involved hundreds of thousands of donors and millions of donations. To have incidence data on repeat donors, it is necessary to have a significant number of voluntary donors who will repeat donations. Therefore, it is unlikely that studies of that sort could be carried out in the countries mentioned here. First, the population of the countries is much smaller; therefore, the number of donations is smaller. For example, in all Central America the number of donations is approximately 210,000 per year. Second, the number of repeat donations from voluntary donors is small. Voluntary donors accounted for 30% and 40% of all donations in Colombia and Costa Rica and 4% to 10% of all donors in Chile, Bolivia, Peru, and Venezuela (2-6). The number of voluntary donors was also small in the remaining countries. In all countries of Central and South America, most donations come from directed donors, relatives or friends of patients. In addition, there is no national registry of donors to allow for follow-up. Using incidence rates for first-time donors instead of repeat donors is not a solution because official incidence rates for HIV or other viruses were not available at the time of this study.

The risk for transfusion-related infection could also be overestimated. Recipients may already be infected. This is especially likely for *T. cruzi* infection in Bolivia, where the seroprevalence in the general population could be higher than 20% (18,19). Another source of overestimation is that only some of the cases detected by screening would be confirmed. In several countries, a confirmatory test is mandatory for HIV, syphilis, and HCV. However, as the primary function of blood banks is donor screening, seropositive donors for any of the diseases mentioned here are usually referred to specialized services or reference laboratories for confirmation of the results of the screening, and if results are confirmed, for treatment and counseling. Results of this confirmatory serology are not often sent back to the blood bank, even when privacy concerns allow for it. Chile was the only country that reported results of confirmatory tests for HIV: results indicated that only 9% of those found positive by the screening were confirmed positive (6). With *T. cruzi*, as there is no confirmatory test, it is assumed that a true positive is a unit that is positive on more than one test. By these criteria, a recent study in Brazil suggested that only one out of five donors positive for *T. cruzi* could be considered a true positive (23). Those facts, however, do not reduce the public health relevance of the problem presented here, although the real numbers of potentially infected units/infected persons may be still lower than our estimates.

Establishment of a screening process in every country will depend on balancing the benefits and costs. Although costs for preventing transfusion of one tainted unit or preventing one infection seem high for some etiologic agents, they are not so. Even in the case of Nicaragua, the country with the lowest HIV prevalence, the cost to prevent the transfusion of one potentially HIV-infected unit (by testing all donors with ELISA) was estimated at US$23,000, while treatment costs (drugs only) for an AIDS patient would be approximately US$12,000 per year.

In general, the risk for an infectious disease through tainted transfusion is not as high as that reported from some countries of Africa (24). Since 1993, donor screening has improved in several countries. Chile, Colombia, Costa Rica, and Venezuela, for example, have made screening for HCV mandatory, and coverage for serology for that infection has increased in those countries, as well as in El Salvador and Honduras. *T. cruzi* screening is now mandatory in Colombia, and the percentage of screened donors not only increased in Colombia but also in Ecuador, El Salvador, and Paraguay. Laws to regulate blood transfusion

practices have been enacted in Bolivia, Guatemala, and Peru. The figures presented, however, underline the need for improvement and stress the importance of an information system that allows assessing the level of screening for infectious diseases in the blood supply. Universal screening of donors for HCV is still a priority in most countries, and increased donor screening for *T. cruzi* is a priority for Bolivia and possibly for Peru.

Continuous collection of the type of information shown here, which has only been partially available (1), provides a baseline against which future achievements can be measured and is essential for obtaining the support needed to maintain or expand the screening of blood donors.

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Calicivirus Emergence from Ocean Reservoirs: Zoonotic and Interspecies Movements

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Caliciviral infections in humans, among the most common causes of viral-induced vomiting and diarrhea, are caused by the Norwalk group of small round structured viruses, the Sapporo caliciviruses, and the hepatitis E agent. Human caliciviruses have been resistant to in vitro cultivation, and direct study of their origins and reservoirs outside infected humans or water and foods (such as shellfish contaminated with human sewage) has been difficult. Modes of transmission, other than direct fecal-oral routes, are not well understood. In contrast, animal viruses found in ocean reservoirs, which make up a second calicivirus group, can be cultivated in vitro. These viruses can emerge and infect terrestrial hosts, including humans. This article reviews the history of animal caliciviruses, their eventual recognition as zoonotic agents, and their potential usefulness as a predictive model for noncultivatable human and other animal caliciviruses (e.g., those seen in association with rabbit hemorrhagic disease).

In vitro cultivation of caliciviruses indicates that these pathogens have been emerging periodically from ocean sources for 65 years (1). The best-documented example of ocean caliciviruses causing disease in terrestrial species is the animal disease vesicular exanthema of swine (VES) (1). Feline calicivirus (the only member of the group with a seemingly ubiquitous and continuous terrestrial presence) also appears to have ocean reservoirs (2). The source of caliciviruses causing gastroenteritis in humans is frequently shellfish, which do not always come from beds contaminated with human waste (3,4). The origins of hepatitis E are often obscure, but water is one suspected source (5). The most recent emerging calicivirus is associated with rabbit hemorrhagic disease (RHD), and although an ocean association has not been reported, the agent readily moves between continents and crosses ocean channels (6). Finally, the only reported in vitro isolation and sequential propagation of a calicivirus pathogenic for humans is a virus residing in the sea (7).

The *Caliciviridae* are divided into five groups, tentatively designated distinct genera, on the basis of sequence relatedness and genomic organization (8). Four are known human pathogens—Sapporo, Norwalk-like small round structured viruses, hepatitis E, and the marine (animal) caliciviruses—while the fifth group, which includes RHD virus, is not yet proven to be a human pathogen. The human Sapporo viruses are more closely related to the marine caliciviruses than to the other human group causing gastroenteritis, the Norwalk-like viruses. On the basis of homology and genomic organization, RHD virus falls between these two groups. In addition, the genomic organization of hepatitis E is most closely related to that of the only other hepatotropic calicivirus currently described, RHD virus (8).

Many marine calicivirus strains in the tentative genus of VES virus-like caliciviruses have been passaged in vitro; their characterization

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has facilitated understanding of calicivirus geographic distribution and host versatility (1). Dozens of serotypes were described on the basis of serum neutralization tests; this antigenic complexity complicated serodiagnosis and hampered studies of effects on host species.

The illnesses associated with two recently discovered viruses classified as *Caliciviridae,* hepatitis E virus and RHD virus, have altered the notion that caliciviruses produce only transient clinical disease but not death (1,9,10). Hepatitis E virus is fatal for 25% of the pregnant women in developing countries who contract hepatitis E (11); RHD can kill 95% of infected rabbits within 24 to 48 hours of exposure (6).

The oceans are reservoirs in which caliciviruses are exposed in a water substrate to life forms from zooplankton to whales and in which they, like other RNA viruses, can amplify to very high numbers with variants occurring in every replicative cycle (12). Such a varied replicative setting has served this parasite well. With the right tools, evidence of previous infection with caliciviruses can often be shown in fish, avian, and many mammalian species, including humans (1). It is not known why some caliciviruses have become potential hemorrhagic agents associated with purpura hemorrhagica in aborted piglets (13), neonatal hemorrhagic syndrome in pinnipeds (A.W. Smith, D.E. Skilling, unpub. data), hemorrhagic disease in fatal hepatitis E in humans (5), and RHD (6). However, it is known that caliciviral diseases can be difficult or impossible to contain and eradicate. Pathogenic caliciviruses can be expected to continue emerging from the sea in unexpected forms at unexpected times in unexpected places. Studying those that have emerged and are compliant to in vitro propagation can provide insights into those that cannot be cell-culture adapted and those yet to be discovered.

History and Its Lessons

The 66-year history of the caliciviruses with ocean reservoirs can be divided into three periods: 1932 to 1972, the species-specific era (14); 1972 to 1982, the new era of virology, during which oceans were first found to be reservoirs of viral disease infecting domestic animals (7,9); and 1976 to the present.

The first evidence of infection with caliciviruses of marine origin can be traced to 1932. A large herd of swine in Orange County, California, was being fed raw garbage collected from restaurants and institutions in the Los Angeles area. When some animals became sick with vesicular lesions on the feet and nose, regulatory veterinarians were notified because vesicular diseases of livestock were reportable. The farm with sick swine and adjacent farms were quarantined for foot-and-mouth disease, and more than 19,000 head of exposed cattle and swine were destroyed and buried in quicklime (14). The outbreak was contained. One year later and 100 miles to the south in San Diego, California, the second known outbreak occurred and was contained (14). This time the disease was found not to be foot-and-mouth disease, because the virus would not infect cattle, but instead was described as a new disease of swine and was called VES (14). In 1934, a third outbreak occurred in San Francisco, California, and VES was again contained (14). In 1935, the events repeated themselves, but from 1936 through mid-December 1939, the disease disappeared and then abruptly reappeared, at times involving 40% of California swine herds. All of these outbreaks in the 1930s and 1940s were shown by cross-infectivity studies to be caused by many distinct but related VES virus strains.

The embargoes placed on raw California pork were successful in containing VES within California until 1952. That year, a passenger train between San Francisco and Chicago served California pork and discarded the raw pork trimmings into the garbage in Cheyenne, Wyoming; the garbage was fed to swine subsequently redistributed by auction sale yard. Within 14 months, all major swine-growing areas in the United States (41 states) had reported VES. For the first time, the federal government, rather than just the California state government, activated eradication and quarantine measures against VES, including enforcement of federal laws requiring garbage to be cooked before it was fed to swine. By 1956, the last reported outbreak of VES had been contained, and the disease was said to have been eradicated. In 1959, VES was declared a foreign animal disease, even though it had never been reported outside the United States (14).

Forty years later, the natural history of this calicivirus still contained few details. Its origins were not known but were said to have been de novo or from some unknown wild animal reservoir, which was extensively sought but not

found (14,15). Swine were the only naturally infected host species; no evidence of human infection had been observed (14). Control of VES had been a notable success story for regulatory veterinary medicine in the United States; within 24 years of its discovery as an entirely new disease, it was said to have been eradicated (14). Internationally accepted animal disease diagnostic tests using swine, horse, and bovine infectivity profiles for vesicular stomatitis virus, foot-and-mouth virus, and VES virus were used routinely; VES virus did not infect cattle, whereas the other viruses did (14,15).

The discovery of a new paradigm in "viral traffic" (16) began the second period of calicivirus history. The movement of a member of the *Caliciviridae* from ocean reservoirs to terrestrial hosts changed the understanding of the natural history of a virus thought to be host specific and eradicated (1,15,17).

The first virus isolate from a pinniped occurred in 1972. The agent, named San Miguel sea lion virus type 1 (SMSV-1), was a calicivirus that caused classic VES in swine (17). Thus began a series of isolating and characterizing viruses in ocean species that were officially designated as "viruses indistinguishable from VES virus" because they were additional VES virus types. They could not be called VES virus (18) since VES had been officially eradicated. Should the VES virus reappear, its status as a foreign animal disease would mandate immediate implementation of eradication measures; eradication was viewed as an impossibility because of the wide range of reservoirs for VES virus, both migratory and ocean species. The 13 marine caliciviruses serotypes (100 $TCID_{50}$ vs. 20 units of neutralizing antibody) isolated from swine before 1956 were called VES viruses; those isolated after 1972 have been designated SMSV-1 through 17 or given more proper nomenclature, e.g., the pygmy chimpanzee isolate (primate calicivirus *Pan paniscus* type 1) (1,19).

By 1982, 11 species of pinnipeds and cetaceans of the North Pacific Ocean and Bering Sea (monk seals, California sea lions, northern sea lions, northern elephant seals, northern fur seals, walrus, gray whales, sei whales, sperm whales, bowhead whales, and Pacific bottlenosed dolphins) were known to be susceptible to calicivirus infection, as was an ocean fish, the opaleye perch (*Girella nigricans*) (20). Furthermore, in many instances, the virus had crossed the intertidal zone to infect terrestrial species (18). On the basis of these data and the established ocean ranges of known calicivirus host species, the shores of Mexico, the United States, Canada, Russia, Korea, Japan, China, and perhaps others bordering the North Pacific Ocean had been regularly exposed to large numbers of marine caliciviruses with unknown host ranges and tissue trophisms (21). By this time, type-specific neutralizing antibodies to two of four serotypes tested were reported in human patients in the United States (22). Cumulatively, these findings lead to the conclusion that fish and perhaps other ocean products provide a vehicle for transmission of these marine caliciviruses to terrestrial animals.

The magnitude of potential exposure to marine caliciviruses from the sea is substantial. For example, a 35-ton gray whale, shown by electron microscopy to have more than 10^6 caliciviruses per gram of feces, can eat 5% or more of its body weight per day and eliminate an equivalent quantity of feces containing an estimated 10^{13} caliciviruses daily. Marine caliciviruses remain viable more than 14 days in 15°C seawater (20).

Although marine mammals were often infected, fish and fish products were more likely to transport the virus from sea to land (23). In contrast to the 1932 to 1936 introductions of VES from raw fish, the rapid and uncontrolled spread of VES virus throughout California after 1939 and then across the United States in 1952 was a pig-to-pig cycle through raw garbage feed. However, new virus serotypes were also introduced through feeding raw fish scraps to swine (1,15). That VES was not a species-specific disease became accepted, but the possibility of human infection, although suspected (22), was largely untested.

During the third historical period (1976 to present), which overlaps with the second, viral traffic across the land/sea interface has been observed repeatedly, as the following examples show. A calicivirus isolated from an opaleye perch and designated SMSV-7 produced fulminating VES in exposed swine and spread from pig to pig by contact transmission (23). A reptilian calicivirus Crotalus-1 was isolated from three species of snakes and one species of amphibian (24) and from three species of marine mammals whose population distributions spanned the North Pacific from Mexico to the Bering Sea (1).

Mink fed a diet of ground-up calicivirus-infected seal meat became infected with VES virus (25). Parasitic nematode larvae from California sea lions in San Diego, California, were used to infect opaleye fish with calicivirus (SMSV-5); when the fish were killed and fed to Northern fur seals on the Pribilof Islands in the Bering Sea 30 days later, the seals developed vesicular disease, and the virus was recovered from the lesions (1). Shellfish resident to the tidal zone were exposed to marine caliciviruses and held at less than 10°C in a continuous flow of sterile seawater. The caliciviruses were reisolated 60 days later in mammalian cell lines (26). When tested with a cDNA calicivirus group-specific hybridization probe from a marine calicivirus (SMSV-5), some shellfish beds on U.S. coasts were positive for caliciviruses of unknown type (26). Feline calicivirus was shown to cause disease not only in dogs, but also in seals (on the basis of 17 of 20 adult sea lions having neutralizing antibody to FCV-F9 with titers of 1:15 to 1:220). Only 11 of 20 of these sea lions had neutralizing antibody to a sea lion isolate, SMSV-13 (titer 1:10). This demonstrates a probable feline calicivirus ocean presence in California sea lions (2). In swine, a so-called mystery pig disease (porcine respiratory reproductive syndrome) was reproduced in pregnant sows in 1992 with a three-plaque passage purified cytolytic calicivirus isolate from stillborn piglets with mystery pig disease (13). A second calicivirus serotype isolated from the same piglets was the same as that isolated from walruses in 1976 (1). A white tern *(Gygis alba rothschildi*), a migratory sea bird sampled in the mid-Pacific (French Frigate Shoals), had a blistering disease caused by a calicivirus (27). In the first fully documented human case of clinical disease caused by a marine calicivirus, SMSV-5 was isolated from blisters on the hands and feet of a patient (7). A second, less well-documented, case involved a field biologist who was handling sea lions and developed severe facial blistering. An untypable calicivirus was isolated in tissue culture (Vero cells) from throat washings (7).

Extent of Exposure

The extent of human disease is not known because test reagents are not readily available and diagnosticians are not alerted to caliciviral causes of human disease, except for diarrhea and occasionally hepatitis. However, evidence of human exposure was shown when 150 serum specimens from normal blood destined for donor use were tested. The samples were antibody-negative for hepatitis B surface and core antigen, HIV-1 and -2, HIV P-24 antigen, human T-cell lymphotropic virus Type 2, and hepatitis C virus. Approximately 19% had antibodies reactive to a polyvalent antigen made up of equal quantities of cesium chloride-banded SMSV-5, 13, and 17. (Figure 1A). To demonstrate that these reactions were not cross-reactions to the human Norwalk calicivirus antibody, serum samples from eight persons with Norwalk virus-induced diarrhea were also tested. Both acute- and convalescent-phase serum specimens were tested by enzyme-linked immunosorbent assay with the same SMSV antigens and Norwalk capsid protein (Figure 1B, C, and D). Although cross-reactivity was not detected, the serum samples may still have been able to cross-react with the calicivirus causing hepatitis E or Sapporo calicivirus, which were not tested. However, sera typed to all 40 marine caliciviruses reacted negatively when tested against Sapporo antigen (data not shown). These results suggest possible human exposure and antigenic response to marine caliciviruses. If that is not the case, such results present a confusing diagnostic picture of calicivirus exposure and diagnosis of human disease.

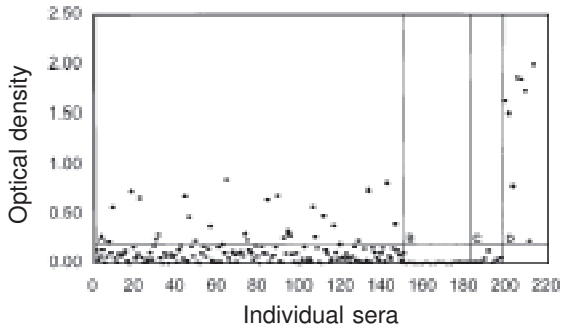

Figure 1. A) 150 blood donor sera tested against a polyvalent antigen containing San Miguel sea lion viruses (SMSVs) 5, 13, and 17 purified by CsCl; B) Eight acute- and eight convalescent-phase sera from a confirmed outbreak of Norwalk gastroenteritis tested against the polyvalent SMSVs 5, 13, 17 antigen; C) The eight acute-phase sera from the same outbreak of Norwalk gastroenteritis tested in B also tested against the baculovirus expressed Norwalk virus capsid protein; D) The eight convalescent-phase sera paired with the acute-phase sera (See C) tested as in C.

Organism Characteristics

The history of marine caliciviruses demonstrates that their biologic properties have great plasticity. The VES virus-like caliciviruses can replicate at temperatures of 15°C to 39°C, have diverse tissue trophisms, and travel by land (terrestrial reptiles, amphibians, and mammals), sea (pinnipeds, cetaceans, teleosts, and perhaps filter-feeding mollusks), and air (pelagic birds, e.g., the white tern). They can persist in nonlytic cycles in many reservoir hosts, and they have a wide diversity of successful antigenic types (1) (more than 40 serotypes on the basis of virus neutralization, e.g., no cross-protection between types). Their cup-like surface morphology is characteristic (Figure 2). Finally, they are zoonotic: this is a paradigm shift (7). No other virus has been shown to have its origins and primary reservoirs in the sea yet emerge to cause disease in humans.

To measure calicivirus adaptivity and preclude strong presumptions of host specificity on the basis of calicivirus type or species of origin, the following list of 16 hosts is given for a single virus serotype, SMSV-5: known natural hosts—five genera of seals, cattle, three genera of whales, donkeys, fox, and humans—and susceptible hosts—opaleye fish, horses, domestic swine, and primates (1). The lists are still growing. SMSV-5 can also persist for 60 days in shellfish, but infectivity has not been measured (26). Feline calicivirus (FCV-F9) has an apparent ocean presence among California sea lions and is not host-specific (2). All members of the family *Felidae* are susceptible to infection, not just domestic cats. In addition, cheetahs are susceptible; the agent has naturally infected and caused disease in dogs and experimentally infected coyotes (28,29). Reports of human antibody against the feline virus suggest zoonotic potential for the feline calicivirus (30).

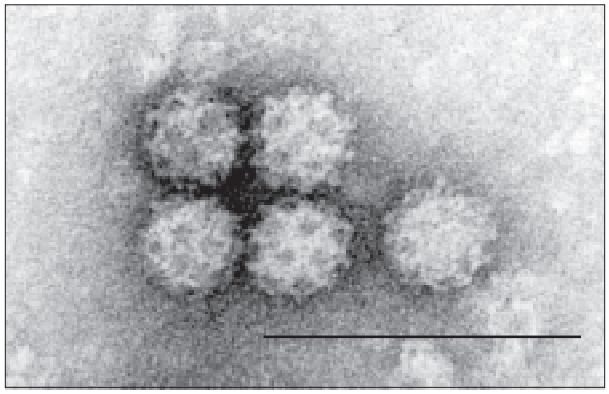

Figure 2. Electron photomicrograph of Cetacean Calicivirus Tursiops - 1 (CCVTur-1). Negative staining using phosphotungstic acid on a carbon-coated grid showing typical surface cup morphologic features as commonly seen by electron microscopy. Bar = 100 nm.

Tissue Trophisms

The broad host range and diverse mechanisms of transmission and survival of marine caliciviruses are expected of an RNA virus quasispecies (12). If structural simplicity associated with a capsid made up of a single protein species and replicative strategies conserved across rather broad tissue and phylogenetic distances is a measure, caliciviruses are primitive RNA viruses. Caliciviral RNA replicative mechanisms are thus expected to generate numerous mutants (perhaps as high as one to 10 per template copy (12), which will come in contact with many pelagic and terrestrial biota. Opportunity exists to form clusters of virus adapted across a diversity of life forms. Actual mutation rates have not been demonstrated for the *Caliciviridae,* but plaque-size reversion studies have found that the mutation rate for this phenomenon is one per 10^6 replicates (14,15). In addition, the expected versatility from RNA virus replicative infidelity and the resulting successful adaptive mechanisms are manifested in the wide spectrum of calicivirus tissue trophisms.

Disease conditions involving calicivirus tissue trophisms include blistering of the skin (particularly on the appendages and around the mouth), pneumonia, abortion, encephalitis, myocarditis, myositis, hepatitis, diarrhea, and coagulation/hemorrhage (1,3,5,7; Table 1). Caliciviruses have the inherent potential and adaptive mechanisms to successfully parasitize essentially all organ systems of the many animal species that have been examined in detail.

The Future

Calicivirus disease manifestations in animals will likely continue but will only become well defined with improved diagnostic means. With cultivatable marine caliciviruses as models, the role of disease-causing caliciviruses can be further defined. Now caliciviruses infecting humans can only be visualized by electron microscopy or histochemistry but cannot be propagated in vitro. Thus, miscarriage and birth defects in human patients, hepatitis other than

Table 1. Calicivirus tissue trophisms

Disease conditions	Species affected	Calicivirus group[a]
Skin blistering	Cattle, cats, dogs, humans, primates, seals, swine	VESV, SMSV, FCV, CCV
Pneumonia	Cats, cattle, swine	FCV, SMSV
Abortion	Seals, swine	VESV, SMSV
Encephalitis	Cats, primates, seals, swine	VESV, SMSV
Myocarditis	Seals, swine	VESV, SMSV
Hepatitis	Humans, rabbits, swine	VESV, RHDV, HEV
Diarrhea	Cattle, dogs, humans, reptiles, swine	VESV, SMSV, CCV, SRSV, Sapporo
Coagulation/ hemorrhage	Humans, rabbits, seals, swine	RHDV, VESV, HEV

[a]The family *Caliciviridae* has been tentatively divided into five groups, each proposed to be a genus. Group 1: Vesicular exanthema of swine (VESV), San Miguel sea lion virus (SMSV), Feline calicivirus (FCV), Canine calicivirus (CCV); Group 2: Sapporo calicivirus (Sapporo); Group 3: Rabbit hemorrhagic disease virus (RHDV); Group 4: Hepatitis E virus (HEV); Group 5: Small round structured virus (SRSV), which includes Norwalk virus.

types A through G, hand-foot-and-mouth–like diseases, viral myocarditis, viral encephalitis of unknown etiology, and joint and muscle disease, for example, should be examined for caliciviruses when other causes of disease are not found.

In the absence of data, extrapolating from cultivatable caliciviruses to predict future effects of poorly characterized caliciviruses should be useful, particularly when there is an urgent need to assess possible human risk. The calicivirus implicated in RHD is a case in point for it might be expected to infect humans.

Additional evidence exists. An anecdotal account mentions a Mexican worker who developed antibodies to RHD while eradicating the disease in Mexico (31). An Australian study designed to assess the risk for illness after RHD escaped from Wardang Island (32) examined a group of 269 persons (153 reporting exposure to rabbits or samples infected with RHD virus and 116 reporting no known RHD virus contact) from two Australian states with the greatest amount of RHD virus activity in rabbits. Exposure was categorized by degree of skin exposure to infected materials. Date of first exposure was noted, but no cumulative exposure index was developed. A "high" exposure category could derive from one exposure, and "low" exposure categories could include multiple exposures, each with low exposure. Symptoms were assessed by recall of illness over the previous 13 months. Because the RHD agent was in high security containment facilities for the first 3 months of the recall period and geographically confined for the following 3 months, that period was considered a low exposure period. Because of the rapid spread of the virus in the two states, the last 6 months of the recall period were considered the high exposure period.

The data (Table 2) show the rate ratios for the occurrence of different illness in the two periods. All rate ratios were considerably greater than 1.00, and the rate ratios for any illness, diarrhea/gastroenteritis, flu/fever, and neurologic illness are significant ($p < 0.005$). Because each group contained health histories for 3 spring months or 3 autumn months, 1 summer month, and 2 winter months, the data are seasonally adjusted; hence, winter illness does not explain the excess symptoms observed in the high exposure group, and RHD virus exposure remains a plausible explanation for increased disease incidence.

It is difficult to produce pure cultures of noncultivatable caliciviruses to carry out Koch's postulates and establish cause and effect for a single pathogen strain or species. For RHD, both a calicivirus and a parvovirus have been identified in ill rabbits, and a parvovirus has been isolated in vitro and reported to fulfill Koch's postulates (33-35). Yet, caliciviruses have been purified from infected organs to the limits of purity by physical means, and those preparations also cause RHD (35). The caliciviruses purified by physical means cannot be proven to be free of

Table 2. Population incidence of rabbit hemorrhagic disease (RHD) virus for seasonally equalized periods (July–December and February–July), derived from Mead et al. (32)

	Jul–Dec 1995	Feb–Jul 1996	Rate Ratio	95% Confidence Interval
Exposure to RHD virus	Low	High		
Any illness	112	210	1.88	1.49–2.36
Flu/fever	94	189	2.01	1.57–2.57
Diarrhea/ gastroenteritis	41	73	1.78	1.21–2.61
Neurologic symptoms	18	49	2.72	1.58–4.67
Rashes/skin	3	10	3.33	0.92–12.1
Bleeding/ hepatitis	2	4	2.00	0.18–22.1

contaminating agents, such as parvovirus (35). If RHD is parvovirus-driven, extrapolation from what is known of other small DNA viruses suggests a rather stable genome and a reduced host range with less likelihood of new host relationships (12). On the other hand, if calicivirus is the primary pathogen, the genomic infidelity that occurs during small RNA virus replication and the documented cross-species transmission of the cultivatable caliciviruses suggest that RHD might also move across species barriers (1,12).

Adequate diagnostic reagents for epidemiologic studies need to be made available; they include antigens, monoclonal antibodies, polymerase chain reaction primer sets, and cDNA probes based on group epitopes. In addition, biotype- or pathotype-specific reagents are needed to differentiate pathogenic from nonpathogenic infections.

The future also holds the confounding problem of vaccines. Although vaccines can be produced, because of calicivirus antigenic diversity, their efficacy would be predictably short-lived and marginal. Other approaches will need to be sought. Conserved traits that render the *Caliciviridae* viable as a virus with certain predictable genomic expressions must be sought, and if they exist, targeted for immune attack.

Conclusions

Only one of the five known calicivirus groups can be grown in vitro and subjected to the full range of host-parasite tests and conditions necessary to more fully define a virus in nature. Therefore, extrapolations developed from this group, the cultivatable marine caliciviruses, should provide insights as a predictive model to help answer questions for the noncultivatable caliciviruses such as small round structured virus, Sapporo virus, hepatitis E virus, and rabbit caliciviruses. From the replicative strategy of the *Caliciviridae* (as RNA viruses), one would predict considerable diversity. In vitro cultivation has shown that caliciviruses exhibit survivability and plasticity in nature. Many of the factors regarding host spectrum, zoonotic potential, disease conditions, transport, intermediate hosts, and abrupt appearance or disappearance, which may be unknown in newly emerging calicivirus diseases (e.g., RHD), may be more reliably predicted with an established model such as the cultivatable marine caliciviruses. New and better biologic tools for diagnostic and epidemiologic assessments must be developed. This should be augmented by recognizing the zoonotic potential of the cultivatable caliciviruses of ocean origin and then examining them as possible models to help solve many unanswered questions for pathogenic *Caliciviridae.*

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Outbreak Investigations—A Perspective

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Outbreak investigations, an important and challenging component of epidemiology and public health, can help identify the source of ongoing outbreaks and prevent additional cases. Even when an outbreak is over, a thorough epidemiologic and environmental investigation often can increase our knowledge of a given disease and prevent future outbreaks. Finally, outbreak investigations provide epidemiologic training and foster cooperation between the clinical and public health communities.

Investigations of acute infectious disease outbreaks are very common, and the results of such investigations are often published; however, surprisingly little has been written about the actual procedures followed during such investigations (1,2). Most epidemiologists and public health officials learn the procedures by conducting investigations with the initial assistance of more experienced colleagues. This article outlines the general approach to conducting an outbreak investigation. The approach applies not only to infectious disease outbreaks but also to outbreaks due to noninfectious causes (e.g., toxic exposure).

How Outbreaks Are Recognized

Possible outbreaks of disease come to the attention of public health officials in various ways. Often, an astute clinician, infection control nurse, or clinical laboratory worker first notices an unusual disease or an unusual number of cases of a disease and alerts public health officials. For example, staphylococcal toxic shock syndrome and eosinophilia myalgia syndrome were first noted by clinicians (3,4). Frequently, it is the patient (or someone close to the patient) who first suspects a problem, as is often the case in foodborne outbreaks after a shared meal and as was the case in the investigation of a cluster of cases of apparent juvenile rheumatoid arthritis near Lyme, Connecticut, which led to the discovery of Lyme disease (5). Review of routinely collected surveillance data can also detect outbreaks of known diseases, as in the case of hepatitis B infection among the patients of an oral surgeon in Connecticut and patients at a weight reduction clinic (6,7). The former outbreak was first suspected when routinely submitted communicable disease report forms for several patients from one small town indicated that all of the patients had recently had oral surgery. However, it is relatively uncommon for outbreaks to be detected in this way and even more uncommon for them to be detected in this way while they are still in progress. Finally, sometimes public health officials learn about outbreaks of disease from the local newspaper or television news.

Reasons for Investigating Outbreaks

The most compelling reason to investigate a recognized outbreak of disease is that exposure to the source(s) of infection may be continuing; by identifying and eliminating the source of infection, we can prevent additional cases. For example, if cans of mushrooms containing botulinum toxin are still on store shelves or in homes or restaurants, their recall and destruction can prevent further cases of botulism.

However, even if an outbreak is essentially over by the time the epidemiologic investigation begins—that is, if no one is being further exposed to the source of infection—investigating the outbreak may still be indicated for many reasons. Foremost is that the results of the investigation may lead to recommendations or strategies for preventing similar future outbreaks. For example, a Legionnaires' disease outbreak investigation may produce recommendations for grocery store misting machine use that may prevent other outbreaks (8). Other reasons for

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investigating outbreaks are the opportunity to 1) describe new diseases and learn more about known diseases; 2) evaluate existing prevention strategies, e.g., vaccines; 3) teach (and learn) epidemiology; and 4) address public concern about the outbreak.

Once a decision is made to investigate an outbreak, three types of activities are generally involved—the epidemiologic investigation; the environmental investigation; and the interaction with the public, the press, and, in many instances, the legal system. While these activities often occur simultaneously throughout the investigation, it is conceptually easier to consider each of them separately.

Epidemiologic Investigation

Outbreak investigations are, in theory, indistinguishable from other epidemiologic investigations; however, outbreak investigations encounter more constraints. 1) If the outbreak is ongoing at the time of the investigation, there is great urgency to find the source and prevent additional cases. 2) Because outbreak investigations frequently are public, there is substantial pressure to conclude them rapidly, particularly if the outbreak is ongoing. 3) In many outbreaks, the number of cases available for study is limited; therefore, the statistical power of the investigation is limited. 4) Early media reports concerning the outbreak may bias the responses of persons subsequently interviewed. 5) Because of legal liability and the financial interests of persons and institutions involved, there is pressure to conclude the investigation quickly, which may lead to hasty decisions regarding the source of the outbreak. 6) If detection of the outbreak is delayed, useful clinical and environmental samples may be very difficult or impossible to obtain.

Outbreak investigations have essential components as follows: 1) establish case definition(s); 2) confirm that cases are "real"; 3) establish the background rate of disease; 4) find cases, decide if there is an outbreak, define scope of the outbreak; 5) examine the descriptive epidemiologic features of the cases; 6) generate hypotheses; 7) test hypotheses; 8) collect and test environmental samples; 9) implement control measures; and 10) interact with the press, inform the public. While the first seven components are listed in logical order, in most outbreak investigations, many occur more or less simultaneously. The importance of these components may vary depending on the circumstances of a specific outbreak.

Case Definition

In some outbreaks, formulating the case definition(s) and exclusion criteria is straightforward; for example, in an outbreak of gastroenteritis caused by Salmonella infection, a laboratory-confirmed case would be defined as a culture-confirmed infection with Salmonella or perhaps with Salmonella of the particular serotype causing the outbreak, while a clinical case definition might be new onset of diarrhea. In other outbreaks, the case definition and exclusion criteria are complex, particularly if the disease is new and the range of clinical manifestations is unknown (e.g., in a putative outbreak of chronic fatigue syndrome). In many outbreak investigations, multiple case definitions are used (e.g., laboratory-confirmed case vs. clinical case; definite vs. probable vs. possible case; outbreak-associated case vs. nonoutbreak-associated case, primary case vs. secondary case) and the resulting data are analyzed by using different case definitions. When the number of cases available for study is not a limiting factor and a case-control study is being used to examine risk factors for becoming a case, a strict case definition is often preferable to increase specificity and reduce misclassification of disease status (i.e., reduce the chance of including cases of unrelated illness or no illness as outbreak-related cases).

Case Confirmation

In certain outbreaks, clinical findings in reported cases should be reviewed closely, either directly, by examining the patients, or indirectly, by detailed review of the medical records and discussion with the attending health-care provider(s), especially when a new disease appears to be emerging (e.g., in the early investigations of Legionnaires' disease, AIDS, eosinophilia myalgia syndrome, and hantavirus pulmonary syndrome) (4,9-11). Clinical findings should also be examined closely when some or all of the observed cases may be factitious, perhaps because of laboratory error (12); a discrepancy between the clinical and laboratory findings generally exists, which may be discernible only by a detailed review of the clinical findings.

Establishing the Background Rate of Disease and Finding Cases

Once it is clear that a suspected outbreak is not the result of laboratory error, a set of activities should be undertaken to establish the

background rate of the disease in the affected population and to find all the cases in a given population in a certain period. This set of activities should prove that the observed number of cases truly is in excess of the "usual" number (i.e., that an outbreak has occurred), define the scope of the outbreak geographically and temporally, find cases to describe the epidemiologic features of those affected and to include them in analytic epidemiologic studies (see below) or, most often, accomplish a combination of these goals.

When hundreds of acute onset diarrhea cases are suddenly seen daily in a single outpatient setting (10), an outbreak is clearly occurring. On the other hand, when too many hospitalized patients are dying unexpectedly of cardiac arrest (13) or the number of cases of listeriosis in a given county in recent months is moderately elevated, it may be necessary to establish the background rates in the population to determine whether an outbreak is occurring. In such situations, the period and geographic areas involved would provide the most useful baseline data, keeping in mind that the labor and time required to collect such information is often directly proportional to the length of the period and the size of the geographic area selected. Because disease incidence normally fluctuates by season, data from comparable seasons in earlier years should be included.

Establishing the background rate of a disease is generally more straightforward if confirmatory tests are available than if laboratory tests are unavailable or infrequently used. The rate of certain invasive bacterial infections (e.g., listeriosis and meningococcal infections) in a given area can be easily documented by reviewing the records of hospital clinical microbiology laboratories; however, cases for which specimens were not submitted to these laboratories for testing will go undetected. When a disease is less frequently laboratory-confirmed because health-care providers may not have considered the diagnosis or ordered the appropriate laboratory tests (e.g., for Legionnaires' disease), establishing the background rate of disease in a community or a hospital suspected of having an outbreak generally requires alternative case-finding strategies and is almost invariably more labor intensive. In an outbreak of a new disease, substantial effort is often necessary to determine whether or not cases of that disease had been occurring but had gone unrecognized.

Once data concerning the background rate of a disease (including case-finding for the current period) have been collected, it is generally possible to determine whether or not an outbreak is occurring or has occurred, although in some situations it may remain unclear whether or not the number of cases observed exceeds the background rate. In part, the problem may relate to how an outbreak is defined. To paraphrase a U.S. Supreme Court justice speaking about pornography, "I can't define an outbreak, but I know one when I see one." Thus, it may be difficult to detect and prove the existence of small outbreaks, but large ones are self-evident.

An outbreak can also be difficult to identify when during the period under study changes occur in the care-seeking behavior and access to care of patients; the level of suspicion, referral patterns, and test-ordering practices of health-care providers; the diagnostic tests and other procedures used by laboratories; and the prevalence of underlying immunosuppressive conditions or other host factors in the population. All these factors, which can affect the apparent incidence of a disease and produce artifactual changes perceived as increases (or decreases) in the actual incidence, need to be considered when interpreting the findings.

Descriptive Epidemiology

By collecting patient data, the case-finding activities provide extremely important information concerning the descriptive epidemiologic features of the outbreak. By reviewing and plotting on an "epidemic curve" the times of onset of the cases and by examining the characteristics (e.g., age, sex, race/ethnicity, residence, occupation, recent travel, or attendance at events) of the ill persons, investigators can often generate hypotheses concerning the cause(s)/source(s) of the outbreak. While linking the sudden onset of gastroenteritis among scores of persons who attended a church supper to the single common meal they shared is generally not a challenge, an otherwise cryptic source can be at least hinted at by the descriptive epidemiologic features of the cases involved. For example, in a particularly perplexing outbreak of *Salmonella* Muenchen infections ultimately traced to contaminated marijuana, the age distribution of the affected persons and of their households was markedly different from that typically seen for salmonellosis (14). Or, similarly, in the outbreak of

legionellosis due to contaminated misting machines in the produce section of a grocery store, before the link to this exposure was even suspected, it was noted that women constituted a substantially higher proportion of the cases usually seen with this disease (5). The shape of the epidemic curve can also be very instructive, suggesting a point-source epidemic, ongoing transmission, or a combination of the two.

Generating a Hypothesis

The source(s) and route(s) of exposure must be determined to understand why an outbreak occurred, how to prevent similar outbreaks in the future, and, if the outbreak is ongoing, how to prevent others from being exposed to the source(s) of infection. In some outbreaks, the source and route are obvious to those involved in the outbreak and to the investigators. However, even when the source of exposure appears obvious at the outset, a modicum of skepticism should be retained because the obvious answer is not invariably correct. For example, in an outbreak of nosocomial legionellosis in Rhode Island, the results of an earlier investigation into a small number of hospital-acquired cases at the same hospital had demonstrated that *Legionella pneumophila* was in the hospital potable water supply, and a sudden increase in new cases was strongly believed to be related to the potable water (15). However, a detailed epidemiologic investigation implicated a new cooling tower at the hospital as the source of the second outbreak.

While the true source of exposure, or at least a relatively short list of possibilities, is apparent in many outbreaks, this is not the case in the more challenging outbreaks. In these instances, hypotheses concerning the source/route of exposure can be generated in a number of ways beyond a detailed review of the descriptive epidemiologic findings. A review of existing epidemiologic, microbiologic, and veterinary data is very useful for learning about known and suspected sources of previous outbreaks or sporadic cases of a given infection or disease, as well as the ecologic niche of an infectious agent. Thus, in an outbreak of invasive *Streptococcus zooepidemicus* infections in New Mexico due to consumption of soft cheese made from contaminated raw milk, the investigation focused on exposure to dairy products and animals because of previous microbiologic and veterinary studies (16).

A review of existing data generally only helps confirm what is already known about a particular disease and is far less helpful in identifying totally new and unsuspected sources or routes of infection (i.e., marijuana as a source of Salmonella). When neither review of the descriptive epidemiologic features of the cases nor review of existing scientific information yields the correct hypothesis, other methods can be used to generate hypotheses about what the patients have in common. Open-ended interviews of those infected (or their surrogates) are one such method in which investigators try to identify all possibly relevant exposures (e.g., a list of all foods consumed) during a given period. For example, in an investigation of *Yersinia enterocolitica* infections in young children in Belgium, open-ended interviews of the mothers of some of the ill children showed that many gave their children raw pork sausage as a weaning food, providing the first clue as to the source of these infections (17). Similarly, in two outbreaks of foodborne listeriosis, a variant of this process led to the identification of the source of the outbreak. In one of these outbreaks, a search of the refrigerator of one of the case-patients who, as a visitor to the area, had had very limited exposure to foods there, suggested cole slaw as a possible vehicle of infection (18). In the other outbreak, an initial case-control study found no differences between cases and controls regarding exposure to a number of specific food items but showed that case households were more likely than control households to buy their food at a particular foodstore chain. To generate a list of other possible food sources of infection, investigators shopped with persons who did the shopping for case households and compiled a list of foods purchased at that foodstore chain that had not been reported in the previous study. This approach implicated pasteurized milk from that chain as the source of the outbreak (19).

In some particularly perplexing outbreaks, bringing together a subset of the patients to discuss their experiences and exposures in a way that may reveal unidentified links can be useful.

Testing the Hypothesis

Whether a hypothesis explaining the occurrence of an outbreak is easy or difficult to generate, an analytic epidemiologic study to test the proposed hypothesis should be considered. While in many instances a case-control study is used, other designs, including retrospective cohort and cross-sectional studies, can be equally or more

appropriate. The goal of all these studies is to assess the relationship between a given exposure and the disease under study. Thus, each exposure of interest (e.g., each of the meals eaten together by passengers on a cruise ship and each of the foods and beverages served at those meals) constitutes a separate hypothesis to be tested in the analytic study. In outbreaks where generating the correct hypothesis is difficult, multiple analytic studies, with additional hypothesis-generating activities in between, are sometimes needed before the correct hypothesis is formed and tested (19).

In interpreting the results of such analytic studies, one must consider the possibility that "statistically significant" associations between one or more exposures and the disease may be chance findings, not indicative of a true relationship. By definition, any "statistically significant" association may have occurred by chance. (When the standard cut point of $p < 0.05$ is used, this occurs 5% of the time.) Because many analytic epidemiologic studies of outbreaks involve testing many hypotheses, the problem of "multiple comparisons" arises often.

While there are statistical methods for adjusting for multiple comparisons, when and even whether to use them is controversial. At a minimum, it is important to go beyond the statistical tests and examine the magnitude of the effect observed between exposure and disease (e.g., the odds ratio, relative risk) and the 95% confidence intervals, as well as biologic plausibility in deciding whether or not a given "statistically significant" relationship is likely to be biologically meaningful. Evidence of a dose-response effect between a given exposure and illness (i.e., the greater the exposure, the greater the risk for illness) makes a causal relationship between exposure and disease more likely. Whether the time interval between a given exposure and onset of illness is consistent with what is known about the incubation period of the disease under study must also be assessed. When illness is "statistically significantly" related to more than one exposure (e.g., to eating each of several foods at a common meal), it is important to determine whether multiple sources of infection (perhaps due to cross-contamination) are plausible and whether some of the noted associations are due to confounding (e.g., exposure to one potential source is linked to exposure to other sources) or to chance.

When trying to decide if a "statistically significant" exposure is the source of an outbreak, it is important to consider what proportion of the cases can be accounted for by that exposure. One or more of the patients may be classified as "nonexposed" for various reasons: incorrect information concerning exposure status (due to poor memory, language barriers); multiple sources of exposure or routes of transmission (perhaps due to cross-contamination); secondary person-to-person transmission that followed a common source exposure; or patients without the suspected exposure, representing background cases of the disease unrelated to the outbreak. The plausibility of each of these explanations varies by outbreak. While there is no cutoff point above or below which the proportion of exposed case-patients should fall before an exposure is thought to account for an outbreak, the lower this proportion, the less likely the exposure is, by itself, the source.

Other possibilities need to be considered when the analytic epidemiologic study finds no association between the hypothesized exposures and risk for disease. The most obvious possibility is that the real exposure was not among those examined, and additional hypotheses should be generated. However, other possibilities should also be considered, particularly when the setting of the outbreak makes this first explanation unlikely (e.g., when it is known that those involved in the outbreak shared only a single exposure or set of exposures, such as eating a single common meal). Two other explanations for failing to find a "statistically significant" link between one or more exposures and risk for illness also need to be considered—the number of persons available for study and the accuracy of the available information concerning the exposures. Thus, if the outbreak involves only a small number of cases (and non-ill persons), the statistical power of the analytic study to find a true difference in exposure between the ill and the non-ill (or a difference in the rate of disease among the exposed and the unexposed) is very limited. If the persons involved in the outbreak do not provide accurate information about their exposure to suspected sources or vehicles of infection because of lack of knowledge, poor memory, language difficulty, mental impairment, or other reasons, the resulting misclassification of exposure status also can

prevent the epidemiologic study from implicating the source of infection. Studies have documented that even under ideal circumstances, memory concerning such exposures is faulty (20). However, given the usually enormous differences in rates of disease between those exposed and those not exposed to the source of the outbreak, even small studies or studies with substantial misclassification of exposure can still correctly identify the source.

Environmental Investigation

Samples of foods and beverages served at a common meal believed to be the source of an outbreak of gastroenteritis or samples of the water or drift from a cooling tower believed to be the source of an outbreak of Legionnaires' disease can support epidemiologic findings. In the best scenario, the findings of the epidemiologic investigation would guide the collection and testing of environmental samples. However, environmental specimens often need to be obtained as soon as possible, either before they are no longer available, as in the case of residual food from a common meal, or before environmental interventions are implemented, as in the case of treating a cooling tower to eradicate Legionella. Because laboratory testing of environmental samples is often expensive and labor-intensive, it is sometimes reasonable to collect and store many samples but test only a limited number. Collaborating with a sanitarian, environmental engineer, or other professional during an environmental inspection or collection of specimens is always beneficial.

While finding or not finding the causative organism in environmental samples is often perceived by the public, the media, and the courts as powerful evidence implicating or exonerating an environmental source, either positive or negative findings can be misleading for several reasons. For example, finding Legionella in a hospital potable water system does not prove that the potable water (rather than a cooling tower or some other source) is responsible for an outbreak of Legionnaires' disease (21). Similarly, not finding the causative organism in an environmental sample does not conclusively rule out a source as the cause of the problem, in part because the samples obtained and tested may not represent the source (e.g., because of error in collecting the specimens, intervening changes in the environmental source) and in part because the samples may have been mishandled. Furthermore, in some outbreaks caused by well-characterized etiologic agents, laboratory methods of detecting the agent in environmental samples are insensitive, technically difficult, or not available, as in the case of recent outbreaks of *Cyclospora* infections associated with eating imported berries (22,23).

Control Measures

Central to any outbreak investigation is the timely implementation of appropriate control measures to minimize further illness and death. At best, the implementation of control measures would be guided by the results of the epidemiologic investigation and possibly (when appropriate) the testing of environmental specimens. However, this approach may delay prevention of further exposure to a suspected source of the outbreak and is, therefore, unacceptable from a public health perspective. Because the recall of a food product, the closing of a restaurant, or similar interventions can have profound economic and legal implications for an institution, a manufacturer or owner, and the employees of the establishments involved, acting precipitously can also have substantial negative effects. The recent attribution of an outbreak of *Cyclospora* infections to strawberries from California demonstrates the economic impact that can result from releasing and acting on incorrect information (22,23). Thus, the timing and nature of control measures are difficult. Balancing the responsibility to prevent further disease with the need to protect the credibility and reputation of an institution is very challenging.

Interactions with the Public and Press

While the public and the press are not aware of most outbreak investigations, media attention and public concern become part of some investigations. Throughout the course of an outbreak investigation, the need to share information with public officials, the press, the public, and the population affected by the outbreak must be assessed. While press, radio, and television reports can at times be inaccurate, overall the media can be a powerful means of sharing information about an investigation with the public and disseminating timely information about product recalls.

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Genetic Diversity of Wild-Type Measles Viruses: Implications for Global Measles Elimination Programs

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Wild-type measles viruses have been divided into distinct genetic groups according to the nucleotide sequences of their hemagglutinin and nucleoprotein genes. Most genetic groups have worldwide distribution; however, at least two of the groups appear to have a more limited circulation. To monitor the transmission pathways of measles virus, we observed the geographic distribution of genetic groups, as well as changes in them in a particular region over time. We found evidence of interruption of indigenous transmission of measles in the United States after 1993 and identified the sources of imported virus associated with cases and outbreaks after 1993. The pattern of measles genetic groups provided a means to describe measles outbreaks and assess the extent of virus circulation in a given area. We expect that molecular epidemiologic studies will become a powerful tool for evaluating strategies to control, eliminate, and eventually eradicate measles.

Until the advent of a live-attenuated vaccine in the early 1960s, measles was an epidemic disease worldwide. Today many countries have controlled measles, but the disease remains endemic on most continents. Development of a live-attenuated measles vaccine and implementation of laws that required proof of vaccination upon school entry dramatically reduced the incidence of measles in the United States. The number of reported cases plummeted from approximately 500,000 before vaccine introduction in 1963 to fewer than 1,500 in 1983. Despite these measures, a reemergence or resurgence of measles in the United States from 1989 to 1991 resulted in more than 55,000 cases of measles and approximately 120 measles-associated deaths (Figure 1; 1). In exploring the reasons for the resurgence, our laboratory genetically characterized measles viruses isolated from wild-type virus-infected persons from the same outbreak or temporally and geographically distinct outbreaks in the United States; in the regions examined, all measles viruses isolated during the period of resurgence were almost identical in nucleotide sequence and genetically distinct from vaccine strains (2).

Measles isolates from many regions of the world have been characterized in parallel studies by our laboratory and by others. In conjunction with classic epidemiologic investigations, these well-characterized viruses have formed a picture of the distribution of wild-type measles viruses in most areas of the world. Eight distinct genotypes have been identified, and undoubtedly more will be added. Some are localized to specific regions, while most are widely distributed. The assembly

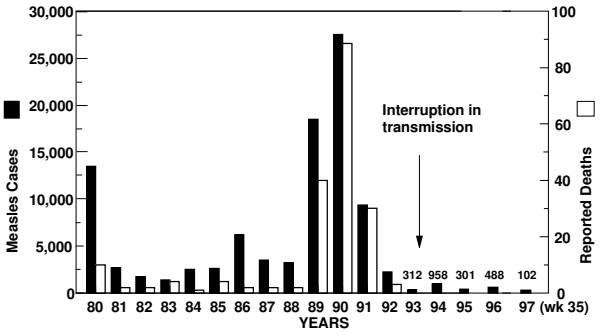

Figure 1. Incidence of U.S. measles cases and measles deaths between 1980 and 1997. Total number of measles cases for years 1993-1997 (week 35) are indicated above each bar.

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of these sequences into a large database, which includes their geographic distribution, has become a new means by which measles transmission pathways can be traced and control measures can be assessed. This "molecular epidemiology" has affected the U.S. measles elimination program, and if used appropriately with standard epidemiologic methods, it will affect global measles elimination and eradication. This article summarizes the status of the known measles genotype distribution throughout the world and describes how molecular epidemiologic information has been used to assess the effectiveness of measles elimination in the United States.

Global Distribution of Measles Genotypes

In most cases, genetic characterization of wild-type measles viruses has been conducted by sequencing the genes coding for the hemagglutinin (H) protein or the nucleoprotein (N). Of the six genes on the viral genome, the H and N genes are the most variable. Over their protein coding regions, the H and N genes vary by up to 7% at the nucleotide level. The single most variable part of the measles genome is the 450 nucleotides that code for the COOH-terminus of the N protein, where nucleotide variability between various wild-type viruses can approach 12%. Several laboratories have analyzed the sequences of wild-type measles viruses and assigned the viruses to various genetic groups (2-14). At present, no widely accepted standard is available for describing genetic groups of measles virus. However, with a few exceptions, the assignment of viruses to a particular group has been consistent between laboratories (10).

Many of our studies have focused on the genetic characterization of measles viruses associated with cases and outbreaks in the United States during the last 10 years (2,11). These viruses can be separated into at least eight distinct genetic groups (Table; Figure 2). Phylogenetic analyses using various computer programs (15-17) indicated good statistical support for each of the groups described below. Actually, more than eight genetic groups are listed when viruses from groups not yet found in the United States are included (e.g., Zambia: 1993, Germany: 1992) (Figure 2). The number of genetic groups is likely to increase since the true extent of genetic heterogeneity among wild-type measles viruses is still unknown, and virologic surveillance has not been conducted or has only just begun in many areas of the world.

Table. Sources of genotypes isolated in the United States, 1995–1997

Group	No. of isolates	Imports from	Also circulating in
4	13	Germany, Spain, United Kingdom, Brazil, Austria, Italy, Greece, Ukraine	Central Europe, Canada, Brazil, United Kingdom
3	9	Japan	Japan, Thailand
5	8	Italy, Germany	Central Europe, United Kingdom, Brazil
7	3	Pakistan, Kenya	South Africa, Canada
8	2	China, Vietnam	China
2	1	Philippines	United States 1989–1992, Micronesia
1	1	Unknown	United Kingdom, Russia, China, Argentina
6	1	Kenya	The Gambia, Cameroon, Gabon, Zambia

Group 1 contains the prototype, Edmonston strain, which was isolated in 1954. This group also contains all vaccine viruses sequenced regardless of whether they were derived from Edmonston (Attenuvax, Edmonston-Zagreb, AIKC, Schwarz) or from temporally and geographically independent wild-type isolates (Shanghai-191: China, Changchun-47: China, CAM-70: Japan, Leningrad-16: Russia) (18). Relatively few wild-type viruses from the prevaccine era are available for molecular characterization. These viruses, which were isolated in Japan, Russia, Finland, Romania, and the United States during the 1950s and 1960s, are in group 1 (9). Therefore, while viruses belonging to the other genetic groups may have been present, group 1 viruses must have had widespread distribution during the prevaccine era. Group 1 viruses continue to circulate, and viruses from group 1 were isolated from patients with clinical measles in the United States, United Kingdom, Russia, China, and Argentina during the last 7 years (5,11,19,20, and unpub. observations). These recent group 1 wild-type

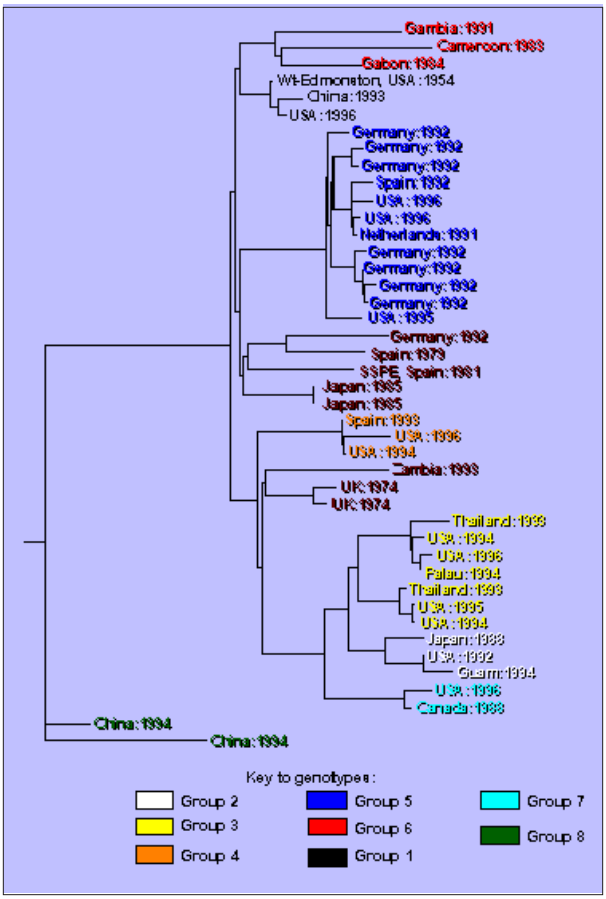

Figure 2. Phylogenetic tree showing genetic relationships between the eight genetic groups of measles virus associated with U.S. outbreaks and cases since 1988. The location and year of isolation is given for each virus. Viruses not assigned to one of the eight groups are labeled in brown. The unrooted tree is based on the sequence of the protein coding region of the H gene (1854 nt). Wt-Edmonston = low passage seed of the original Edmonston isolate. SSPE = sequences obtained from cases of subacute sclerosing panencephalitis.

viruses have several nucleotide substitutions that distinguish them from vaccine viruses. In contrast, measles vaccine viruses reisolated from immunosuppressed patients with giant cell pneumonia had nucleotide sequences nearly identical to those of the vaccine virus found in the vaccine vial (unpub. observations). This suggests that vaccine viruses are very stable even after prolonged replication in a human host. Therefore, it is unlikely that the group 1 wild-type viruses represent laboratory contamination of cultures with vaccine virus or reisolation of vaccine virus from recently vaccinated persons. Sequence studies have failed to identify a distinct set of genetic markers that consistently differentiate wild-type and presumably virulent viruses from attenuated viruses. Current studies are focusing on the analysis of the noncoding regions of the viral genome. More studies are needed to compare attenuated strains with their more virulent or reactogenic precursors. The recent development of an infectious clone for measles (21) will, no doubt, contribute to those studies.

Group 2 viruses were associated with the resurgence of measles in the United States between 1989 to 1991, an epidemic that had an unusually high incidence of deaths and hospitalizations (Figure 1). The circulation of group 2 viruses within the United States was interrupted in 1993, and this will be described in more detail below. Among these viruses, the Illinois-1 (Chicago-1) strain has become a representative of recent wild-type viruses, and almost the entire genome has been sequenced. Group 2 viruses were first isolated in Japan during the early 1980s; more recently, they were isolated in Japan, the Philippines, and Micronesia (2,11-13,22).

The group referred to as group 3 viruses can actually be divided into two distinct groups with a common geographic distribution. These viruses have been isolated from outbreaks in Japan and Thailand and from sporadic cases following importation into North America and Europe (2,11). Although virus from groups 2 and 3 cocirculated in Japan during the late 1980s and early 1990s, the group 3 viruses have recently become the predominant genotype (23).

Groups 4 and 5 appear to be circulating widely in many countries in western Europe, particularly Germany, Spain, and the United Kingdom, where virologic surveillance has been conducted (6,7,24). Viruses from this group are also circulating in France, Italy, Austria, and Greece since they have been associated with multiple importations from these areas into the United States (2,11; Table).

All representatives of the group 6 viruses have been isolated in the African countries of The Gambia (4), Cameroon, Gabon, and Zambia or associated with importations into the United States from Kenya. There is more genetic variability within the group 6 viruses than among most of the other genetic groups, yet all group 6 viruses contain a subset of nucleotide substitutions that places them on this African lineage.

Relatively few viruses from central Africa, where most measles infections are occurring, have been isolated for genetic analysis, and it will be interesting to determine if other genotypes are also present in this area.

Group 7 viruses were first isolated during an epidemic in Montreal, Canada, in 1988 (2,11). Group 7 was the predominant group among a number of recently isolated viruses from Johannesburg, South Africa (9,25,26). The identification of a group 7 virus in association with an importation to the United States from Pakistan suggests (11) that the viruses in this group may be circulating widely in Africa and Asia.

The group 8 viruses form a highly distinct group isolated in four provinces within the People's Republic of China during the early 1990s (19). Like group 6, group 8 viruses have more nucleotide variability within the group (up to 3%) than the other groups. Recent evidence also suggests that group 8 viruses are circulating in other parts of China (Hong Kong) and in Vietnam.

Several recently isolated viruses do not fit into the eight genetic groups that, so far, contain most recent isolates (Figure 2). Some outliers represent single isolations of a unique genetic type. However, preliminary analysis of a number of wild-type viruses from Zambia indicates that these viruses belong to a genetic group that is distinct from the eight groups described thus far (unpub. observations).

If viruses isolated during the early to mid-1980s were included into the genetic analysis (not shown), it would be apparent that more genetic groups exist. However, viruses representing these groups have not been isolated in the last 10 to 15 years, and it must be assumed that these groups are circulating in restricted geographic regions, are circulating at such a low frequency as to escape surveillance, or are extinct.

A summary of genetic groups (Figure 3) represents a static picture that simply identifies where particular genetic groups have been isolated, with no accounting for frequency of isolation or source of the virus. Certain regions of the world (including much of Africa and most of southern Asia) are still vastly underrepresented. A survey of recent Australian isolates is in progress. The pattern of genotypes in the United Kingdom and the United States is very complex because of relatively good strain surveillance and the frequency of international travel to these locations.

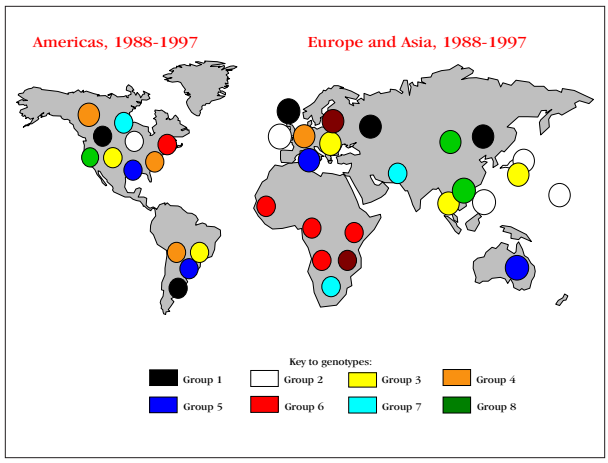

Figure 3. Global distribution of measles genetic groups. Colored circles indicate areas where measles viruses from various genetic groups have been isolated. Viruses not assigned to one of the eight groups are labeled in brown.

Molecular Epidemiology

Measles has long been considered one of the most communicable of diseases. The resurgence of disease from 1989 to 1991 in the United States (Figure 1) provides a good example of the rapid transmissibility of the virus. During this resurgence only group 2 viruses were isolated, and the sequences from these viruses were highly related (2). With continued molecular surveillance, we were able to document the interruption of transmission of the group 2 viruses and monitor the change in measles genotypes associated with outbreaks and sporadic cases from 1994 to the present. Molecular surveillance of measles viruses was most useful when the change in genotypes was observed over time. Without that information, it would not have been possible to describe the transition from an apparently "indigenous" lineage to importation of multiple lineages (Figure 4). This is in contrast to the situation in South and Central America. In these areas, viral surveillance was not conducted before mass vaccination campaigns were initiated, so the identity of the prevailing genotype could not be determined. Therefore, it is difficult to interpret the genetic data obtained from viruses currently causing outbreaks in these regions.

The molecular surveillance of wild-type viruses in the United States between 1989 and

1997 provides the best example of dynamic molecular surveillance (Figure 4). Viruses isolated over a 4-year period from major outbreaks in New York, Philadelphia, Chicago, Los Angeles, Houston, and southern Texas varied by less than 0.4% at the nucleotide level in the H and N genes (2). Analysis of the few wild-type measles viruses isolated in the United States before 1988 indicates lineages other than group 2. This suggests that the group 2 viruses were probably imported during the late 1980s and were rapidly transmitted to the entire country. While numerous importations occurred during the resurgence, apparently these viruses did not circulate widely enough to be detected by molecular surveillance. Perhaps the number of measles-susceptible persons in the U.S. population during the resurgence was high enough to sustain continuous transmission without accumulation of variants or displacement by other imported viruses.

More aggressive childhood vaccination programs, the introduction of a two-dose schedule, and successful mass vaccination campaigns conducted by the Pan American Health Organization in South and Central America greatly reduced the number of reported measles cases in the United States in 1993 (Figure 1). During a 6-week period at the end of 1993, no indigenous cases of measles were reported (27). Molecular surveillance of measles viruses associated with cases and outbreaks in the United States during 1994, 1995, 1996, and 1997 documented this interruption of transmission of what had been the indigenous genotype (2,11). Only one group 2 virus was detected in the United States after 1993, and this was directly linked to importation from the Philippines (11).

Molecular surveillance data allow us to draw several conclusions about the transmission of measles virus in the United States. The first is that increasing the level of population immunity by vaccination can interrupt the transmission of measles virus. This is hardly new information, and interruption of transmission was described for The Gambia in 1983 and more recently in

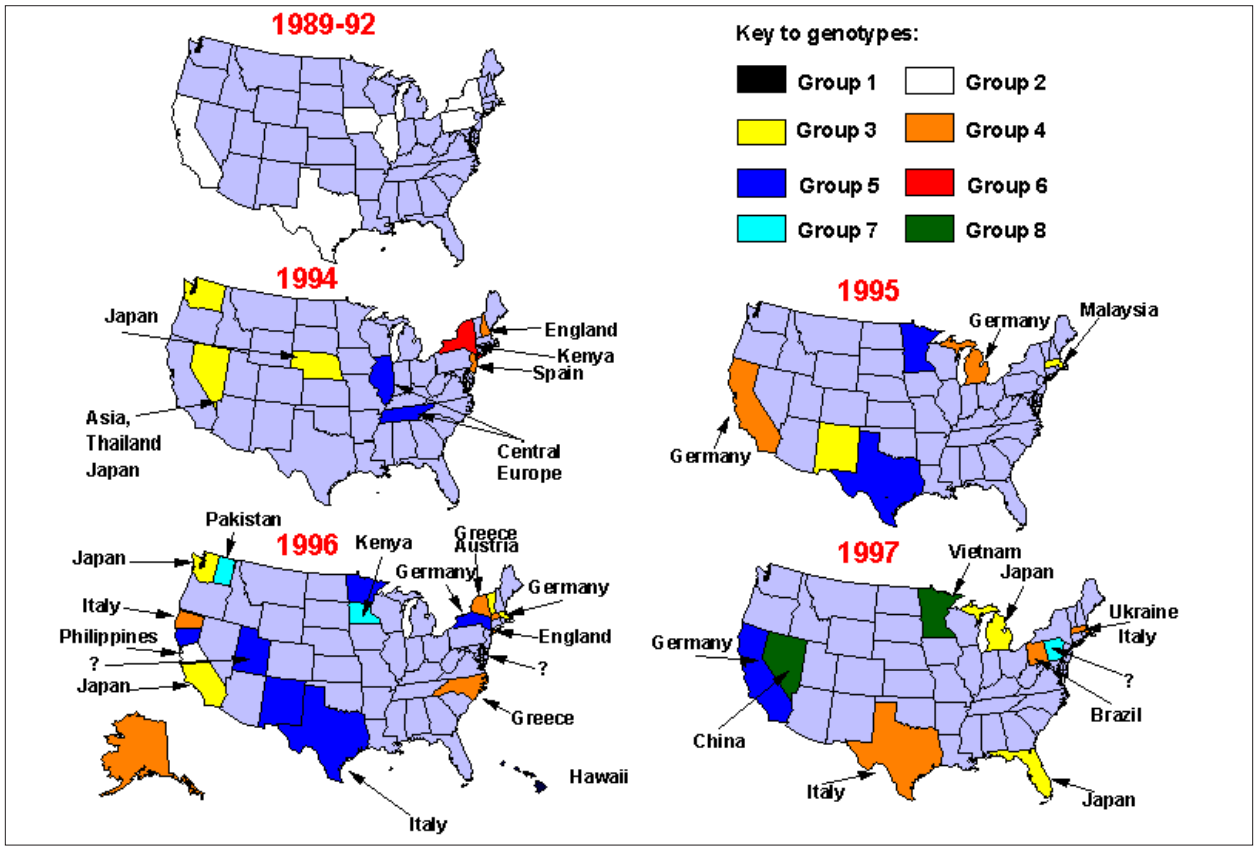

Figure 4. Change in genetic groups of measles viruses associated with U.S. cases and outbreaks between 1988 and September 1997. Arrows indicate sources of virus, if known.

Finland. However, our studies are the first in which genetic analysis of measles strains has been used to document interruption of transmission. Secondly, long-term asymptomatic transmission of virus is unlikely since no group 2 viruses were detected in the United States after 1993 that were not directly linked to importation. Finally, measles will not be fully controlled anywhere until it is controlled globally. Virus introduced by importation will continue to fuel sporadic outbreaks and epidemics even in areas with relatively good control measures. These observations should strengthen our resolve to accelerate measles control activities on a global level.

The molecular data imply that under conditions of continuous indigenous transmission of measles virus, the number of circulating genotypes is limited. As population immunity increases, the pattern of genotypes becomes more complex to reflect the multiple sources of imported virus. We hope to test this model further by conducting molecular surveillance of wild-type measles viruses circulating in areas that still have endemic measles.

Conclusions

Genetic characterization of wild-type measles viruses provides a valuable means to measure the level of virus circulation in areas just beginning to implement measles control plans. In areas that already achieved good measles control, molecular epidemiologic studies provide a means to describe outbreaks and cases. Identifying the source of the virus can lead to improved control measures. To be maximally effective, molecular epidemiologic studies must include surveys of viral genetic groups from all areas of the world. Specimens for viral isolations should be obtained from as many chains of transmission as possible. Obtaining specimens must become an integral part of measles surveillance and be included in the standard operating procedures for investigating measles cases. If we can establish a large database to describe the indigenous genetic groups before large-scale control measures are enacted, we can closely monitor the ability of these control measures to reduce or interrupt transmission of measles. Molecular epidemiology will greatly enhance measles elimination and eradication efforts.

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Diversity among Multidrug-Resistant Enterococci

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Enterococci are associated with both community- and hospital-acquired infections. Even though they do not cause severe systemic inflammatory responses, such as septic shock, enterococci present a therapeutic challenge because of their resistance to a vast array of antimicrobial drugs, including cell-wall active agents, all commercially available aminoglycosides, penicillin and ampicillin, and vancomycin. The combination of the latter two occurs disproportionately in strains resistant to many other antimicrobial drugs. The propensity of enterococci to acquire resistance may relate to their ability to participate in various forms of conjugation, which can result in the spread of genes as part of conjugative transposons, pheromone-responsive plasmids, or broad host-range plasmids. Enterococcal hardiness likely adds to resistance by facilitating survival in the environment of a multidrug-resistant clone, thus enhancing potential spread from person to person. The combination of these attributes within the genus *Enterococcus* suggests that these bacteria and their resistance to antimicrobial drugs will continue to pose a challenge.

Enterococci, which have been known as a cause of infective endocarditis for close to a century, more recently have been recognized as a cause of nosocomial infection and "superinfection" in patients receiving antimicrobial agents (1). The enterococcus is now receiving increased attention because of its resistance to multiple antimicrobial drugs, which probably explains a large part of its prominence in nosocomial infections. The most common enterococci-associated nosocomial infections are infections of the urinary tract, followed by surgical wound infections and bacteremia (1-3). Enterococci are often present in intraabdominal and pelvic infections, although not all patients with such infections require specific antienterococcal therapy. Other enterococcal infections include infections (including meningitis and bacteremia) in very ill neonates; central nervous system infections in adults, typically with a history of central nervous system surgery or intrathecal chemotherapy; and rarely, osteomyelitis and pulmonary infections. Enterococci frequently arise from colonization of indwelling T tubes, causing liver or biliary infection in liver transplant patients (1).

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Antimicrobial Resistance

Most enterococci have naturally occurring or inherent resistance to various drugs, including cephalosporins and the semisynthetic penicillinase-resistant penicillins (e.g., oxacillin) and clinically achievable concentrations of clindamycin and aminoglycosides. Compared with streptococci, most enterococci are relatively resistant to penicillin, ampicillin, and the ureidopenicillins, with MICs of 1 µg/ml to 8 µg/ml for most *Enterococcus faecalis* and even higher for most *E. faecium*. Many enterococci are also tolerant to the killing effects of cell-wall active agents, including ampicillin and vancomycin; recent data suggest that this property may not be inherent, but rather acquired after exposure to antibiotics (4). Inherent in vivo resistance of *E. faecalis* to trimethoprim-sulfamethoxazole may explain the lack of efficacy in animal models. In vitro, trimethoprim-sulfamethoxazole readily inhibits most enterococci at low concentrations, but this activity is lessened by exogenous folates (5). Moreover, bactericidal activity against *E. faecalis* seems unreliable and very method dependent (6). In animal models, this combination has not shown good activity and is not generally accepted

as an effective antienterococcal therapy, especially for systemic infections (7,8).

In addition to natural resistance to many agents, enterococci have also developed plasmid- and transposon-mediated resistance to tetracycline (as well as minocycline and doxycycline), erythromycin (plus the newer compounds azithromycin and clarithromycin), chloramphenicol, high levels of trimethoprim, and high levels of clindamycin.

The propensity of *E. faecalis* to acquire multiple antibiotic-resistance traits may result from a variety of distinctly different mechanisms for conjugation, i.e., bacterial mating. The best studied system of conjugation involves oligopeptides called pheromones and pheromone-responsive plasmids (9; Figure 1). Briefly, strains of *E. faecalis* typically secrete into the culture medium a number of different small peptide sex pheromones specific for different types of plasmids. When a cell containing a pheromone-responsive plasmid (the potential donor cell) comes into contact with its corresponding pheromone, transcription of a gene on the plasmid is turned on, resulting in the synthesis of a sticky substance (called aggregation substance) on its surface. When the donor cell bumps into another *E. faecalis*, aggregation substance, which contains two Arg-Gly-Asp motifs, sticks to the binding substance on the surface of most *E. faecalis* cells, causing them to clump together. In the test tube, clumps of cells actually fall to the bottom of the tube, resulting in a visible aggregate. By a process not yet well understood, the pheromone-responsive plasmid can then transfer from the donor bacterium to the other (recipient) bacterium. Once the recipient cell has acquired this particular plasmid, the synthesis of the corresponding sex pheromone is shut off to prevent self-clumping. This system of conjugation, which occurs primarily in *E. faecalis,* is highly efficient and results in transfer of plasmids in both filter and broth matings.

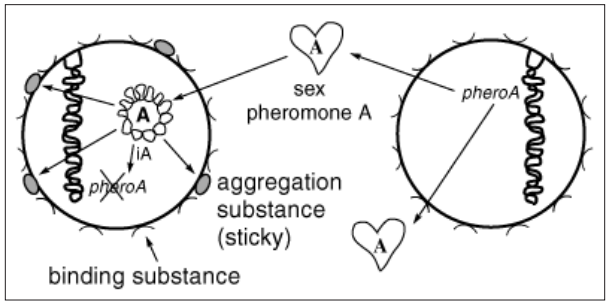

Figure 1. *Enterococcus faecalis* pheromone-responsive conjugative system.
Pheromone A released from the potential recipient cell (right) interacts with plasmid A in the potential donor cell (left) to induce synthesis of aggregation substance. Attachment of aggregation substance to binding substance causes the cells to clump into visible aggregates. Once the pheromone-responsive plasmid A has transferred from donor to recipient cell, synthesis of pheromone A is shut off.

Another system of conjugation, also not well understood, involves broad host-range plasmids that can transfer among species of enterococci and other gram-positive organisms such as streptococci and staphylococci (10). The transfer frequency is generally much lower than with the pheromone system and is much more efficient with filter than with broth matings. Since staphylococci, streptococci, and enterococci share a number of resistance genes, these broad host-range plasmids may be a mechanism by which some of these resistance genes have spread among different genera.

A third type of conjugation, which involves conjugative transposons, may also explain the spread of resistance genes to many different species (11). As opposed to ordinary transposons, which can jump within a cell from one DNA location to another, conjugative transposons also encode the ability to bring about conjugation between different bacterial cells. Since plasmids typically require rather complex machinery for replication (often depending on successful interactions with host proteins) and must face additional problems of surface exclusion and incompatibility, conjugative transposons (which do not replicate, but instead insert into the chromosome or into a plasmid of the new host) appear to be an even more efficient and far-reaching way of disseminating a resistance gene. This may explain why the *tetM* gene of the conjugative transposon Tn*916* has spread beyond the gram-positive species into gram-negative organisms, including gonococci, meningococci, and *Haemophilus ducreyi,* as well as into mycoplasma and ureaplasma, among others (12,13). Other resistance genes, including those encoding resistance to erythromycin and kanamycin, are also found on conjugative transposons; these frequently contain or are related to Tn*916*. Such transposons may have evolved from a Tn*916* ancestor; their emergence suggests the

possibility of further dissemination of resistance among gram-positive organisms. Particularly ominous are reports of the *vanB* gene cluster within large conjugative chromosomal elements that appear similar, at least in function, to conjugative transposons (14).

High-Level Aminoglycoside Resistance

Although some acquired resistance of enterococci is not clinically important because the agents involved are not commonly used, other resistance greatly affects enterococcal therapy; high-level resistance (HLR) to aminoglycosides is an example. This resistance is added onto the normal low-level resistance of enterococci to aminoglycosides and typically results in MICs of ≥ 2,000 µg/ml. This degree of resistance predicts, without exception, resistance to synergism between cell-wall active agents and the aminoglycoside to which the organism is highly resistant (1). High-level aminoglycoside resistance is most often due to aminoglycoside-modifying enzymes; HLR to streptomycin can also be ribosomal, that is, due to a mutation that results in ribosomes resistant to streptomycin inhibition. HLR to kanamycin (without gentamicin) is a fairly common trait and is due to the production of a 3'-phosphotransferase, APH(3')-III. This enzyme is important because it also eliminates synergism between cell-wall active agents and amikacin (through phosphorylation of the 3'-hydroxyl group), although it does not necessarily confer HLR to amikacin. HLR to gentamicin results from the bifunctional protein (AAC(6')-I/APH(2")-I), encoded by a single gene with two active sites, one with 6'-acetyltransferase activity and the other, 2"-phosphotransferase activity (15). The combination of these activities results in HLR or resistance to synergism for all commercially available aminoglycosides except streptomycin, which is not modified by this enzyme. However, HLR to streptomycin (due to either ribosomal resistance or a streptomycin adenylyltransferase) is also common and can coexist with the gene(s) for HLR to other aminoglycosides. Spectinomycin is also not modified by the bifunctional enzyme, but this agent, which is not a true aminoglycoside, is not generally bactericidal against enterococci and does not appear to show synergism with cell-wall active agents.

Strains of enterococci from patients with endocarditis and other serious infections for whom combination therapy is desired should be screened for HLR to streptomycin and gentamicin. HLR screening for tobramycin is not generally performed or advisable It could, in principle, be used for *E. faecalis*, but *E. faecium* isolates have a chromosomally encoded, naturally occurring gene for a 6'-acetyltransferase that eliminates synergism with tobramycin, although it does not cause HLR (MICs are typically 128-500 µg/ml); a probe for this gene has been used to confirm the identification of *E. faecium* isolates to species. In addition, HLR of an *E. faecium* isolate (without HLR to gentamicin) to tobramycin, due to an adenylyltransferase, was recently described (16). Therefore, the use of tobramycin for possible synergism in serious enterococcal infections would need to be preceded by screening for HLR to tobramycin (a test that is not commonly available), as well as for identification to species, neither of which is practical.

More recently, veterinary and human isolates resistant to moderate levels of gentamicin (256 µg/ml) were found to have a new gentamicin-modifying enzyme encoded by a gene designated *aph(2")-Ic* (17). This gene conferred resistance to synergism between gentamicin and cell-wall active agents and may be less easily detected than strains producing the bifunctional enzyme.

Beta-Lactamase– and non-Beta-Lactamase–Associated Penicillin Resistance

The first known penicillinase-producing isolate of enterococcus was an isolate of *E. faecalis* recovered from a patient in Houston, Texas, in 1981 (18). Although rare, these isolates have been reported from the United States (Texas, Florida, North Carolina, Delaware, Pennsylvania, New York, Massachusetts, and Connecticut), Lebanon, Canada, and Argentina (19). Like other enterococci, beta-lactamase-producing strains have been found as colonizers, as in the large "outbreak" of colonization in a Boston children's hospital, but they have also been associated with true infections, as was demonstrated by cases at a Virginia Veterans Administration hospital, by isolates from Argentina, and in other reports (20,21). The enterococcal penicillinase gene, identical to the gene encoding staphylococcal type A penicillinase, almost always occurs in strains with HLR to gentamicin and is often found on a transferable plasmid that also contains *aph(2")-Ia/aac(6')-Ie*. The relatively low levels of beta-lactamase produced by enterococci

result in a marked inoculum effect with these strains so that at low and even moderate inocula (10^3-10^5 CFU/ml), penicillinase-producing enterococci usually appear no more resistant than other enterococci, while at high inocula ($\geq 10^7$ CFU/ml), these organisms are usually highly resistant to penicillin, ampicillin, and ureidopenicillins. The activity of the penicillinase is reversed by the beta-lactamase inhibitors clavulanate, sulbactam, and tazobactam; in animal models of endocarditis, beta-lactamase inhibitors have been shown to markedly enhance the therapeutic efficacy of ampicillin or penicillin. In the clinical laboratory, penicillinase-producing enterococci are generally not detected by routine laboratory susceptibility testing, such as MICs or disk diffusion. For this reason, if a penicillin is to be used for therapy, enterococcal isolates from patients with endocarditis or other serious infections should be tested for penicillinase production by using a specific beta-lactamase test such as the chromogenic cephalosporin nitrocefin.

Nonpenicillinase-producing, penicillin-resistant enterococci have been reported for decades and usually are *E. faecium*. Until recently, MICs of penicillin typically ranged from 8 µg/ml to 64 µg/ml, with an occasional isolate having higher levels of resistance. However, increasingly, strains with much higher levels of penicillin resistance have been reported (22). Whether a large number of strains have converted from low-level to high-level resistance or a more limited number of strains have been disseminated is unclear. The mechanisms involved in this resistance are overproduction of a low-affinity penicillin-binding protein (a cell-wall synthesis enzyme) and a further decrease in the affinity of one of these enzymes for penicillin (23). As a possible explanation for why many vancomycin-resistant *E. faecium* also have very high levels of resistance to ampicillin, Rice and colleagues (24) showed that transfer of vancomycin resistance from one strain to another was linked to transfer of ampicillin resistance.

Vancomycin Resistance

Most surprising in recent years has been the emergence among enterococci of acquired resistance to vancomycin. Vancomycin had been in clinical use since the 1950s, although it was not heavily used until the late 1970s and particularly the 1980s. Because multiple genes are involved in generating vancomycin resistance, the development of resistance was neither easy nor recent. Three phenotypes of vancomycin resistance (types A, B, and C) are now well described; a fourth, type D, has been recently reported (25). VanA-type strains are typically highly resistant to vancomycin and moderately to highly resistant to teicoplanin. This phenotype is often plasmid or transposon mediated and is inducible (i.e., exposure of bacteria to vancomycin results in the induction of the synthesis of several proteins that together confer resistance) (26).

In vancomycin-susceptible enterococci, D-alanyl-D-alanine (formed by an endogenous D-alanine-D-alanine ligase) is added to a tripeptide precursor to form a pentapeptide precursor. The D-Ala-D-Ala terminus is the target of vancomycin; once vancomycin has bound, the use of this pentapeptide precursor for further cell-wall synthesis is prevented. In the VanA phenotype, one of the proteins whose synthesis is induced by exposure of bacterial cells to vancomycin is called VanA; VanA is a ligase and resembles the D-alanine-D-alanine ligase from *Escherichia coli* and other organisms, including vancomycin-susceptible enterococci (27). VanA generates D-Ala-D-X, where X is usually lactate; the formation of D-lactate is due to the presence of VanH, a dehydrogenase encoded by *vanH*. The depsipeptide moiety, D-Ala-D-Lac, is then added to a tripeptide precursor, resulting in a depsipentapeptide precursor. Vancomycin does not bind to the D-Ala-D-Lac terminus, so this depsipentapeptide can be used in the remaining steps of cell-wall synthesis. However, when the normal pentapeptide precursor ending in D-Ala-D-Ala is also present, cells are not fully vancomycin resistant, despite the presence of D-Ala-D-Lac containing precursors. This apparent problem is taken care of in large part by *vanX*, which encodes a dipeptidase, VanX, that cleaves D-Ala-D-Ala, preventing its addition to the tripeptide precursor. Should any D-Ala-D-Ala escape cleavage and result in a normal pentapeptide precursor, *vanY* encodes an ancillary or back-up function. That is, it codes for a carboxypeptidase, VanY, which cleaves D-alanine and D-lactate from D-Ala-D-Ala and D-Ala-D-Lac termini, respectively, resulting in tetrapeptide precursors, to which vancomycin does not bind. The other genes involved in the VanA resistance complex include *vanR* and *vanS*, whose encoded proteins are involved in somehow sensing the presence of extracellular vancomycin or

its effect and signaling intracellularly to activate transcription of *vanH*, *vanA*, and *vanX* (27). A final gene in the *vanA* cluster is *vanZ,* which encodes VanZ, the role of which is not known.

VanB, encoded by *vanB* in the *vanB* gene cluster, is also a ligase that stimulates the formation of D-Ala-D-Lac. The VanB phenotype is typically associated with moderate to high levels of vancomycin resistance but is without resistance to teicoplanin. This is explained by the observation that vancomycin, but not teicoplanin, can induce the synthesis of VanB and of $VanH_B$ and $VanX_B$. However, because mutants resistant to teicoplanin can readily be selected from VanB strains on teicoplanin-containing agar, clinical resistance would likely occur among VanB strains if teicoplanin were widely used. Most of the proteins encoded by the *vanA* gene cluster have homologues encoded by the *vanB* gene cluster, except for VanZ. The *vanB* gene cluster has an additional gene, *vanW,* of unknown function.

The VanC phenotype (low-level resistance to vancomycin, susceptible to teicoplanin) is an inherent (naturally occurring) property of *E. gallinarum* and *E. casseliflavus*. This property is not transferable and is related to the presence of species-specific genes *vanC-1* and *vanC-2*, respectively (28); a third possible species, *E. flavescens* and its gene *vanC-3*, are so closely related to *E. casseliflavus* and *vanC-2* that different names are probably not warranted (29). These species appear to have two ligases; the cell-wall pentapeptide, at least in *E. gallinarum*, ends in a mix of D-Ala-D-Ala and D-Ala-D-Ser (29,30). The genes *vanC-1* and *vanC-2* apparently lead to the formation of D-Ala-D-Ser containing cell-wall precursors, while D-Ala-D-Ala ligases, also present in these organisms, result in D-Ala-D-Ala. The presence of both D-Ala-D-Ala and D-Ala-D-Ser precursors may explain why many isolates of these species test susceptible to vancomycin and why even those isolates with decreased susceptibility display only low-level resistance.

VanD-type glycopeptide resistance has been recently described in an *E. faecium* isolate from the United States (25). The organism was constitutively resistant to vancomycin (MIC ≥ 64 µg/ml) and to low levels (4 µg/ml) of teicoplanin. Following polymerase chain reaction amplification with primers that amplify many D-Ala-D-Ala ligases, a 605-bp fragment was identified whose deduced amino acid sequence showed 69% identity to VanA and VanB and 43% identify to VanC.

Molecular Epidemiology of Newer Resistance Traits

High-Level Gentamicin Resistance

The DNA sequence of the gene encoding HLR to gentamicin in *E. faecalis* is the same as the sequence of the gentamicin resistance gene of staphylococci (15). Since this gene was well established in staphylococci by the 1970s but HLR to gentamicin was not reported in enterococci until 1979, the seemingly obvious conclusion is that this gene spread from staphylococci to enterococci rather than vice versa or, at least, staphylococci acquired it first. However, the disk diffusion method used in the 1970s and microtiter dilution MICs done later are capable of detecting gentamicin resistance in staphylococci but do not distinguish enterococci with high-level gentamicin resistance from those with low-level, inherent resistance. Therefore, since laboratories were not screening enterococci by special techniques for high-level gentamicin resistance, it cannot be definitively stated that this resistance did not appear in enterococci earlier or coincident with its emergence in staphylococci. However, several observations support the likelihood that gentamicin resistance appeared and disseminated in staphylococci before it did in enterococci. In 1971, Moellering et al. reported the lack of high-level gentamicin resistance among enterococci (31). Watanakunakorn reported the absence of high-level gentamicin resistance among 126 enterococci from 1980 to 1984, with HLR subsequently appearing in 1985 (32). Phillips et al. from the United Kingdom reported no highly gentamicin-resistant strains in 1969 to 1979 or 1980 to 1985 and appearance of strains in 1986 (33). Zervos et al. reported that only one (0.04%) of 269 isolates of *E. faecalis* had high-level gentamicin resistance in 1981; this figure gradually increased to 7.7% in 1984 (34). High-level gentamicin resistance in *E. faecium* appears to have occurred after its appearance in *E. faecalis*, with the first report occurring in 1988 (35).

Delineation of the molecular epidemiology of strains of enterococci was limited in the past by the lack of an easy, reliable, and widely accessible method for subspecies strain differentiation. Zervos and Schaberg reported the use of plasmid patterns in enterococci to suggest the intrahospital spread of strains with high-level gentamicin resistance. Pulsed-field gel electrophoresis (PFGE)

of *E. faecalis* with HLR to gentamicin found that different isolates from both the same and different locations had markedly different restriction endonuclease digestion patterns. That is, it found no evidence of a common strain or strains that predominated among gentamicin-resistant organisms (36). Strains isolated between 1981 and 1984 at the University of Michigan demonstrated that plasmids encoding high-level gentamicin resistance were heterogeneous, which again argues against clonal dissemination of a limited number of strains or plasmids to account for spread of this property (34,37). We have subsequently shown that gentamicin resistance in enterococci can be encoded on a transposon identical to that in staphylococci (38). In addition, the enterococcal gentamicin-resistance gene has been found in other genetic settings, one of which has also been found in North American *Staphylococcus aureus* with gentamicin resistance (39). Since all enterococci with HLR to gentamicin (MIC ≥ 2,000 µg/ml) that have been tested have hybridized with the same gene probe, this property could be termed a "gene epidemic." However, by the time gentamicin resistance was discovered in enterococci, this gene was already widespread with no evidence of either a common plasmid or a common or predominant strain.

Other genetic elements encoding HLR to gentamicin have also been described. Thal et al. have described a 27-kb element designated Tn*924* that encodes HLR to gentamicin and could be mobilized from the chromosome of an *E. faecalis* by a coresident plasmid (40). Rice et al. have described a large (ca. 60 kb) transferable element, tentatively named Tn*5385*, which appears to contain within it an 18-kb conjugative transposon (Tn*5381*) encoding tetracycline resistance and a 26-kb IS*256*-based composite transposon (Tn*5384*) encoding resistance to gentamicin and to erythromycin (41).

Penicillin-Resistant Penicillinase-Producing Enterococci

PFGE analyses of penicillinase-producing enterococci have shown that a common penicillinase-producing strain (or "clone"), defined as having an identical or related chromosomal digestion pattern, was present in Texas, Florida, North Carolina (unpub. observation), Delaware, Pennsylvania, and Virginia, which had a large outbreak with numerous infections (42,43). Moreover, at each of these locations, all isolates of penicillinase-producing *E. faecalis* examined were derivatives of this strain; in the hospital in which this strain has been endemic for many years, a single penicillinase-producing isolate of *E. faecium*, the only such isolate ever reported, was also found (44). Among numerous non-Bla+ *E. faecalis,* a PFGE pattern similar to that of Bla+ *E. faecalis* has been found only once from an isolate from a Philadelphia hospital that had one of the Bla+ isolates (43 and unpub. obs.). Penicillinase-producing isolates from Connecticut, Boston, Canada (unpub. observations), Lebanon, and Argentina represent different strains (19). However, clonal relatedness of three isolates with an almost identical pattern was demonstrated in Connecticut (despite one of these lacking HLR to gentamicin) (45), and all six isolates in Argentina appeared to be a single strain. The Boston isolates have not been studied by PFGE, but a number of isolates from the same hospital had a single shared plasmid, suggesting that these also represent clonal dissemination of a single strain. While all of these penicillinase-producing *E. faecalis* could be detected by nitrocefin, a vancomycin-resistant *E. faecalis* that tested negative for penicillinase by nitrocefin but showed a marked inoculum effect with penicillin and destroyed penicillin in a bioassay has been reported (46). Thus, in contrast to high-level gentamicin resistance in enterococci, which appeared to be widely disseminated on different plasmids and in different strains by the time this phenotype was studied, penicillinase production in enterococci is still largely associated with a limited number of strains; moreover, in locations known to have more than one isolate, oligoclonal spread within each setting remains the rule.

Nonpenicillinase-Producing Penicillin-Resistant Strains

PFGE analyses of highly penicillin-resistant *E. faecium* from the Medical Colleges of Virginia and Pennsylvania have shown that within each location, most highly resistant strains represented a single clone (47). Analysis of the PFGE patterns also raised the possibility, on the basis of similarities between patterns of isolates in these two different locations, that a strain may have spread from one institution to the other (47); this conclusion was supported by the finding that these isolates belonged to the same multilocus

enzyme cluster (unpub. observations). Circumstantial evidence of intrahospital spread of highly penicillin-resistant *E. faecium* also comes from the study of Grayson et al. (22), who reported a sudden increase in more highly penicillin-resistant isolates of *E. faecium* at the Massachusetts General Hospital in 1988. Although no genetic analysis was done, the fact that gentamicin resistance also simultaneously appeared and most of the *E. faecium* highly resistant to penicillin were also highly resistant to gentamicin suggests clonal dissemination of one or a few strains within that hospital (22).

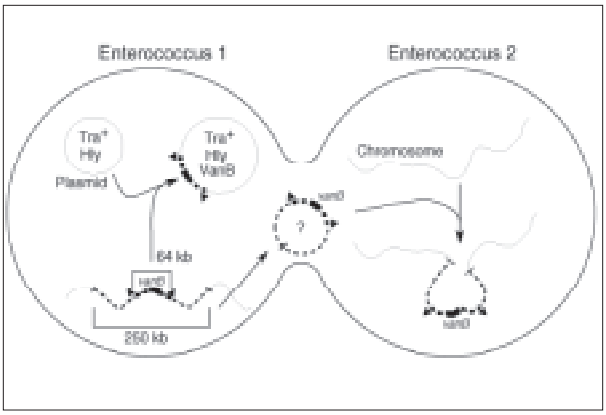

Figure 2. Potential modes of spread of vancomycin-resistant genes. Adapted in part from Quintiliani and Courvalin (14).
The *vanB* gene cluster (shown on the left) on a 64-kb transposon is part of a 250-kb mobile element shown to move from the chromosome of one enterococcus and insert into the chromosome of another. Although not demonstrated, circularization of the *vanB* containing large mobile elements resembles the mechanism described for conjugative transposons that can excise from the chromosome of one strain, circularize, transfer from one enterococcus to another, and reinsert into the chromosome of the recipient (such as the one on the right). The 64-kb transposon shown on the left can also jump to another plasmid within the host enterococcus. If it is a conjugative (Tra+) plasmid, that plasmid can then transfer by conjugation to other bacteria, taking the *van* resistance genes with it. In one instance, the vancomycin resistance transposon was shown to transpose to a plasmid encoding the virulence factor hemolysin (Hly).

Vancomycin Resistance

Vancomycin resistance in enterococci is heterogeneous on many levels. For example, three different, well-described types of vancomycin resistance are known, each associated with different ligase genes, *(vanA, vanB, vanC1,* and *vanC2)*, and a fourth type, VanD, has been reported recently (25). VanA and VanB type resistance is encoded by gene clusters that are acquired (i.e., not part of the normal genome of enterococci) and are often transferable. In contrast, *vanC1* and *vanC2* are normally occurring genes that are endogenous species characteristics of *E. gallinarum* and *E. casseliflavus*, respectively, and are not transferable. The acquired gene clusters associated with *vanA* and *vanB* are found in different genetic surroundings. The *vanA* gene cluster has been found in a small Tn*3*-like transposon, Tn*1546*, and in elements that appear to be closely related (e.g., Tn*5488*, which has an insertion sequence [IS*1251*] within Tn*1546* [48,49]) or lacking *vanZ* (50). These elements have in turn been found on both transferable and nontransferable plasmids, as well as on the chromosome of the host strain. VanB type resistance was initially not found to be transferable, but at least in some instances, the *vanB* gene cluster has been found on large (90 kb to 250 kb) chromosomally located transferable elements, one of which contains within it a 64-kb composite transposon (Tn*1547*) (Figure 2; 14). More recently, *vanB* has been found as part of plasmids.

In addition to being found in different genetic surroundings, the *vanA* and *vanB* gene clusters have also been found in a number of different bacterial species. *vanA* has been found in multiple enterococcal species as well as in lactococci, *Orskovia*, and *Arcanobacteria* (51). The distribution of the *vanB* gene cluster seems somewhat more restricted, having been found primarily in *E. faecium* and *E. faecalis*, although it has recently been found in *Streptococcus bovis* (52).

When vancomycin-resistant enterococci (VRE) from patients in a given hospital have been examined, particularly after the first recovery of VRE, evidence is often found of a single or predominant strain (53-58). Finding isolates with identical or highly related PFGE patterns in different hospitals indicates interhospital clonal transmission (59-61). Some reports do not find a single or a predominant strain, especially when VRE have been present in a hospital or area for some time (62,63). This was also true of two reports from France in which all of 16 and all of 24 vancomycin-resistant *E. faecium* were different (50,64,65). The reports from France likely reflect another observation regarding the diversity of

vancomycin resistance. v*anA* has also been shown to be present in normal fecal enterococci of healthy, nonhospitalized persons in different parts of Europe (66-68); in one study, 20 different strains (identified by PFGE) of vancomycin-resistant *E. faecium* were found in the fecal flora of 17 persons in two areas in Belgium (68). *vanA* containing *E. faecium* have also been found in the feces of healthy animals as well as from animal products in Europe (50,67,69-71); in one study, VRE were found in the feces of healthy meat-eaters but not vegetarians (72). VRE have not, however, been found as part of the normal fecal flora in the United States (73) possibly because glycopeptides, often used in animal feed in Europe, are not used in the United States. For example, 24,000 kg annually of the glycopeptide avoparcin were reportedly used in recent years in Denmark (74). Reports of VRE in the feces of animals on farms using glycopeptides, but not in those without such use, support this hypothesis (71,75). While oral glycopeptide use markedly increases the numbers of VRE per gram of stool in humans (68) and by analogy, presumably does so in animals, glycopeptide use does not explain the origin of these gene clusters.

The problem of multidrug-resistant enterococci promises to be with us for the foreseeable future. The enterococcus has likely emerged as a major nosocomial pathogen in part because of its resistance to multiple antibiotics, which allows it to survive and subsequently infect patients. With its propensity to acquire new traits, such as high-level gentamicin, penicillin, and vancomycin resistance, the enterococcus continues to create new therapeutic problems and dilemmas; its ability to transfer some of its plasmids to streptococci and staphylococci and the implications of a possible spread of penicillin and vancomycin resistance to these and other gram-positive species are also of concern.

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Proteases of Malaria Parasites: New Targets for Chemotherapy

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The increasing resistance of malaria parasites to antimalarial drugs is a major contributor to the reemergence of the disease as a major public health problem and its spread in new locations and populations. Among potential targets for new modes of chemotherapy are malarial proteases, which appear to mediate processes within the erythrocytic malarial life cycle, including the rupture and invasion of infected erythrocytes and the degradation of hemoglobin by trophozoites. Cysteine and aspartic protease inhibitors are now under study as potential antimalarials. Lead compounds have blocked in vitro parasite development at nanomolar concentrations and cured malaria-infected mice. This review discusses available antimalarial agents and summarizes experimental results that support development of protease inhibitors as antimalarial drugs.

Hundreds of millions of cases of malaria occur annually, and infections with *Plasmodium falciparum*, the most virulent human malaria parasite, cause more than one million deaths per year (1). Despite extensive control efforts, the incidence of the disease is not decreasing in most malaria-endemic areas of the world, and in some it is clearly increasing (2). Malaria also remains a major risk to travelers from industrialized to developing countries. Because malaria parasites are increasingly resistant to antimalarial drugs, appropriately counseled travelers to malaria-endemic regions are more likely to contract malaria now than they were 40 years ago.

Malaria control efforts include attempts to develop an effective vaccine, eradicate mosquito vectors, and develop new drugs (2,3). However, the development of a vaccine has proven very difficult, and a highly effective vaccine will probably not be available in the near future (4). Efforts to control *Anopheles* mosquitoes have had limited success, although the use of insecticide-impregnated bed nets does appear to reduce malaria-related death rates (5). In addition, methods to replace natural vector populations with mosquitoes unable to support parasite development are under study and may contribute to malaria control in the long term (6). However, the current limitations of vaccine and vector control, as well as the increasing resistance of malaria parasites to existing drugs, highlight the continued need for new antimalarial agents.

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Established Antimalarial Drugs

Antimalarial drugs have been used for centuries. Early natural products, including the bark of the cinchona tree in South America and extracts of the wormwood plant in China, were among the first effective antimicrobial agents to be used. Cinchona bark was used in Europe beginning in the 17th century, and upon its isolation from bark in 1820, quinine became widely used. In the last 50 years, extensive efforts, including the screening of hundreds of thousands of compounds, have led to the development of a number of effective synthetic antimalarial drugs. The most important of these, chloroquine, has been the mainstay of antimalarial chemotherapy for the last 50 years. The compound eradicates parasites rapidly, has minimal toxicity, is widely available at low cost throughout the world, and needs to be taken only once a week for chemoprophylaxis. However, resistance to chloroquine has been steadily increasing since the drug's initial use in South America and Southeast Asia in the late 1950s. Chloroquine resistance is now widespread in most *P. falciparum*-endemic areas of the world (3). Thus, the use of chloroquine for presumptive treatment of falciparum malaria

or for chemoprophylaxis is usually no longer appropriate (7). Moreover, resistance to chloroquine of *P. vivax*, the second most lethal human malaria parasite, is increasing in South Asia (8).

No other antimalarial drug (9-12) is as efficacious and safe as chloroquine (Table 1). The best antimalarial drug for treating chloroquine-resistant falciparum malaria remains quinine (or intravenous quinidine), which is fairly toxic; quinine resistance is increasing in Southeast Asia, particularly in the border areas of Thailand (9). Amodiaquine, used to treat chloroquine-resistant malaria in developing countries, is also quite toxic, and resistance to it is also common (13). Mefloquine (14) is widely used for chemoprophylaxis against chloroquine-resistant *P. falciparum*, but its use is limited by toxicity (15) and (in the developing world) high cost. Mefloquine is not approved for treatment of malaria in the United States because of the neurotoxicity of doses required for the treatment. Fansidar, a combination of sulfadoxine and pyrimethamine, is no longer recommended for chemoprophylaxis because of its dermatologic toxicity (15). Fansidar is also not an ideal drug for treatment because it is slow acting, but it is increasingly important in treating chloroquine-resistant malaria in developing countries because economic constraints limit the use of other agents (16). The use of both mefloquine and Fansidar will increasingly be limited by drug resistance, already widespread in parts of Southeast Asia (9,17).

Other antimalarial drugs have specialized uses. Tetracyclines and some other antibiotics (clindamycin, sulfas) are slow acting and generally best used as an adjunct to quinine therapy in treating falciparum malaria (9). Doxycycline is also used for chemoprophylaxis in regions with high levels of drug resistance, especially Southeast Asia (10,17). Other drugs for chemoprophylaxis include proguanil, which remains effective in combination with chloroquine in many areas other than Southeast Asia, and Maloprim, a combination of dapsone and pyrimethamine (10,17). Resistance to these drugs is fairly common, however. Primaquine has a well-defined specific role: eradicating chronic liver stages of *P. vivax* and *P. ovale* after treating the acute blood infection with chloroquine.

New Antimalarial Drugs

Relatively few new antimalarial drugs are undergoing clinical testing (Table 2). Halofantrine, identified in the 1940s, was not developed until

Table 1. Established antimalarial drugs[a]

Drug	Role	Best Feature(s)	Limitations
Chloroquine	TX of and CP against non-Pf and sensitive Pf parasites	Very safe; low cost; long half-life	Widespread R
Quinine/quinidine	Best TX for Pf malaria; low cost	Limited R; rapidly acting	Fairly toxic (cinchonism, cardiac)
Amodiaquine[b]	TX of R Pf malaria	Low cost	Toxicity (bone marrow, liver); R common
Mefloquine	CP against R Pf malaria; not approved for TX in United States	Relatively little R, though increasing; long half-life	Moderately toxic (mostly CNS); high cost; R in SE Asia
Fansidar	TX of Pf malaria; no longer recommended for CP	Relatively low cost; long half-life	Skin toxicity (can be fatal); increasing R
Primaquine	Eradication of chronic liver stage Pv, Po malaria	Only drug for this indication	Hemolysis with G6PD deficiency; increasing R
Proguanil[b]	CP only (often with chloroquine)	Low cost; nontoxic	R common
Maloprim[b]	CP only (often with chloroquine)	Low cost	R common; skin rashes
Tetracyclines	CP; TX of Pf malaria in combination with quinine	Low cost	Skin and gastrointestinal toxicity

[a]TX, therapy; CP, chemoprophylaxis; R, resistance/resistant; Pf, *Plasmodium falciparum;* Pv, *P. vivax;* Po, *P. ovale;* CNS, central nervous system; G6PD, glucose 6-phosphate dehydrogenase.
[b]Not available in the United States.

Table 2. New antimalarial drugs

Drug	Role	Best Feature(s)	Limitations
Halofantrine	TX of Pf malaria; not approved for CP	Usually effective against R Pf malaria	Variable bioavailability, cardiac toxicity
Artemisinin and related compounds[a]	TX of Pf malaria	Rapidly acting; effective against multidrug-R strains	Recurrence after TX fairly common
Atovaquone	? TX of Pf malaria; ? CP (probably in combination with proguanil)	Limited toxicity	Limited studies so far show frequent recurrence after TX
Pyronaridine[a]	? TX of Pf malaria	Effective against R strains	Studies limited to date
Desferrioxamine	? TX of severe Pf malaria	Well tolerated when used for iron overload	Studies limited to date
Azithromycin	? CP	Limited toxicity	Studies limited to date

For abbreviations, see Table 1, footnote a.
[a]Not available in the United States.

the 1980s; its use has been limited by variable oral absorption and cardiac toxicity (12,18). The drug is approved in the United States for treatment of chloroquine-resistant *P. falciparum* infection, although in most cases quinine (or intravenous quinidine) is preferable. The most effective new drugs are artemisinin and related compounds. Artemisinin was isolated in 1972 from *Artemisia annua*, a plant used in China for centuries to treat fever (19). Artemisinin derivatives (artesunate, artelinate, artemether, arteether, dihydroartemisinin) have been synthesized and are undergoing extensive clinical testing. These compounds, which are already widely used in some areas, are potent, rapidly acting antimalarials that are effective against chloroquine-resistant *P. falciparum* (20). Because recrudescences of infection after treatment are common, however, artemisinin and related compounds might best be used in combination with another drug.

Other compounds are under evaluation. Atovaqone (21), which is approved for treating patients with *Pneumocystis* infections, appears to be effective against malaria in combination with proguanil (22), but its use has been limited by recrudescence after treatment. Pyronaridine, an acridine derivative used to treat malaria in China, has shown efficacy against falciparum malaria (23). The iron chelator desferrioxamine enhances the clearance of parasites in mild malaria (24) and, in conjunction with quinine and Fansidar, hastens recovery from deep coma in severe falciparum malaria (25). Azithromycin, a quinolone antibiotic, appears efficacious in malaria chemoprophylaxis (26).

Malarial Proteases: New Targets for Chemotherapy

The limitations of antimalarial chemotherapy underscore the need for new drugs, ideally directed against new targets. Potential targets for chemotherapy include malarial proteases (27). The erythrocytic life cycle, which is responsible for all clinical manifestations of malaria, begins when free merozoites invade erythrocytes. The intraerythrocytic parasites develop from small ring-stage organisms to larger, more metabolically active trophozoites and then to multinucleated schizonts. The erythrocytic cycle is completed when mature schizonts rupture erythrocytes, releasing numerous invasive merozoites. Proteases appear to be required for the rupture and subsequent reinvasion of erythrocytes by merozoite-stage parasites and for the degradation of hemoglobin by intraerythrocytic trophozoites (Figure).

Proteases and Erythrocyte Rupture and Invasion

The rupture of erythrocytes by mature schizonts and the subsequent invasion of erythrocytes by free merozoites appear to require malarial protease activity, possibly to breach the erythrocyte cytoskeleton, a complex network of proteins. In addition, a number of malarial proteins are proteolytically processed during the late schizont and merozoite life-cycle stages; for example, merozoite surface protein-1 is processed in a manner inhibited by serine protease inhibitors (28), presumably to facilitate the complex series of events involved in erythrocyte

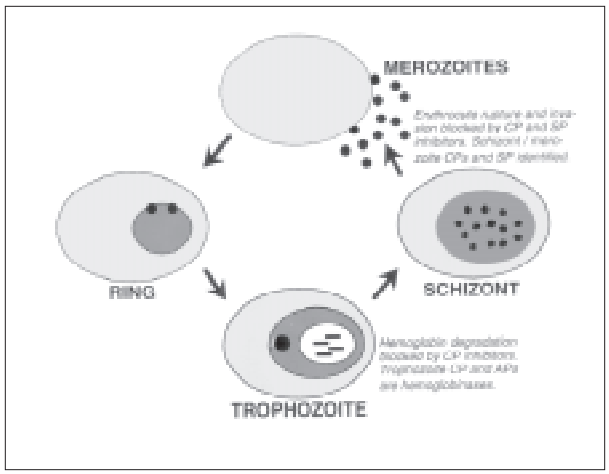

Figure. Protease targets in erythrocytic malaria parasites. The *Plasmodium falciparum* erythrocytic life cycle is shown schematically, and data supporting cysteine (CP), serine (SP), and aspartic (AP) proteases of the different parasite stages as chemotherapeutic targets are provided in italics.

rupture and invasion (29). Although the specific roles of different classes of proteases are not completely clear, inhibitors of cysteine and serine proteases have consistently blocked erythrocyte rupture and invasion (27).

Candidate *P. falciparum* rupture/invasion proteases have been identified, but none has been fully characterized biochemically or molecularly: 1) a 68 kD cysteine protease was identified in schizonts and merozoites and localized to the merozoite apex, suggesting that it may be released from the rhoptry organelle during invasion (30); 2) a cysteine protease of mature schizonts and a serine protease of merozoites were identified in highly synchronized parasites (31); 3) a serine protease was shown to be bound to the schizont/merozoite membrane by a glycosylphosphatidylinositol anchor, to be activated by phosphatidylinositol-specific phospholipase C during the merozoite stage, and to be capable of cleaving the erythrocyte cytoskeletal protein band 3 (32,33); 4) another protease, inhibited by both cysteine and serine protease inhibitors, hydrolyzed the erythrocyte cytoskeletal proteins spectrin and band 4.1 (34); and 5) the serine repeat antigen (35,36) and the related protein SERP H (37), both expressed in mature schizonts, have important similarities in their sequences with cysteine proteases. Further research should identify the specific biologic roles of the proteases mentioned and better characterize these enzymes, thus fostering the development of specific inhibitors.

Host proteases may also play a role in erythrocyte rupture by *P. falciparum*. In recent studies, host urokinase was shown to bind to the surface of *P. falciparum*-infected erythrocytes, and the depletion of urokinase from parasite culture medium inhibited erythrocyte rupture by mature schizonts (38). This inhibition was reversed by exogenous urokinase.

Drug Development Efforts

Synthetic peptide inhibitors of the *P. falciparum* schizont cysteine protease Pf 68 inhibited erythrocyte invasion by cultured parasites (39,40). The most effective peptide, GlcA-Val-Leu-Gly-Lys-NHC_2H_5, inhibited the protease and blocked parasite development at high micromolar concentrations (40; Table 3). Although these results do not demonstrate levels of inhibition expected to be therapeutically relevant, they suggest that a specific protease activity is required for erythrocyte invasion by malaria parasites and thus is a potential target for antimalarial drugs.

Proteases and Malarial Hemoglobin Degradation

Extensive evidence suggests that the degradation of hemoglobin is necessary for the growth of erythrocytic malaria parasites, apparently to provide free amino acids for parasite protein synthesis (27,50). In *P. falciparum*, hemoglobin degradation occurs predominantly in trophozoites and early schizonts, the stages at which the parasites are most metabolically active. Trophozoites ingest erythrocyte cytoplasm and transport it to a large central food vacuole. In the food vacuole, hemoglobin is broken down into heme, a major component of malarial pigment (51), and globin, which is hydrolyzed to its constituent amino acids. The food vacuole is an acidic organelle analogous to lysosomes. Several lysosomal proteases are well characterized, including cysteine (cathepsins B, H, and L) and aspartic (cathepsin D) proteases (52), and malaria parasites contain analogous food vacuole proteases that degrade hemoglobin. At least two aspartic proteases and one cysteine protease have been isolated from purified *P. falciparum* food vacuoles (53).

Malarial aspartic protease activities have been identified (54-60). Two recently characterized aspartic proteases (plasmepsin I and

Table 3. Protease targets for chemotherapy

Protease	Biologic role	Effective inhibitors[a] Compound (Reference)	In vitro[b] (IC_{50}; µM)	In vivo[c] (mg/kg/day)
Pf68	Erythrocyte invasion	GlcA-Val-Leu-Gly-Lys-NHC_2H_5 (40)	900	
Plasmepsin I	Hemoglobin degradation	SC-50083 (41)	2-5	
		Ro 40-4388 (42)	0.25	
Plasmepsin II	Hemoglobin degradation	Compound 7 (43)	20	
Falcipain	Hemoglobin degradation	Z-Phe-Arg-CH_2F (44)	0.064	
		Mu-Phe-HPh-CH_2F (45)	~0.03	400
		Mu-Leu-HPh-VSPh (46)	0.01	
		Oxalic bis ((2-hydroxy-1-naphthylmethylene)hydrazide) (47)	7	
		1-(2,5-dichlorophenyl)-3-(4-quinolinyl)-2-propen-1-one (48)	0.23	
		7-chloro-1,2-dihydro-2-(2,3-dimethoxy-phenyl)-5,5-dioxide-4-(1H,10H)-phenothiazinone (49)	2	

[a]The structures of these compounds and details of the described studies are in the references noted.
[b]Assays compared the development of new ring-form parasites or the uptake of [^{3}H]hypoxanthine by treated and control parasites.
[c]Cure of *Plasmodium vinckei*-infected mice.

plasmepsin II) are located in the food vacuole, have acid pH optima, and share sequence homology with other aspartic proteases (41,53,61,62). Furthermore, the aspartic proteases can cleave hemoglobin. One of the enzymes, plasmepsin I, cleaves native hemoglobin (53,59). Plasmepsin II appears to prefer denatured globin as a substrate (53). On the basis of these data, plasmepsin I is thought to be responsible for initial cleavages of hemoglobin after the molecule is transported to the food vacuole (53).

Incubation of cultured *P. falciparum* parasites with the protease inhibitor leupeptin caused trophozoite food vacuoles to fill with apparently undegraded erythrocyte cytoplasm (63-65). Analysis of the leupeptin-treated parasites showed that they contained large quantities of undegraded globin, while minimal globin was detectable in control parasites (64,66). Leupeptin inhibits both cysteine and some serine proteases, but the highly specific cysteine protease inhibitor E-64 also caused undegraded globin to accumulate. After parasites were incubated with inhibitors of other classes of proteases including the aspartic protease inhibitor pepstatin (63-67), globin did not accumulate. More recent studies that used nondenaturing electrophoretic methods demonstrated that cysteine protease inhibitors not only blocked malarial globin hydrolysis, but also inhibited earlier steps in hemoglobin degradation, including denaturation of the hemoglobin tetramer and the release of heme from globin (68). Another study showed that E-64, but not pepstatin, inhibited the production of hemozoin (the malarial end product of heme) by cultured parasites (69). These results suggest that a cysteine protease is required for initial steps in hemoglobin degradation by *P. falciparum*.

A *P. falciparum* trophozoite cysteine protease with biochemical features expected for a food vacuole hemoglobinase has been identified (31) and biochemically (70-72) and molecularly (73) characterized. This protease, called falcipain, degraded denatured and native hemoglobin in vitro; its acid pH optimum, substrate specificity, and inhibitor sensitivity indicated that it was a papain family cysteine protease (64,70,71). Specific inhibitors of falcipain blocked hemoglobin degradation and prevented parasite development. The degree of inhibition of falcipain by fluoromethyl ketones (44) and vinyl sulfones (46) correlated with their inhibition of hemoglobin degradation and parasite development, supporting the hypothesis that falcipain is the cysteine protease required for hemoglobin degradation.

The specific mechanism for hemoglobin degradation in the malarial food vacuole remains unclear. As noted above, both the aspartic protease plasmepsin I and the cysteine protease falcipain have been identified in parasite food vacuoles and shown to cleave denatured and native hemoglobin in vitro (53,71). Results showing that only cysteine protease inhibitors block hemoglobin processing and globin hydrolysis in cultured parasites suggest that falcipain is required for initial steps of hemoglobin degradation (66-68,74). However, other studies have

shown that native hemoglobin is cleaved by plasmepsin I, but not falcipain, in nonreducing conditions that may be present in the food vacuole (53,59,72). In any event, regardless of the exact sequence of hemoglobin processing, multiple enzymes, including at least the three proteases already identified, appear to participate in the degradation of hemoglobin. These proteases are thus logical targets for antimalarial drug development.

Aminopeptidase activity has also been described in malaria parasites (75-77). This activity, with a neutral pH optimum, was not found in food vacuole lysates (77). When these lysates were incubated with hemoglobin, discrete peptide fragments, but not free amino acids, were identified (77). These results suggest that hemoglobin is degraded to small peptides in the food vacuole, that these peptides are transported to the parasite cytosol, and that additional processing of hemoglobin peptides is mediated by cytosolic aminopeptidase activity (77).

Drug Development Efforts

Both the cysteine protease inhibitor E-64 and the aspartic protease inhibitor pepstatin blocked *P. falciparum* development (63-67). Administered together, the two inhibitors acted synergistically (67). However, only E-64 blocked globin hydrolysis (64-67). Numerous peptide-based cysteine protease inhibitors, including fluoromethyl ketones (44,70,78) and vinyl sulfones (46), inhibited falcipain at low nanomolar concentrations and inhibited *P. falciparum* development and hemoglobin degradation at concentrations below 100 nanomolar (Table 3). In a malaria animal model, a fluoromethyl ketone that inhibited falcipain at low nanomolar concentrations blocked *P. vinckei* protease activity in vivo after a single subcutaneous dose, and, when administered for 4 days, cured 80% of murine malaria infections (45). Thus, despite the theoretical limitations of potentially rapid degradation in vivo and inhibition of host proteases, peptide protease inhibitors show promise as candidate antimalarial drugs. Fluoromethyl ketones have subsequently shown toxicity in animal studies, but evaluations of related, apparently nontoxic inhibitors of falcipain as antimalarial drugs are under way.

A computer model for the structure of falcipain was used to identify nonpeptide inhibitors (47). Screening of potential nonpeptide inhibitors identified a low micromolar lead compound (47; Table 3). Subsequent synthesis and testing of small molecules based on the structure of the lead compound have identified biologically active falcipain inhibitors, including chalcones that block parasite metabolism at submicromolar concentrations (48) and phenothiazines that block parasite metabolism and development at low micromolar concentrations (49).

Peptidelike aspartic protease inhibitors are potent inhibitors of plasmepsins I and II. In independent studies SC-50083 (41), Ro 40-4388 (42), and "compound 7" (43) inhibited plasmepsin I or II at nanomolar concentrations and blocked parasite development at high nanomolar to micromolar concentrations (Table 3). Drug development efforts should be assisted by the recent determination of the structure of plasmepsin II (43). Inhibitors of aspartic and cysteine proteases have synergistic effects in inhibiting the growth of cultured malaria parasites (67), and these proteases also act synergistically to degrade hemoglobin in vitro (41). Therefore, the combination of inhibitors of malarial cysteine and aspartic proteases may provide the most effective chemotherapeutic regimen and best limit the development of parasite resistance to protease inhibitors. Ultimately, a better understanding of the biochemical properties and biologic roles of malarial proteases will foster the development of protease inhibitors that specifically inhibit parasite enzymes and thus are the most suitable candidates for chemotherapy.

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Zoonotic Tuberculosis due to *Mycobacterium bovis* in Developing Countries

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The World Health Organization (WHO) estimates that human tuberculosis (TB) incidence and deaths for 1990 to 1999 will be 88 million and 30 million, respectively, with most cases in developing countries. Zoonotic TB (caused by *Mycobacterium bovis)* is present in animals in most developing countries where surveillance and control activities are often inadequate or unavailable; therefore, many epidemiologic and public health aspects of infection remain largely unknown. We review available information on zoonotic TB in developing countries, analyze risk factors that may play a role in the disease, review recent WHO activities, and recommend actions to assess the magnitude of the problem and control the disease in humans and animals.

Tuberculosis (TB), one of the most widespread infectious diseases, is the leading cause of death due to a single infectious agent among adults in the world. *Mycobacterium tuberculosis* is the most common cause of human TB, but an unknown proportion of cases are due to *M. bovis* (1). In industrialized countries, animal TB control and elimination programs, together with milk pasteurization, have drastically reduced the incidence of disease caused by *M. bovis* in both cattle and humans. In developing countries, however, animal TB is widely distributed, control measures are not applied or are applied sporadically, and pasteurization is rarely practiced. The direct correlation between *M. bovis* infection in cattle and disease in the human population has been well documented in industrialized countries. Whereas little information is available from developing countries (2,3), risk factors for *M. bovis* in both animals and humans are present in the tropics.

TB is a major opportunistic infection in HIV-infected persons (4). The vast majority of people carrying this dual infection live in developing countries; however, dual HIV and *M. bovis* infection has been reported in industrialized countries (5-11). The epidemic of HIV infection in developing countries, particularly countries in which *M. bovis* infection is present in animals and the conditions favor zoonotic transmission, could make zoonotic TB a serious public health threat to persons at risk (3,12-14).

We summarize available epidemiologic information on TB and zoonotic TB, examine risk factors that can influence the occurrence of zoonotic TB in developing countries, and describe the most recent TB activities of the World Health Organization (WHO) (15-18).

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Human TB: Global Situation and Trends

The global incidence of TB is greatly underestimated. In 1995, 3.3 million cases were reported to the Global Tuberculosis Programme of WHO, whereas a more likely number is 8.8 million. Of the reported cases, 62% occurred in the Southeast Asian and Western Pacific regions, 16% in sub-Saharan Africa, and 7% to 8% in each of the regions of the Americas, Eastern Mediterranean, and Europe. Many countries, especially those with few resources, are unable to report all TB cases because of difficulties in identifying suspected cases, establishing a diagnosis, and recording and reporting cases.

In 1995, an estimated 8.8 million new TB cases occurred—5.5 million (62%) in the Southeast Asian and Western Pacific regions and 1.5 million (17%) in sub-Saharan Africa. The annual global incidence is predicted to increase to 10.2 million by the year 2000, an increase of 36% from 1990. Southeast Asia, Western Pacific regions, and sub-Saharan Africa will account for 81% of these new cases (Table 1). For 1990 to 1999, in the absence of effective control, global TB incidence and deaths will reach 88 million and 30 million, respectively (19); 70% of the new cases will occur in patients 15 to 59 years of age, the most economically productive segment of the population.

As a result of the HIV epidemic, the crude incidence rate of TB is expected to increase in sub-Saharan Africa from 191 cases per 100,000 in 1990 to 293 in 2000. However, the total number of new cases will double by the year 2000. Because of the HIV epidemic, the decline of the crude incidence rate in the Southeast Asian and Central and South American regions is expected to be slower than in previous years. In industrialized countries, a small increase in crude incidence rate and total cases is expected as the result of immigration from countries with a high prevalence of dual HIV and TB infection.

The worldwide incidence of HIV-attributable TB cases is estimated to increase from 315,000 (4% of the total TB cases) in 1990 to 1.4 million (14% of the total TB cases) by the year 2000. In 2000, approximately 40% of these HIV-attributable cases will occur in sub-Saharan Africa and 40% in Southeast Asia. Ten percent of the total number of TB cases expected during 1990 to 1999 are estimated to be attributable to HIV infection.

While demographic factors, such as population growth and changes in population structure, will largely account for the expected increase in TB incidence worldwide, the HIV epidemic in sub-Saharan Africa will have a greater role than demographic factors.

By the year 2000, 3.5 million persons will be dying of TB annually, an increase of 39% from 1990. In Southeast Asia alone, 1.4 million deaths will occur annually. During 1990 to 1999, an estimated 30 million will die of TB, with 9.7% of the cases attributable to HIV infection. *M. tuberculosis* will be largely responsible for the new TB

Table 1. Estimated human tuberculosis and HIV-attributable tuberculosis cases in 1990, 1995, and 2000, by region (19)

	1990			1995			2000		
Region	TB cases	Rate[a]	HIV-attributed	TB cases	Rate	HIV-attributed	TB cases	Rate	HIV-attributed
Southeast Asia	3,106,000	237	66,000	3,499,000	241	251,000	3,952,000	247	571,000
Western Pacific[b]	1,839,000	136	19,000	2,045,000	140	31,000	2,255,000	144	68,000
Africa	992,000	191	194,000	1,467,000	242	380,000	2,079,000	293	604,000
Eastern Mediterranean	641,000	165	9,000	745,000	168	16,000	870,000	168	38,000
Americas[c]	569,000	127	20,000	606,000	123	45,000	645,000	120	97,000
Eastern Europe[d]	194,000	47	1,000	202,000	47	2,000	210,000	48	6,000
Industrialized countries[e]	196,000	23	6,000	204,000	23	13,000	211,000	24	26,000
Total TB cases	7,537,000	143	315,000	8,768,000	152	738,000	10,222,000	163	1,410,000
Attributed to HIV			4.2%			8.4%			13.8%
Increase since 1990				16.3%			35.6%		

[a]Rate: incidence of new cases per 100,000 population.
[b]Western Pacific Region of WHO except Japan, Australia, and New Zealand.
[c]American Region of WHO, except USA and Canada.
[d]Eastern European countries and independent states of the former USSR.
[e]Western Europe, USA, Canada, Japan, Australia, and New Zealand.

cases and deaths, but an unknown, and potentially important, proportion will be caused by *M. bovis*.

Bovine TB in Developing Countries

Although prevalence data on animal TB in developing countries are generally scarce, information on bovine TB occurrence and control measures exists (20,21).

Africa

Of 55 African countries, 25 reported sporadic/low occurrence of bovine TB; six reported enzootic disease; two, Malawi and Mali, were described as having a high occurrence; four did not report the disease; and the remaining 18 countries did not have data (Figure 1).

Of all nations in Africa, only seven apply disease control measures as part of a test-and-slaughter policy and consider bovine TB a notifiable disease; the remaining 48 control the disease inadequately or not at all (Figure 2). Almost 15% of the cattle population are found in countries where bovine TB is notifiable and a test-and-slaughter policy is used. Thus, approximately 85% of the cattle and 82% of the human population of Africa are in areas where bovine TB is either partly controlled or not controlled at all.

Asia

Of 36 Asian nations, 16 reported a sporadic/low occurrence of bovine TB, and one (Bahrain) described the disease as enzootic; ten did not report bovine TB; and the remaining nine did not have data (Figure 3). Within the Asian region, seven countries apply disease control measures as part of a test-and-slaughter policy and consider bovine TB notifiable. In the remaining 29 countries, bovine TB is partly controlled or not controlled at all (Figure 4).

Of the total Asian cattle and buffalo populations, 6% and less than 1%, respectively, are found in countries where bovine TB is notifiable and a test-and-slaughter policy is used; 94% of the cattle and more than 99% of the buffalo populations in Asia are either only partly controlled for bovine TB or not controlled at all. Thus, 94% of the human population lives in countries where cattle and buffaloes undergo no control or only limited control for bovine TB.

Latin American and Caribbean Countries

Of 34 Latin American and Caribbean countries, 12 reported bovine TB as sporadic/low occurrence, seven reported it as enzootic, and one (Dominican Republic) described occurrence as

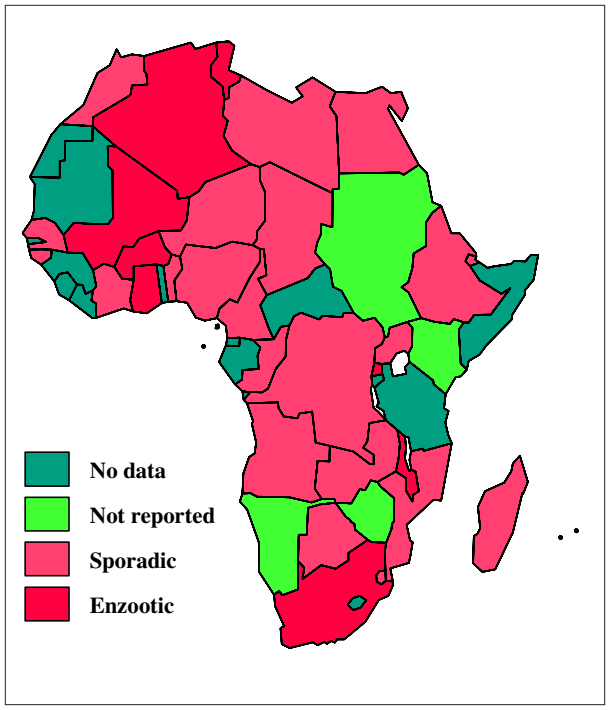

Figure 1. Bovine tuberculosis occurrence, Africa (21).

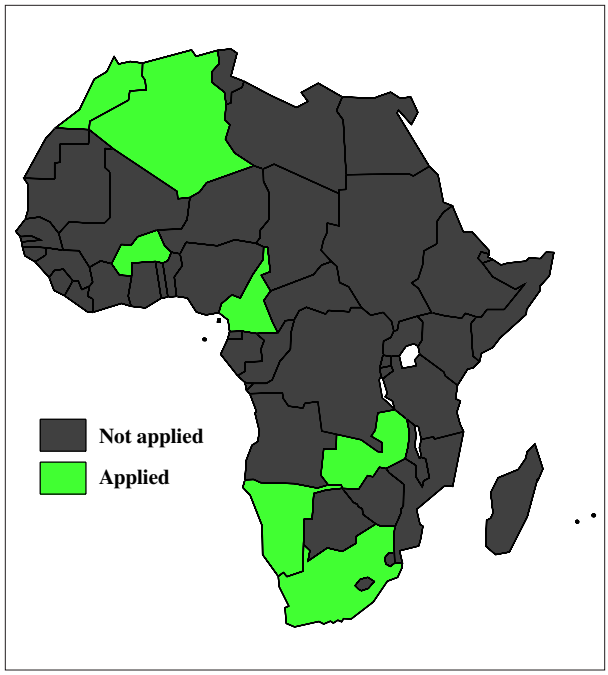

Figure 2. Control measures for bovine tuberculosis based on test-and-slaughter policy and disease notification, Africa (21).

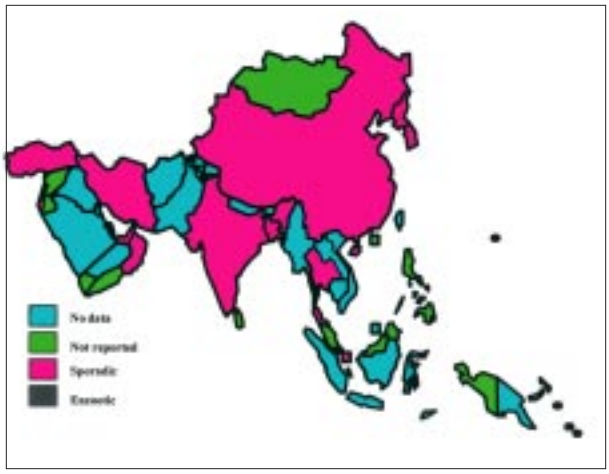

Figure 3. Bovine tuberculosis occurrence, Asia (21).

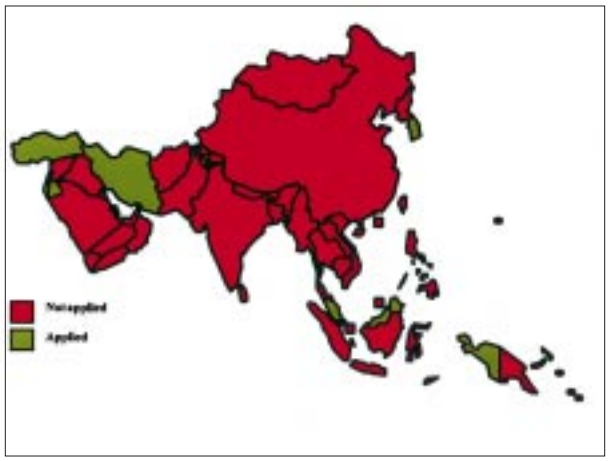

Figure 4. Control measures for bovine tuberculosis based on test-and-slaughter policy and disease notification, Asia (21).

high. Twelve countries did not report bovine TB. No data were available for the remaining two countries (Figure 5).

In the entire region, 12 countries apply disease control measures as part of a test-and-slaughter policy and consider bovine TB a notifiable disease. In the remaining 22 nations, the disease is partly controlled or not controlled at all (Figure 6). The regional prevalence of bovine TB has been estimated at 1% and higher in 67% of the total cattle population and 0.1% to 0.9% in a further 7%; the remaining 26% are free of the disease or are approaching the point of elimination (22).

Of the total Latin American and Caribbean cattle population, almost 76% is in countries where bovine TB is notifiable and a test-and-slaughter

Figure 5. Bovine tuberculosis occurrence, Latin America and the Caribbean (21).

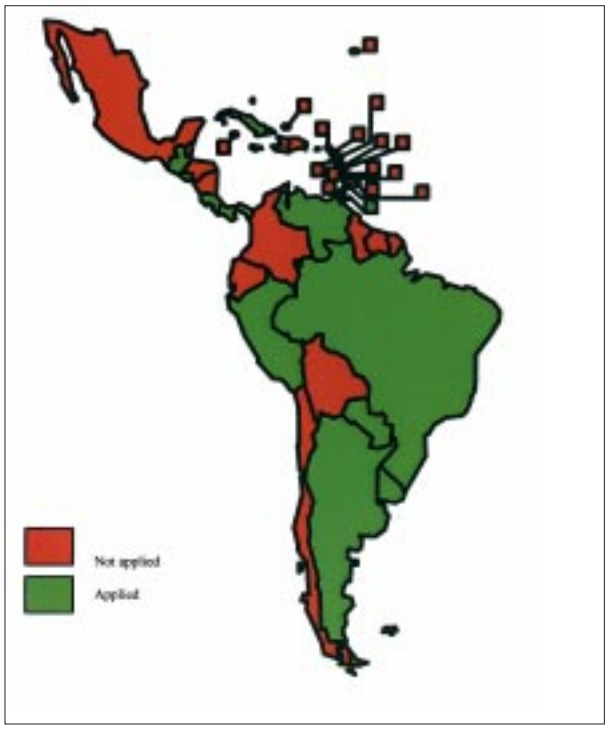

Figure 6. Control measures for bovine tuberculosis based on test-and-slaughter policy and disease notification, Latin America and the Caribbean (21).

policy is used. Thus, approximately 24% of the cattle population in this region is either only partly controlled for bovine TB or not controlled at all. It is also estimated that 60% of the human population live in countries where cattle undergo no control or only limited control for bovine TB.

Zoonotic TB in Humans

TB caused by *M. bovis* is clinically indistinguishable from TB caused by *M. tuberculosis*. In countries where bovine TB is uncontrolled, most human cases occur in young persons and result from drinking or handling contaminated milk; cervical lymphadenopathy, intestinal lesions, chronic skin TB (lupus vulgaris), and other nonpulmonary forms are particularly common. Such cases may, however, also be caused by *M. tuberculosis*. Little is known of the relative frequency with which *M. bovis* causes nonpulmonary TB in developing nations because of limited laboratory facilities for the culture and typing of tubercle bacilli.

Agricultural workers may acquire the disease by inhaling cough spray from infected cattle; they develop typical pulmonary TB. Such patients may infect cattle, but evidence for human-to-human transmission is limited and anecdotal.

In regions where bovine TB has been largely eliminated, a few residual cases occur among elderly persons as a result of the reactivation of dormant lesions. These are fewer than 1% of all TB cases. Surveys in the United States, Scandinavia, and South England have shown that approximately half of these postprimary cases are pulmonary, a quarter involve the genitourinary tract (a rare occurrence in primary disease), and the remainder involve other nonpulmonary sites, notably cervical lymph nodes (23). In the same regions, approximately 10% of cases caused by *M. tuberculosis* are nonpulmonary, although, for reasons that are not clear, the incidence is higher, approximately 20%, in ethnic minority populations.

Information on human disease due to *M. bovis* in developed and developing countries is scarce. From a review of a number of zoonotic tuberculosis studies, published between 1954 and 1970 and carried out in various countries around the world, it was estimated that the proportion of human cases due to *M. bovis* acccounted for 3.1% of all forms of tuberculosis: 2.1% of pulmonary forms and 9.4% of extrapulmonary forms (24). Table 2 summarizes the findings of more recent reports of TB caused by *M. bovis* in industrialized countries.

Table 2. Human tuberculosis due to *Mycobacterium bovis*, industrialized countries

		Cases		
Country (ref.)	Years	No.	% of total TB	Pulmonary (% of total *M. bovis*)
Australia (25)	1970-94	240	0.43-3.1	71.6[a]
England (23)	1977-90	232	1.2	40.0
Germany (26)	1975-80	236	4.5	73.7
Ireland				
Rural (27)	1986-90	17	6.4	70.6
Urban (28)	1982-85	9	0.9	88.8
New Zealand (29)	1983-90	22	7.2	31.8
Spain (30)	1986-90	10	0.9	50.0
Sweden (17)	1983-92	96	2.0	-
Switzerland (31)	1994	18	2.6	-
U.S. (32)	1954-68	6	0.3	33.3
U.S. (9)	1980-91	73	3.0	52.0[b] 12.0[c]

[a] Overall percentage includes 80.6 % males and 51.2% females.
[b]Adults.
[c]Children.

Human disease caused by *M. bovis* has been confirmed in African countries. In an investigation by two Egyptian health centers, the proportions of sputum-positive TB patients infected with *M. bovis*, recorded during three observations, were 0.4%, 6.4%, and 5.4% (33). In another study in Egypt, nine of 20 randomly selected patients with TB peritonitis were infected with *M. bovis,* and the remaining with *M. tuberculosis* (34).

Isolation of *M. bovis* from sputum samples of patients with pulmonary TB has also been reported from Nigeria. Of 102 *M. tuberculosis* complex isolates, 4 (3.9%) were *M. bovis* (35). Another study in Nigeria reported that one of 10 mycobacteria isolated from sputum-positive cultures was *M. bovis* (36).

In a Zaire study, *M. bovis* was isolated from gastric secretions in two of five patients with pulmonary TB (37). In the same study, the prevalence of the disease in local cattle was approximately 8% by tuberculin testing and isolation of *M. bovis*.

In a recent investigation in Tanzania, seven of 19 lymph node biopsies from suspected extrapulmonary TB patients were infected with *M. tuberculosis* and four with *M. bovis* (14). No mycobacteria were cultured from the remaining

Table 3. Isolates from suspected extrapulmonary tuberculosis patients, Tanzania, 1994 (14)

Occupation	No. of samples	*M. tuberculosis*	*M. bovis*	Neg.
Livestock keeper	4	0	2	2
Farmers	6	2	1	3
Children	3	2	1	0
Unknown	6	3	0	3
Total	19	7	4	8

eight (Table 3). Although the number of samples was low, the high proportion (36%) of *M. bovis* isolates is of serious concern.

In an epidemiologic study in Zambia (38), an association between tuberculin-positive cattle and human TB was found. Households that reported a TB case within the previous 12 months were approximately seven times more likely to own herds containing tuberculin-positive cattle (odds ratio = 7.6; p = 0.004). Although this could be explained by zoonotic TB transmission, other factors such as transient sensitivity to tuberculin of cattle exposed to TB patients and coincidental environmental factors favoring both human clinical TB and sensitivity to bovine tuberculin should also be considered.

In Latin America, a conservative estimate would be that 2% of the total pulmonary TB cases and 8% of extrapulmonary TB cases are caused by *M. bovis.* These cases would therefore account for 7,000 new TB cases per year, a rate of nearly 2 per 100,000 inhabitants. From a nationwide study in Argentina during 1982 to 1984, 36 (0.47%) of 7,672 mycobacteria cultured from sputum samples were *M. bovis* (39). However, in another study in Santa Fe province (where most of the dairy cattle industry is concentrated) during 1984 to 1989, *M. bovis* caused 0.7% to 6.2% of TB cases (40).

Very limited data on the zoonotic aspects of *M. bovis* are available from Asian countries. However, cases of TB caused by *M. bovis* were not reported in early investigations in India (41).

Epidemiology

Much information on the epidemiologic patterns of zoonotic TB has been obtained in this century from industrialized countries. However, some striking epidemiologic differences related to both animal and human populations in developing countries require particular attention.

Risk Factors: Animal Population

Animal reservoirs. The widespread distribution of *M. bovis* in farm and wild animal populations represents a large reservoir of this microorganism. The spread of the infection from affected to susceptible animals in both industrialized and developing countries is most likely to occur when wild and domesticated animals share pasture or territory (42). Well-documented examples of such spread include infection in badgers (*Meles meles*) in the United Kingdom and possums (*Trichosurus vulpecula*) in New Zealand. Wild animal TB represents a permanent reservoir of infection and poses a serious threat to control and elimination programs.

Milk production and animal husbandry. Milk production has increased in most developing countries as a consequence of greater demand for milk for human consumption (43; Figure 7). This increased demand for milk—estimated at 2.5% per year for 1970 to 1988 for sub-Saharan Africa (44)—led to increases in the number of productive animals and milk imports and intensification of animal production through the introduction of more productive exotic breeds.

Although the prevalence of the disease within a country varies from area to area, the highest incidence of bovine TB is generally observed

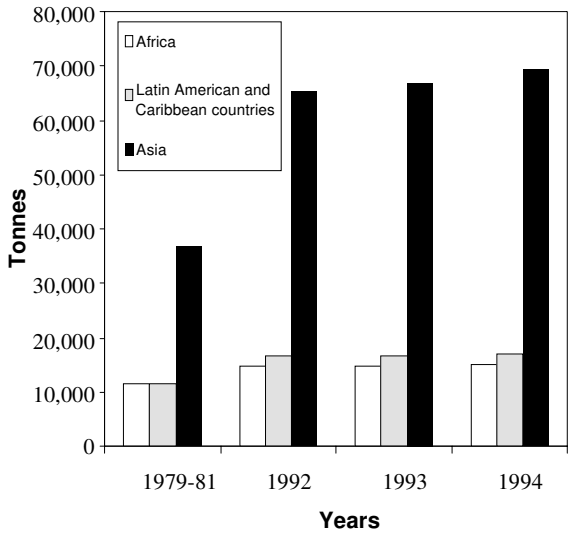

Figure 7. Cow milk production by region (43).

where intensive dairy production is most common, notably in the milksheds of larger cities (1). This problem is exacerbated where there is inadequate veterinary supervision, as is the case in most developing countries. In addition, in some industrialized countries such as the United States, where bovine TB is close to elimination, large dairy herds (i.e., 5,000 or more cows) that are crowded together represent the main source of infection (45).

In developing countries, bovine TB infects a higher proportion of exotic dairy breeds (*Bos taurus*) than indigenous zebu cattle (*Bos indicus*) and crossbred beef cattle (1). However, under intensive feedlot conditions, a death rate of 60% and depression of growth have been found in tuberculous zebu cattle (46). In those areas where extensive management is more common, animal crowding (e.g., near watering ponds, dip tanks, markets, and corrals) still plays a major role in the spread of the disease.

Control measures and programs. The basic strategies required for control and elimination of bovine TB are well known and well defined (47). However, because of financial constraints, scarcity of trained professionals, lack of political will, as well as the underestimation of the importance of zoonotic TB in both the animal and public health sectors by national governments and donor agencies, control measures are not applied or are applied inadequately in most developing countries.

Successful conduct of a test-and-slaughter policy requires sustained cooperation of national and private veterinary services, meat inspectors, and farmers, as well as adequate compensation for services rendered. Only a few developing countries can adhere to these requirements.

In addition, bovine TB does not often justify the emergency measures required for other zoonotic diseases (e.g., Rinderpest, East Coast fever, and foot and mouth disease). The full economic implications of zoonotic TB are, however, overlooked in many developing nations where the overall impact of the disease on human health and animal production needs to be assessed. According to recent estimates, annual economic loss to bovine TB in Argentina is approximately 63 million US dollars (48). In a study recently conducted in Turkey, the estimated socioeconomic impact of bovine TB to both the agriculture and health sectors was approximately 15 to 59 million US dollars per year (49).

Several Latin American countries, through agreements between governments and cattle owners associations, have made the decision to control and eliminate bovine TB. Where foot and mouth disease has been eliminated, bovine TB and other existing infections such as brucellosis become important because of their impact on the meat and live animal export trade. Bovine TB and brucellosis also limit the development of the dairy industry and its expansion at the regional level.

Risk Factors: Human Population

Close physical contact. Close physical contact between humans and potentially infected animals is present in some communities, especially in developing regions. For example, in many African countries cattle are an integral part of human social life; they represent wealth and are at the center of many events and, therefore, gatherings. In addition, with 65% of African, 70% of Asian, and 26% of Latin American and Caribbean populations working in agriculture, a significant proportion of the population of these regions may be at risk for bovine TB.

Food hygiene practices. Consumption of milk contaminated by *M. bovis* has long been regarded as the principal mode of TB transmission from animals to humans (1). In regions where bovine TB is common and uncontrolled, milkborne infection is the principal cause of cervical lymphadenopathy (scrofula) and abdominal and other forms of nonpulmonary TB. Although proper food hygiene practices could play a major role in controlling these forms of TB, such practices are often difficult to institute in developing countries.

In all countries of sub-Saharan Africa, there is active competition between large-scale, often state-run, processing and marketing enterprises and the informal sector. The informal sector can ignore standards of hygiene and quality, and producers often sell directly to the final consumers. In addition, an estimated 90% of the total milk produced is consumed fresh or soured (44). Although it has been stated that Africans generally boil milk and that the souring process destroys *M. bovis* (44), other sources strongly contradict these statements (39). *M. bovis* was

isolated from seven (2.9%) of 241 samples of raw milk in Ethiopia (17). Both *M. bovis* and *M. tuberculosis* have also been found in milk samples in Nigeria (36) and Egypt (34). Thus, serious public health implications of potentially contaminated milk and milk products should not be underestimated.

HIV/AIDS. According to recent WHO global estimates, of the 9.4 million people infected with both HIV and TB in mid-1996, 6.6 million (70%) live in sub-Saharan Africa (4). The greatest impact of HIV infection on TB is in populations with a high prevalence of TB infection among young adults. The occurrence of both infections in one person makes TB infection very likely to progress to active disease.

In many developing countries, TB is the most frequent opportunistic disease associated with HIV infection. HIV seroprevalence rates greater than 60% have been found in TB patients in various African countries (4). Persons infected with both pathogens have an annual risk of progression to active TB of 5% to 15%, depending on their level of immunosuppression; approximately 10% of non-HIV infected persons newly infected with TB become ill at some time during their lives. In the remaining 90%, effective host defenses prevent progression from infection to disease.

TB cases due to *M. bovis* in HIV-positive persons also resemble disease caused by *M. tuberculosis*. Thus, they manifest as pulmonary disease, lymphadenopathy, or, in the more profoundly immunosuppressed, disseminated disease.

M. bovis has been isolated from HIV-infected persons in industrialized countries. In France, *M. bovis* infection accounted for 1.6% of TB cases in HIV-positive patients. All isolated strains were resistant to isoniazid (7). Taking into consideration the intrinsic resistance of *M. bovis* to pyrazinamide, two of the first-line anti-TB drugs were not effective. WHO-recommended standard treatment for new TB cases includes, in the initial phase, isoniazid, rifampicin, pyrazinamide, and streptomycin or ethambutol. In situations of high primary resistance to isoniazid and streptomycin, the intrinsic resistance of *M. bovis* to pyrazinamide may severely limit the efficacy of treatment of TB caused by *M. bovis*.

In a Paris hospital, a source patient with pulmonary TB due to a multidrug-resistant strain of *M. bovis* led to active disease in five patients. Disease occurred 3 to 10 months after infection (10). This observation led to three concerns: 1) human-to-human *M. bovis* transmission leading to overt disease, 2) a short interval between infection and overt disease, and 3) disseminated multidrug-resistant *M. bovis.*

In another study, conducted in San Diego, California, one of 24 adults with pulmonary TB and 11 of 24 adults with nonpulmonary TB due to *M. bovis* had AIDS. One of 25 children, a 16-year-old boy with abdominal TB, was also HIV-positive (9).

It is commonly believed that *M. bovis* is less virulent than *M. tuberculosis* in humans and therefore less likely to lead to overt postprimary disease and that human-to-human transmission leading to infectious disease is rare. However, if the apparent difference in virulence is the result of differences in responsiveness of the host defense mechanisms, HIV-induced immunosuppression could well lower host defenses leading to overt disease after infection.

Surveillance of TB due to *M. bovis*

The use of direct smear microscopy as the only method for diagnosis of suspected TB, although an essential requirement of any national TB program, could partly explain the relatively low notification rate of disease caused by *M. bovis* in developing countries. Direct smear microscopy does not permit differentiation between species of the *M. tuberculosis* complex; in addition, culture and speciation are often not carried out, and even when culture facilities are available, *M. bovis* grows poorly in standard Löwenstein-Jensen medium, one of the most widely used culture media (50). In some countries, human disease caused by *M. bovis* is merely reported as TB to avoid inquiries from disease control agencies, which might generate problems of patient confidentiality (2).

The collection of representative data on the incidence of TB due to *M. bovis* from most laboratories in developing countries has additional problems. For example, the location and coverage of laboratories are often biased towards city populations; sputum specimens may predominate, with relatively few specimens from extrapulmonary lesions, particularly among children. Specimens from children with TB are frequently negative on culture, and biopsies are difficult to take from lesions.

Recent outbreaks of multidrug-resistant TB in some parts of the world underscore the need for

surveillance through wider application of reliable culture and drug susceptibility tests.

Control Measures and Programs in Developing Countries

Bovine TB can be eliminated from a country or region by implementing a test-and-slaughter policy, if no other reservoir host of infection exists. While the test-and-slaughter policy is likely to remain the backbone of national elimination bovine TB programs, the policy has numerous constraints in developing countries. Alternative strategies (e.g., programs based on slaughterhouse surveillance and traceback of tuberculous animals to herds of origin) may be technically and economically more appropriate in these countries.

Measures to prevent transmission of infection should be the primary objective to be achieved with trained public health personnel, public education, and proper hygienic practices. Test-and-slaughter programs may be feasible and appropriate in areas with low bovine TB prevalence and effective control of animal movement.

Animal Vaccination and Research Developments

Although not usually considered relevant to elimination programs in livestock (47), vaccination of animals against TB would be a viable strategy in two disease control situations: in domesticated animals in developing countries and in wildlife and feral reservoirs of disease in industrialized countries where test-and-slaughter programs have failed to achieve elimination of the disease.

Many issues need to be addressed before vaccination becomes a realistic option for control of disease in cattle and other animals. First, a highly effective vaccine needs to be developed. The results obtained globally with bacillus Calmette-Guérin (BCG) have been suboptimal, and efficacy has varied considerably from region to region (42,51). Secondly, the delivery of the vaccine poses few problems in domesticated animals, but it is fraught with difficulties in wild animals. Thirdly, vaccination may compromise diagnostic tests. A vaccine that induces tuberculin reactivity would invalidate the key diagnostic tool used in control programs. Fourthly, short of performing lengthy and expansive field studies, evaluation of the protective efficacy of a new vaccine will pose serious difficulties. Traditionally, the guinea pig and mouse have been used for this purpose, but the information gained has been of little value. Recent work has, however, indicated that deer may well prove a suitable mammal for evaluating new vaccines and optimum delivery systems (52).

Enzyme-linked immunosorbent assay and gamma-interferon tests may prove to be more sensitive and specific than the tuberculin test and may facilitate diagnostic procedures. Nucleic acid-based technology, notably polymerase chain reaction and related methods, may provide more rapid, sensitive, and specific diagnostic tools. Multicenter studies of the applicability of these techniques to the diagnosis of human TB have, however, shown that their sensitivity and specificity are not as high as originally expected and that many problems need to be solved before the techniques are introduced into routine laboratory practice (53). Restriction fragment length polymorphism analysis (DNA fingerprinting) could be useful in epidemiologic studies that trace the spread of disease between cattle, other animals, and humans (54) or in the rapid differentiation of *M. bovis* within the *M. tuberculosis* complex (55). The use of these techniques is limited by resources in most developing countries.

WHO and Zoonotic TB

The public health importance of animal TB was recognized early by WHO, which in its 1950 report of the Expert Committee on Tuberculosis (56) stated: "The committee recognizes the seriousness of human infection with bovine tuberculosis in countries where the disease in cattle is prevalent. There is the danger of transmission of infection by direct contact between diseased cattle and farm workers and their families, as well as from infected food products." Since then, TB in animals has been controlled and almost eliminated in several industrialized countries but in very few developing countries.

More recently, WHO has been involved in zoonotic TB through the activities of the Division of Emerging and other Communicable Diseases Surveillance and Control at WHO in Geneva (WHO/EMC) and the Veterinary Public Health program of the WHO Regional Office for the Americas, Pan American Health Organization (PAHO/HCV).

WHO/EMC has organized and coordinated a working group of experts from countries

worldwide (15-17). Their subjects are epidemiology, public health aspects, control, and research on zoonotic TB. In addition, a joint WHO, Food and Agriculture Organization of the United Nations (FAO), and Office International des Epizooties (OIE) Consultation on Animal Tuberculosis Vaccines was held to review current knowledge on TB vaccine development for humans and animals and make recommendations for animal TB vaccine research and development (57). Promising results of cattle vaccination with low doses of BCG were reported. It is also planned for field trial cattle vaccination to commence early in 1998 in Madagascar in collaboration with national and international research institutions, OIE and WHO. In the framework of the working group activities, the guidelines for speciation within the *Mycobacterium tuberculosis* complex (50) have been prepared to respond to the growing need for reliable differentiation between *M. tuberculosis*, *M. africanum,* and *M. bovis* and to promote and strengthen surveillance.

A Plan of Action for the Eradication of Bovine Tuberculosis in the Americas (18) has been developed by PAHO in collaboration with member countries of the region. PAHO/HCV, in cooperation with the Pan American Institute for Food Protection and Zoonosis (INPPAZ), Buenos Aires, Argentina, and other technical institutions (e.g., FAO), provides technical support to the regional plan. PAHO/HCV activities train specialists in diagnosis, reporting, surveillance systems, and quality control of reagents, as well as supporting the planning and implementation of national programs. INPPAZ acts as a reference center for these activities. The first phase of the regional plan is expected to lead, in the next 10 years, to the elimination of bovine TB from countries with more advanced national programs. In the remaining countries, the objectives will be to strengthen epidemiologic surveillance, defining areas at risk and setting up control and elimination programs.

Conclusions

Although the epidemiology of bovine TB is well understood and effective control and elimination strategies have been known for a long time, the disease is still widely distributed and often neglected in most developing countries. Its public health consequences, although well documented from the past experiences of industrialized countries, have scarcely been investigated and are still largely ignored in these regions. Because of the animal and public health consequences of *M. bovis,* disease surveillance programs in humans should be considered a priority, especially in areas where risk factors are present. The increase of TB in such areas calls for stronger intersectoral collaboration between the medical and veterinary professions to assess and evaluate the scale of the problem, mostly when zoonotic TB could represent a significant risk, for example, in rural communities and in the workplace.

Industrialized countries, where the test-and-slaughter policies have not completely eliminated infection in cattle because of wild animal reservoirs, are now reconsidering wild animal vaccination. Any vaccination research and development program should therefore also take into account the possible application of vaccines to cattle, particularly in developing countries.

In developing countries, where HIV and bovine TB are likely to be common, particularly in young persons, the ability of HIV infection to abrogate any host factors that prevent the progression of infection by *M. bovis* to overt disease may lead to higher incidence and case-fatality rates for human TB caused by this species and increased human-to-human transmission of this disease. This should be of great concern in those developing countries where bovine TB is present and measures to control spread of infection are not applied or are applied inadequately. Research is needed to determine when *M. bovis* is of zoonotic importance and what the underlying mechanisms of transmission are. Locally operative risk factors for zoonotic TB should therefore be identified to determine persons at risk and develop appropriate control measures. International cooperation in all aspects of zoonotic TB remains essential in the fight against this disease.

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What Makes *Cryptococcus neoformans* a Pathogen?

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Life-threatening infections caused by the encapsulated fungal pathogen *Cryptococcus neoformans* have been increasing steadily over the past 10 years because of the onset of AIDS and the expanded use of immunosuppressive drugs. Intricate host-organism interactions make the full understanding of pathogenicity and virulence of *C. neoformans* difficult. We discuss the current knowledge of the characteristics *C. neoformans* must possess to enter the host and establish progressive disease: basic growth requirements and virulence factors, such as the polysaccharide capsule; shed products of the organism; melanin production; mannitol secretion; superoxide dismutase; proteases; and phospholipases.

Cryptococcus neoformans is an encapsulated fungal organism (Figure 1) that can cause disease in apparently immunocompetent, as well as immunocompromised, hosts (1,2). Most susceptible to infection are patients with T-cell deficiencies (1,2). *C. neoformans* var. *neoformans* causes most cryptococcal infections in humans, so this review will focus on information from the *neoformans* variety of this basidiomycetous fungus. *C. neoformans* var. *neoformans* is found worldwide; its main habitats are debris around pigeon roosts and soil contaminated with decaying pigeon or chicken droppings (1,3). Not part of the normal microbial flora of humans, *C. neoformans* is only transiently isolated from persons with no pathologic features (2,4). It is generally accepted that the organism enters the host by the respiratory route in the form of a dehydrated haploid yeast or as basidiospores. After some time in the lungs, the organism hematogenously spreads to extrapulmonary tissues; since it has a predilection for the brain, infected persons usually contract meningoencephalitis (1). If untreated, cryptococcal meningoencephalitis is 100% fatal, and even when treated with the most effective antifungal drugs, cryptococcal infections can be fatal if the host does not have adequate T-cell-dependent immune function (2).

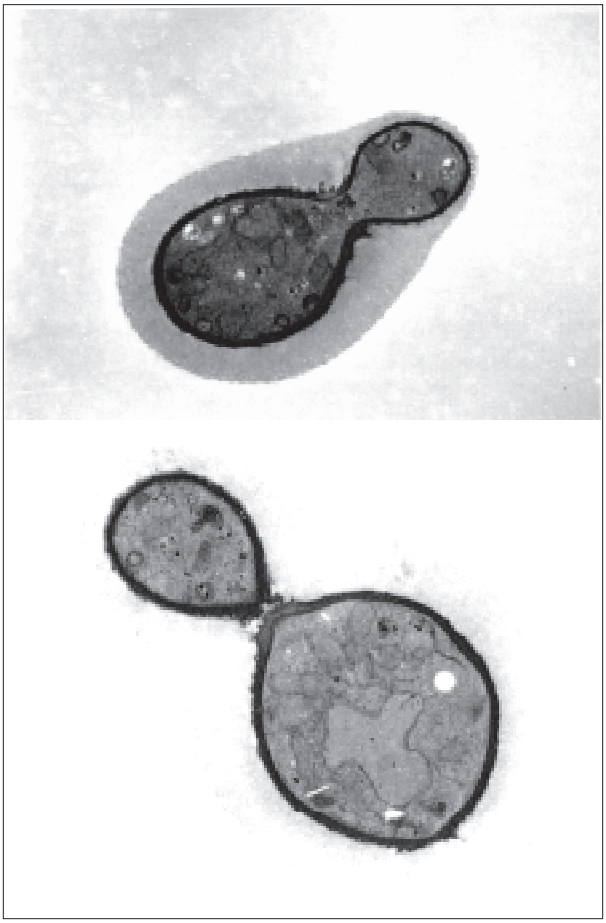

Figure 1. Transmission electron micrograph of budding *C. neoformans* showing the characteristic polysaccharide capsule.

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To be classified as a pathogen, an organism must be able to cause infection under certain conditions. By this definition, *C. neoformans* can certainly be classified as a pathogen. Because the immunodeficient are more susceptible than the immunocompetent to infection with this yeast-like organism, *C. neoformans* is frequently referred to as an opportunistic pathogen. The factors that make *C. neoformans* a pathogen can be divided into two major groups. The first comprises the basic characteristics needed to establish an infection and survive in the human host; the second comprises the virulence factors that affect the degree of pathogenicity.

Basic Characteristics for Pathogenicity

The Infectious Particle

To enter the alveolar spaces of the lungs and establish pulmonary infection, an organism must produce viable forms smaller than 4 µm in diameter. The typical vegetative form of *C. neoformans* is the yeast form with a cell diameter of 2.5 µm to 10 µm. The organism can also undergo sexual reproduction, and since it is a basidiomycete (*Filobasidiella neoformans*), it forms basidiospores. Sexual reproduction appears to occur much less frequently in nature than asexual or vegetative reproduction. The sexual spores (basidiospores) are approximately 1.8µm to 3µm in diameter and result from crosses of the α- and a-mating types on an appropriate medium (1). Although the exact nature of the infectious particles of *C. neoformans* is not known, they are presumed to be the dehydrated yeast cells or basidiospores of the appropriate size range to get into the lungs. Once inside the lungs, the yeast cells become rehydrated and acquire the characteristic polysaccharide capsule (Figure 2). In the case of basidiospores, they would convert to encapsulated blastoconidia.

Recently, Wickes et al. (5) reported that the α-mating type of *C. neoformans* can produce monokaryotic hyphae on a solid medium without a nitrogen source or water. The monokaryotic hyphae contain clamp connections and basidia with short chains of basidiospores and are similar in all respects except in nuclei number to the dikaryotic hyphae of the sexual forms (5). The haploid fruiting bodies formed by nitrogen-starved α-mating type isolates also produce abundant amounts of blastoconidia, which on the appropriate medium can produce haploid hyphae (5).

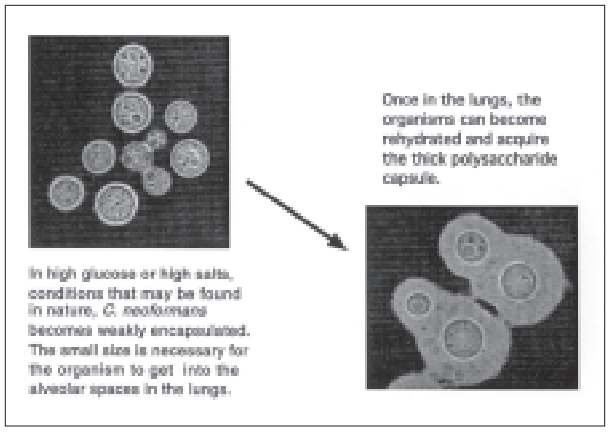

Figure 2. Proposed means of infection by *C. neoformans*.

None of the a-mating type strains tested produced the haploid fruiting bodies. Backcross studies indicated that the ability to undergo haploid fruiting lies within (or is tightly linked to) the *MAT*α locus, which is responsible for the α-mating type phenotype (5). These results suggest basidiospores from the monokaryotic hyphae may be another source of the infectious particle in nature. Regardless of the nature of the infectious particle (yeast cell or basidiospore), if a cryptococcal isolate is not able to produce small infectious particles, it cannot be pathogenic under the usual conditions for establishing the disease.

Mating Types

Kwon-Chung and Bennett (6) surveyed the mating types of *C. neoformans* isolates from environmental and clinical sources and found a 30- to 40-fold higher frequency of α-mating type than a-mating type. The skewed ratio of α-mating type to a-mating type was not thought to be due to a genetic preponderance towards α-mating type progeny because crosses between two test strains resulted in a 1:1 ratio of α-mating and a-mating type progeny (7). To examine the reason for the disparate ratio, Kwon-Chung and colleagues (8) constructed a pair of congenic strains of *C. neoformans* that differed only in their mating type. Survival studies with these congenic strains demonstrated that the α-mating type (B-4500) was significantly more virulent than the a-mating strain (B-4476) when injected intravenously into mice (8). Eighty percent of mice infected with 10^6 B-4500 (α-mating type) were

dead within 36 days; whereas, it took 93 days for 80% of mice infected with 10^6 B-4476 (a-mating type) to die (8). Although the mating type locus of *C. neoformans* has been cloned and partially characterized, the genes or gene products that contribute to the increased virulence of α-mating type isolates are not known.

The mating type locus (*MAT*α) cloned from an α-mating type *C. neoformans* isolate is 35 kb to 45 kb long (9). Moore and Edman (9) demonstrated that introducing a 2.1-kb fragment of the *MAT*α locus into an a-mating strain isolate stimulated filament formation on starvation medium. Basidia and basidiospores were not produced on the filaments. In contrast, electroporation of an α-mating strain with the same *MAT*α DNA fragment was ineffective in stimulating filament formation under the same growth conditions (9). Sequence analysis of the 2.1-kb fragment from the *MAT*α locus identified a 114-bp open reading frame that encodes a 38-amino acid propheromone peptide (9). The nature and the effects of the active pheromone peptide on mating and possibly virulence of *C. neoformans* are unknown; consequently, how the mating type relates to virulence at the genetic level is also unknown. However, the observation of haploid fruiting by α-mating type isolates of *C. neoformans* on nitrogen- and water-depleted medium (5) may help explain why the α-mating type isolates from clinical specimens are more frequent than the a-mating type strains (6).

Growth In Vivo

To cause infection in humans, a *C. neoformans* isolate must grow at 37°C in an atmosphere of approximately 5% CO_2 and at a pH of 7.3 to 7.4. To survive at 37°C, the organism must have an intact gene that encodes the *C. neoformans* calcineurin A catalytic subunit (10). Calcineurin is a serine-threonine specific phosphatase that is activated by Ca^{2+}-calmodulin and is involved in stress responses in yeasts (10). Although calcineurin A mutant strains of *C. neoformans* can grow at 24°C, they cannot survive in vitro at 37°C, in 5% CO_2, or at alkaline pH (10). Since these are similar to conditions in the host, one would predict that the calcineurin A mutant would not survive in the human host. In support of that prediction, Odom et al. have shown that such mutants are not pathogenic for immunosuppressed rabbits (10). Calcineurin A appears to be a basic requirement for *C. neoformans* survival in the host and consequently is a necessary factor for the pathogenicity of the organism.

Virulence Factors

Virulence factors increase the degree of pathogenicity of a microbe. *C. neoformans* has a number of virulence factors; generally, the virulence of an isolate cannot be attributed to any single factor, but rather it is attributed to many working in unison to cause progressive disease. As virulence factors go, those of *C. neoformans* would be considered low-grade. We will discuss each virulence factor separately; however, the severity of the host's disease results from a combination of several virulence factors superimposed on the host's innate and immune resistance status. The virulence factors that will be discussed are capsule, cryptococcal products, melanin production, mannitol production, and potential factors such as superoxide dismutase, proteases, phospholipase B, and lysophospholipase. The polysaccharide capsule and the soluble extracellular constituents of *C. neoformans* (referred to here as cryptococcal products) are probably the dominant virulence factors.

Capsule

C. neoformans has a capsule composed primarily of a high molecular weight polysaccharide that has a backbone of α-1,3-D-mannopyranose units with single residues of ß-D-xylopyranosyl and ß-D-glucuronopyranosyl attached (11-15). This polysaccharide is referred to as glucuronoxylomannan (GXM) (11) and has four serotypes: A and D, produced by *C. neoformans* var. *neoformans,* and B and C, produced by *C. neoformans* var. *gattii*. The evidence indicates that the capsule is a key virulence factor for *C. neoformans;* acapsular mutants are typically avirulent, whereas encapsulated isolates have varying degrees of virulence (16-19). Two capsular genes, *CAP59* and *CAP64,* have been described (20-22). Complementation of an acapsular, avirulent mutant with *CAP59* resulted in a virulent transformant with a capsular phenotype, and deletion of *CAP59* by homologous integration caused the encapsulated organism to become acapsular and avirulent (20). Similar observations have been made with the *CAP64* gene (21,22). Although their biochemical functions have not been defined, it appears that the two genes are essential for capsule formation and virulence.

Chemotactic Effects on Leukocytes

Some properties of the *C. neoformans* capsule enable the host to more effectively clear the organism from tissues; however, others protect the organism from host defenses. The capsules of serotype A and D isolates are chemotactic for neutrophils (23). Moreover, complement is fixed by cryptococcal capsules by the alternative pathway (24), and this process produces chemotactic peptides such as C5a (23). Chemotaxis of leukocytes induced by either of these mechanisms would be advantageous to the host.

Effects of Complement Interactions

Complement fixing by *C. neoformans* in tissue would result in chemotactic factor production and attraction of leukocytes into the infection site. Once in the infected tissue, the leukocytes would interact with and kill the organism. As complement is fixed, C3b and C3bi are deposited on the surface of the cryptococci (24). The capsule can mask the C3b and C3bi deposits (24-26); however, if they are not completely masked, the deposited complement components facilitate binding of the cryptococci to CR3 receptors on leukocytes (27). Such binding interactions are advantageous to the host; they enhance the opportunity for the leukocytes to kill the cryptococci either extracellularly or after phagocytosis. The organism can also be opsonized by antibodies to GXM, but the capsule may block the Fc portion of the antibody and prevent it from binding to Fc receptors on the phagocytic host cells (28). Some of these functions of the capsule favor the host; however, if the capsule is very large, the organism is protected (24-26). The cryptococci could deplete complement in the host, creating an environment that favors the cryptococci (29).

Effects on Phagocytosis

All considered, the capsule is more beneficial to the organism than to the host. Encapsulated *C. neoformans* cells are not phagocytized or killed by neutrophils, monocytes, or macrophages to the same degree as acapsular mutants (25,30-33). Encapsulated *C. neoformans* have a stronger negatively charged surface than acapsular cells or *Saccharomyces cerevisiae* cells (34). The high negative charge could cause electrostatic repulsion between the organism and the negatively charged host effector cells and reduce intimate cell-cell interactions required for clearance of the cryptococci (34).

Altered Antigen Presentation

The inability of macrophages to ingest the encapsulated organisms could also diminish antigen presentation to T cells and consequently reduce immune responses. This speculation has been experimentally demonstrated by Collins and Bancroft (35). Other studies with human alveolar macrophages have confirmed that antigen presentation is not as effective with encapsulated cryptococci as with acapsular strains (33,36). Unlike acapsular cells, encapsulated isolates cannot stimulate proliferative responses in T cells because of the reduced secretion of interleukin-1 (IL-1) by the antigen-presenting cells stimulated with the encapsulated yeasts (36).

Effects on Cytokine Production

In addition to being antiphagocytic, more resistant to killing, and less able to stimulate T-cell proliferation (25,30-33,35,36), highly encapsulated isolates of *C. neoformans* opsonized with a heat-labile serum component, presumably complement, do not stimulate monocytes and macrophages to produce proinflammatory cytokines such as TNFα, IL-1ß, and IL-6 as effectively as similarly opsonized nonencapsulated or weakly encapsulated isolates (37-41). The signal for induction of cytokine production can be a direct result of the attachment of the monocyte or macrophage to the acapsular cryptococci or can be an outcome of the phagocytic process, induced by acapsular cryptococci. Since the capsule blocks phagocytosis, any cytokines induced by the phagocytic process would not be induced by the encapsulated *C. neoformans* cells. If the cryptococcal cell wall materials must be exposed to induce cytokine production, the capsule would block the direct induction of cytokine production. Regardless of the mechanism of stimulation, the lack of production of proinflammatory cytokines could have a bearing on protection. TNFα is necessary for the induction of the protective immune response against *C. neoformans* (42). Consequently, the lack of or reduced production of TNFα in infections with highly encapsulated isolates of *C. neoformans* would prevent the induction of protective immunity, resulting in progressive disease. The roles of IL-1ß and IL-6 in protecting against *C. neoformans* have not been defined, but it is highly probable that the lack of these two cytokines could compromise the protective responses of the host and give cryptococci the advantage.

In contrast to the reduced TNFα, IL-1ß, and IL-6 produced by stimulating monocytes and macrophages with highly encapsulated cryptococci, IL-10 produced by these host cells increases after interaction with encapsulated strains (43). Neutralization of IL-10 with anti-IL-10 in cocultures of human peripheral blood mononuclear cells and encapsulated cryptococci increased the amounts of TNFα and IL-1ß produced, which indicates that the induction of IL-10 production by stimulating macrophages with encapsulated *C. neoformans* downregulates the production of the proinflammatory cytokines TNFα and IL-1ß (43). One might predict that the induction of high levels of IL-10 would also preferentially stimulate the induction of a T-helper 2 (Th2) response rather than a Th1 response in the T cells (44). Since the Th1 response is associated with protection against *C. neoformans* (45), increased levels of IL-10 would dampen induction of the protective immune response.

Encapsulated cryptococcal cells do not affect the different types of leukocytes in the same manner. Although encapsulated isolates do not stimulate macrophages to produce proinflammatory cytokines, they do stimulate neutrophils to produce proinflammatory cytokines and the chemotactic factor IL-8 more effectively than weakly encapsulated or acapsular organisms (46). As with the stimulation of macrophages by acapsular cryptococci to produce proinflammatory cytokines, serum is required for the encapsulated organisms to induce neutrophils to produce cytokines (46). In the case of cytokine production by neutrophils in response to encapsulated *C. neoformans,* it appears that the opsonization process releases a factor into the supernatant that induces the neutrophils to produce the cytokines (46). How these opposing in vitro observations with macrophages and neutrophils relate to the in vivo system or pathogenicity is yet to be determined.

Cryptococcal Products

In disseminated cryptococcosis, measurable levels of cryptococcal products are present in the body fluids of the patients (47). Although GXM is the major cryptococcal component in body fluids, it is highly probable that the organism also sheds galactoxylomannan (GalXM) and mannoproteins (MP) in vivo. This speculation is based on indirect evidence from in vivo studies and on the fact that GalXM and MP are in culture medium when the organism is grown in vitro (15,48). Cryptococcal antigens in serum or spinal fluid are diagnostic for cryptococcosis (47). Furthermore, if disseminated cryptococcosis patients have high cryptococcal antigen titers at the onset of therapy, they are less likely to respond to therapy or more likely to die before therapy is completed than patients with low cryptococcal antigen titers (49). The direct relationship of cryptococcal antigen levels in body fluids and the severity of disease suggests that the cryptococcal antigens in the host's circulatory system or spinal fluid may have adverse effects on host defenses.

Effects on Leukocyte Migration

It has been recently demonstrated in the mouse model that intravascular cryptococcal antigens inhibit the migration of leukocytes from the bloodstream into an inflammatory site (50). Intravascular cryptococcal antigens significantly reduce leukocyte infiltration into a site of acute inflammation induced by such stimuli as cryptococcal culture filtrate antigen, TNFa, or the chemotactic peptide FMLP (formylmethionyl leucyl phenylalanine) or into a delayed-type hypersensitivity reaction site induced by purified protein derivative of *Mycobacterium tuberculosis* or by *C. neoformans* antigen(s) (50). Each of the identified cryptococcal products, GXM, GalXM, and MP, when given intravenously to mice, can inhibit leukocyte migration (50). Considering that GXM is the dominant cryptococcal component in the host's circulation, one might predict that GXM is mainly responsible for the reduced leukocyte migration into inflammatory sites (50). These observations imply that pulmonary cryptococcosis patients, who have low to no cryptococcal antigen concentrations in their serum, would have a normal influx of leukocytes into pulmonary sites of infections. On the other hand, in severe pulmonary infections or in disseminated cryptococcosis patients who have high levels of circulating cryptococcal antigens, minimal inflammation would be expected in the infected tissues. In fact, for years investigators have commented on the minimal host tissue responses observed in patients with disseminated cryptococcosis (3). These recent data demonstrate that the circulating cryptococcal antigens are responsible, at least in part, for the lack of host tissue response.

Cryptococcal antigen(s) can potentially prevent leukocytes from migrating into an inflammatory site in two nonexclusive ways (51,52). First, GXM can stimulate neutrophils to shed L-selectin, a surface molecule necessary for the first step in neutrophil movement into tissues (51). Without L-selectin the neutrophils do not slow down and roll along the inflamed endothelial cells lining the blood vessels. With this first step in extravasation blocked, the numbers of neutrophils in infected tissues would be greatly reduced. Second, GXM and GalXM can bind to CD18, the beta chain of the adhesion molecule LFA-1, and prevent the binding of anti-CD18 to LFA-1 (52). Consequently, this binding of GXM and GalXM could prevent LFA-1 on the neutrophils from binding to the LFA-1 ligand, ICAM-1 on the inflamed endothelial cell surface. If leukocytes are inhibited from entering tissues by either or both of these mechanisms, the organism is not effectively eliminated, and the disease is more severe.

Induction of Immunomodulatory Cells

Cryptococcal antigens injected into the bloodstream of experimental animals can induce regulatory T cells, which dampen or ablate the anticryptococcal humoral as well as cell-mediated immune responses (53-71). Some clinical correlates support the concept that the antigenemia seen in disseminated cryptococcosis downmodulates the immune responses (72,73).

A long-lasting, specific immunologic unresponsiveness has been reported in persons cured of cryptococcal meningitis (72-74). Although they made antipneumococcal polysaccharide antibodies to the same degree as control volunteers after vaccination with pneumococcal polysaccharide, cured patients could not make antibodies to cryptococcal polysaccharides after vaccination with cryptococcal antigens (72,73). Henderson et al. (72,73) speculated that the intense, prolonged antigenemia associated with the cryptococcosis may account for the observed unresponsiveness. It is not unusual for patients with disseminated cryptococcosis to have depressed cell-mediated immune (CMI) responses to cryptococcal antigens (75,76). However, sufficient information is not available to determine whether the patients had a generalized defect in CMI function or had a specifically depressed CMI response because of the cryptococcal antigenemia.

Experimental animal models demonstrate convincingly that cryptococcal antigens given intravenously can induce immunoregulatory T cells that downmodulate the anticryptococcal CMI response (56-71). Serum from *C. neoformans*-infected mice with a cryptococcal antigen titer of 10^{-4} when transferred intravenously to naive mice induces regulatory T cells that specifically depress the anticryptococcal CMI response (67). Similar immunoregulatory T cells are induced by simulating the antigenemia with intravenous injection of cryptococcal culture filtrate antigen (56,57,60-63,66-69). The immunoregulatory $CD4^+$ T cells, which appear in the lymph nodes of the mice 7 days after antigen injection, diminish the induction of T cells responsible for the anticryptococcal delayed-type hypersensitivity response and reduce the ability of the mice to clear cryptococci from tissues (56,57,60,68). A second immunoregulatory T cell, a $CD8^+$ cell, has been found in spleens of mice (57). The $CD4^+$ immunoregulatory cells do not alter the numbers or types of leukocytes that infiltrate an anticryptococcal delayed-type hypersensitivity reaction; however, they do have a downregulating effect on IL-2 and IFNγ production at the site (77). In contrast, the $CD8^+$ immunoregulatory cells appear to affect the numbers of neutrophils that infiltrate the delayed-type hypersensitivity reaction site (Murphy, unpub. data). The details of the characteristics and functions of these immunoregulatory T cells have been reviewed (45). The available data strongly support the notion that cryptococcal products in the circulation induce an array of immunoregulatory T cells, which depress the anticryptococcal immune responses and protection.

More consideration should be given to the impact of high levels of cryptococcal antigen in the cerebrospinal fluid (CSF) on progression of disease. As suggested by Denning et al. (78), high cryptococcal antigen concentrations could change the osmolality of the CSF, thereby affecting its outflow and adsorption and increasing intracranial pressure. The increased pressure may cause headaches, visual loss, and early death (78). It is also possible that release of mannitol by *C. neoformans* contributes to increased intracranial pressure in cryptococcal meningitis patients (78).

Melanin Synthesis

One characteristic that differentiates pathogenic isolates of *C. neoformans* from nonpathogenic isolates and other *Cryptococcus* species is the organism's ability to form a brown to black

pigment on a medium (such as birdseed or caffeic acid agar) that contains diphenolic compounds (1). This pigment, first described by Staib (79), is a melaninlike compound produced by *C. neoformans* isolates with phenoloxidase activity (80). The importance of melanin production in *C. neoformans* virulence was first demonstrated in the early 1980s. Rhodes, Polacheck, and Kwon-Chung (81) reported that naturally occurring *C. neoformans* mutants lacking melanin (Mel^-) were less virulent in mice than melanin-producing strains. Others (82-84), using chemically induced mutants or isogenic pairs of *C. neoformans,* have confirmed and extended this observation.

Biochemical analyses suggest that in *C. neoformans* melanogenesis is accomplished by conversion of dihydroxyphenols such as 3,4-dihydroxyphenylalanine (DOPA) to dopaquinone (Figure 3). This conversion is catalyzed by a phenoloxidase and is the rate-limiting step, presumably because subsequent steps in the pathway, such as dopaquinone rearranging to dopachrome and ultimately autoxidation to melanin, are spontaneous (85). *C. neoformans* lacks a tyrosinase enzyme required for endogenous production of dihydroxyphenols (83); thus to produce melanin, a *C. neoformans* isolate must be able to acquire diphenolic compounds from its growth environment, and it must have the enzyme phenoloxidase to catalyze conversion of these compounds into the subsequent melanin intermediates. The brain is a tissue rich in catecholamines such as DOPA and is a favorite target for infection by *C. neoformans*. However, the organism cannot use catecholamines as a sole carbon source, which suggests that the brain is not a preferred nutritional niche for growth of

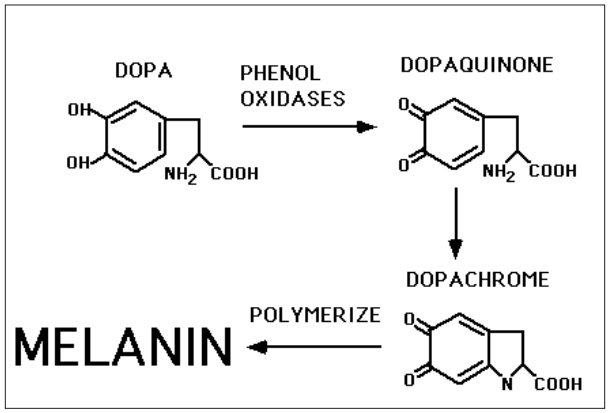

Figure 3. Proposed pathway for melanin synthesis by *C. neoformans* (85).

C. neoformans (86); rather, it may serve as a survival niche as described below.

Polacheck et al. (86) reported that melanin-producing isolates of *C. neoformans* were resistant to damage by an in vitro epinephrine oxidative system, whereas mutants lacking phenoloxidase activity were highly susceptible, as evidenced by decreased survival. Jacobson and Tinnell (87) found that melanin protected *C. neoformans* from damage by hypochlorite and permanganate but not by hydrogen peroxide. In these experiments, hypochlorite was 100 times more fungicidal than hydrogen peroxide, and *C. neoformans* could produce sufficient levels of melanin to effectively protect the organism from oxidative compounds produced by macrophages (87). Wang and Casadevall (88) extended these findings by examining survival of *C. neoformans* in the presence of nitric oxide and reactive nitrogen intermediates as well as in the epinephrine oxidative system described by Polacheck et al. (86). Wang and Casadevall cultured *C. neoformans* cells with L-DOPA to melanize the yeast cells (88). Melanized cryptococci survived damage by both nitrogen- and oxygen-derived oxidants significantly better than nonmelanized organisms of the same strain. These results support the hypothesis that *C. neoformans* uses catecholamines in the brain to make melanin, thereby protecting the organism from oxidative damage by scavenging free radicals (86).

Recently, cryptococcal diphenoloxidase has been purified, and the gene *CNLAC1* encoding this enzyme was cloned (89). The glycosylated protein has a molecular weight of 75 kDa and contains copper. The substrate specificity of the enzyme indicates that it is a laccase. *CNLAC1* contains 14 introns, a 624 amino acid open reading frame; transcriptional activity is derepressed in the absence of glucose (89), which confirms an earlier report of low glucose requirements for melanin formation (90). Disruption of *CNLAC1* resulted in loss of virulence of *C. neoformans*, whereas complementation with *CNLAC1* increased virulence of Mel^- mutants in mice (84); these results suggest that the laccase (phenoloxidase) encoded by *CNLAC1* is a potential virulence factor of *C. neoformans*. Furthermore, transcripts of the *CNLAC1* gene have been detected by reverse transcription-polymerase chain reaction (RT-PCR) in *C. neoformans* yeast cells isolated from CSF in a rabbit model of cryptococcal meningitis (84).

Besides acting as an antioxidant, melanin production may help *C. neoformans* survive in the host in other ways. Melanized yeast cells are less susceptible to amphotericin B than nonmelanized yeast cells, and this may contribute to the inability to effectively treat infections in immunocompromised hosts (91). Furthermore, phagocytosis of melanized *C. neoformans* by a macrophage cell line in the presence of an anti-GXM antibody was decreased, which suggests that melanin deposition in the cell wall may inhibit opsonization by specific antibodies (92). Recently, Huffnagle and coinvestigators (93) reported that melanized heat-killed *C. neoformans* strain 145 yeast cells stimulated less TNFα production by alveolar macrophages and less antigen-specific T-cell proliferation than nonmelanized heat-killed 145 strain cells. The authors suggest that melanin "cloaks" *C. neoformans* from recognition by host effector cells and inhibits induction of a protective T-cell-mediated immune response (93); however, it is possible that scavenging of host-produced oxidants and inhibition of phagocytosis by melanin may contribute to the decreased TNFα production and lymphoproliferation observed.

Although melanin production is important in the virulence of *C. neoformans*, there is very little evidence demonstrating the presence of melanin in vivo. One report indicates that phenoloxidase activity in *C. neoformans* is greatly diminished at 37°C compared with activity at 25°C, which suggests that melanin production may be limited in vivo (94). Detection of melanin in vivo is hampered by the lack of specific antibodies or stains. A modified Fontana-Masson stain has been used to detect a brown pigment in the cell wall of *C. neoformans* cells in histologic brain sections; however, the stain is not specific because *Cryptococcus laurentii*, which is Mel$^-$, also stains with Fontana-Masson (95).

In summary, accumulating evidence indicates that melanin production is an effective survival (virulence) factor of *C. neoformans,* and melanin may serve multiple roles in protecting this medically important fungus from host defenses. Two caveats should be noted in labeling melanin as a major virulence factor for *C. neoformans*. The *C. neoformans* isolate must be able to internalize the melanin precursors, and the phenoloxidase enzyme must make a sufficient amount of melanin precursor at 37°C. Consequently, much more work is needed to delineate the role of melanin production in virulence of *C. neoformans,* especially in light of the apparent temperature sensitivity of the cryptococcal phenoloxidase enzyme.

Mannitol Production

Accumulating evidence suggests that production of the hexitol D-mannitol may contribute to survival of *C. neoformans* in the host. Wong et al. (96) reported that of the 12 human isolates of *C. neoformans* examined, all produced and secreted D-mannitol into growth medium. Further, by using a rabbit cryptococcal meningitis model, they showed that *C. neoformans* can produce D-mannitol in vivo. CSF from rabbits treated with cortisone and infected intracisternally with *C. neoformans* contained more D-mannitol than CSF from controls, cortisone-treated uninfected rabbits, or rabbits infected with *C. neoformans* but not treated with cortisone (96). In the latter group, cryptococcal infection was limited. The levels of D-mannitol in infected rabbit CSF correlated well with both the numbers of culturable *C. neoformans* and the cryptococcal antigen titer of the CSF, which suggests that levels of D-mannitol in CSF may be prognostic (96); however, it is not known whether different isolates of *C. neoformans* vary in mannitol production. These authors suggested two means by which mannitol production may contribute to *C. neoformans* pathogenesis. First, high concentrations of D-mannitol in the CNS may contribute to brain edema. Second, mannitol is a potent scavenger of hydroxyl radicals, and cryptococcal-produced D-mannitol may help protect the organism from oxidative damage (96).

To investigate the role of mannitol in cryptococcosis, Chaturvedi and co-workers (97) isolated a low mannitol producing mutant after UV irradiation of *C. neoformans* strain H99. The mutant, mannitol low producer (MLP), was similar to the parent strain H99 in many growth and morphology characteristics and in known virulence factors such as capsule production and phenoloxidase activity (97). However, the mutant MLP strain was more susceptible to growth inhibition and killing by heat and high salt than the parent strain. In addition, the mutant MLP strain was significantly less virulent than the parent strain (MLP LD_{50} = 3.7 X 10^6 CFU, H99 LD_{50} = 6.9 X 10^2) (97). Further comparisons of H99 and MLP strains showed that polymorphonuclear leukocytes (PMNL) killed MLP significantly

better than H99 at several effector-to-target ratios (98). Addition of superoxide dismutase, mannitol, or dimethyl sulfoxide inhibited PMNL killing of both strains, but addition of catalase did not alter killing, which suggests that reactive oxygen intermediates such as the hydroxyl radical and hypochlorous acid are potent effector molecules against *C. neoformans* and mannitol may protect against oxidative killing by scavenging such compounds (98).

The results above indicate that mannitol production by *C. neoformans* correlates with increased resistance to heat stress, osmotic stress, and damage by reactive oxygen intermediates, as well as increased pathogenicity of this fungal agent. Additional studies are required to determine the role of mannitol production in virulence of *C. neoformans*. Isogenic strains lacking the enzyme required for mannitol production, namely mannitol dehydrogenase, are not available because the gene for this enzyme has not been found. However, a gene (*Mtl*) has been isolated from *C. neoformans* that can induce expression of the *S. cerevisiae* mannitol dehydrogenase gene (99). The 346 amino acid product encoded by the *Mtl* gene is thought to be involved in regulating mannitol production in *C. neoformans* and may be beneficial in future studies of mannitol production (99).

Other Potential Virulence Factors

Superoxide Dismutase

Jacobson and coinvestigators (100) examined superoxide dismutase (SOD) production by *C. neoformans* to determine whether SOD levels increased at 37°C to compensate for possible decreases in melanin production at this temperature. These investigators observed an increase in SOD levels at 37°C, which suggests that SOD may participate in free radical scavenging at this higher temperature in vivo (100); however, there is no evidence that SOD production serves as a virulence factor for *C. neoformans*.

Proteases

Muller and Sethi (101) reported that *C. neoformans* grown in culture produced a protease that could digest human plasma proteins, and Brueske (102) reported that *C. neoformans* culture supernatants contained a protease capable of digesting casein. However, neither of these studies determined whether the proteases were manufactured in vivo. Limited evidence indicating in vivo production of proteases has been presented by Salkowski and Balish (103). These investigators observed skin lesions on T-cell–deficient mice after intravenous infections with *C. neoformans* strain SLHA (103). Microscopic examination of the lesions suggested that the cryptococcal yeast cells were degrading collagen bundles in the dermis (103). Supernatants of SLHA strain cultures liquefied gelatin in vitro indicating that this cryptococcal strain secreted a collagenaselike protein (103). Thus, *C. neoformans* proteases possibly serve as virulence mechanisms by initiating invasion of host tissues; however, more studies with isogenic strains of *C. neoformans* are required before proteases can be listed as virulence factors.

Phospholipases

Recently, Chen and co-workers (104,105) reported phospholipase, lysophospholipase, and lysophospholipase-transacylase activity of *C. neoformans* grown on egg yolk agar. Of 50 strains tested, 49 had phospholipase activity (104), due to phospholipase B secreted into cultures (105). Analysis of four strains with varying levels of phospholipase activity indicated a correlation between phospholipase activity and virulence in BALB/c mice (104). The authors suggest that extracellular phospholipase activity produced by *C. neoformans* may disrupt mammalian cell membranes and allow the yeast cells to penetrate into host tissues (104,105); however, further investigations are necessary to establish the role, if any, of these types of products in the virulence of *C. neoformans*.

Regulation of Virulence

Regulation of virulence factors such as capsule production and melanin formation is not well understood. However, the gene GPA1, which encodes a G-protein α-subunit, is involved in the regulation of these virulence factors as well as in *C. neoformans* mating (106). Disruption of *GPA1* resulted in defects in mating in response to nitrogen starvation, capsule production in response to iron limitation, and melanin synthesis in response to glucose starvation (106). Furthermore, *gpa1* mutants were much less virulent in a rabbit model of cryptococcal meningitis (106). Reconstitution of the *gpa1* mutant with wild-type *GPA1* restored mating, capsule production, melanin synthesis, and virulence. Addition of cyclic AMP

also restored these phenotypes, which suggests that *C. neoformans GPA1* regulates these factors by sensing the nutritional signals of the environment and regulating cyclic AMP metabolism in the organism (106). These findings along with results of other molecular studies are intriguing and represent the initial steps in defining at the molecular level the factors controlling virulence in *C. neoformans.*

Conclusions

Virulent isolates of *C. neoformans* must be able to produce small particles that can get into the alveolar spaces, must be able to grow at 37°C at a pH of 7.3 to 7.4 in an atmosphere of approximately 5% CO_2, must have an intact calcineurin pathway, and (possibly) must be an α-mating type. The ability to produce a large capsule and shed great amounts of capsular material into the body fluids makes the organism highly virulent. Other factors, such as melanin, mannitol, superoxide dismutase, protease, and phospholipase production, may enhance the pathogenicity of *C. neoformans*. The effectiveness of many of these cryptococcal virulence factors depends on the status of the host's defensive mechanisms. Although we have learned much about the pathogenicity and virulence of *C. neoformans*, many gaps still remain in our knowledge.

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Hantavirus Infection in Children in Argentina

Clinical hantavirus infection was diagnosed in five Argentine children ages 5 to 11 years by immunoglobulin M (IgM)- capture enzyme-linked immunosorbent assay using Sin Nombre virus (SNV) antigens. Death in three of the children was associated with absence of detectable IgG to SNV antigens. An additional two cases in healthy children were studied: one, a breast-fed 15-month-old whose mother died of suspected hantavirus pulmonary syndrome (HPS) 8 months previously, had hantavirus IgG (≥ 1:6400); a second, whose mother survived HPS during month three of pregnancy, apparently had maternal antibodies no longer detectable 1 year after birth.

In May 1993, a new hantaviral illness, hantavirus pulmonary syndrome (HPS), was recognized in the southwestern region of the United States (1). HPS is a viral zoonosis characterized by a febrile prodrome in young, healthy adults; the disease progresses to respiratory failure with the clinical picture of adult respiratory distress syndrome (ARDS). The striking pulmonary involvement differentiates HPS from a previously described hantaviral disease known as hemorrhagic fever with renal syndrome.

In the first 100 HPS cases in the United States, the average age was 34.9 years (range 11 to 69); eight cases were in children or adolescents under 16 years of age (2). In Argentina, from 1987 to July 1997, 114 cases were diagnosed in three areas of the country where several strains of new world hantaviruses are known to cause HPS diseases (3,4). Before 1995, no cases were detected in Argentine children under 12 years of age. Ten cases were reported among adolescents (13 to 19 years) with a case-fatality rate of 30% (Instituto Nacional de Enfermedades Virales Humanas, [INEVH], unpub. data).

The initial case definition referred to ARDS and included adults and young adults (5) as the affected population. The lack of HPS cases among children in the original outbreaks led to a circulating hypothesis that children were not at risk or were at a very low risk for HPS. Another hypothesis was that children were protected from pulmonary involvement, perhaps by immune system immaturity or a lack of other risk factors (such as cigarette smoking) for lung injury.

In this report we describe five cases in children; in all of them the etiologic diagnosis was established by the presence of immunoglobulin M (IgM) antibody to Sin Nombre virus (SNV) antigens. Serologic results for two of the children were also positive for SNV IgG antibody. Serum samples were tested for IgM and IgG antibodies to SNV by enzyme-linked immunosorbent assay (ELISA) (6). An ELISA titer greater than or equal to 1:400 was considered positive (Table 1).

Patient 1 was identified during the study of the first outbreak in southern Argentina in 1995 (5). Four patients in this outbreak were from the same family. During interviews of the family members, we found that a 9-year-old boy had a febrile disease without respiratory involvement, beginning on April 19. Serology performed on May 3, 14 days after the onset of symptoms, demonstrated IgM and IgG antibodies to SNV antigens.

Table 1. Hantavirus infection in children, Argentina, 1995–1997

				Serology[a] Date			
Case	Sex	Age (yrs)	Date of onset	IgM IgG	Area[b]	Outcome
1	M	9	4-19-95	5-3-95 >6400 1600	South	Alive
2	F	5	3-21-97	3-23-97 >6400 Neg	North	Dead
3	M	9	3-30-97	4-3-97 1600 400	North	Alive
4	F	11	4-14-97	4-16-97 1600 Neg	North	Dead
5	M	5	4-27-97	4-28-97 1600 Neg	Central	Dead

[a]Titer expressed as the reciprocal of the serum dilution reactive in enzyme-linked immunosorbent assay.
[b]Area of origin in Argentina.

The other four cases in children were identified during routine surveillance. From 1995 to 1997, samples from 25 children (ages 3 months to 12 years; mean = 5.8 years) were sent to INEVH for diagnosis. Table 2 summarizes the main clinical and laboratory findings. None of the children had renal failure; patient 2 had uremia of 0.30 g/l, and patient 4 had a serum creatinine level of 1.40 g/l. All patients who later died received supplemental oxygen as part of their treatment.

Two special situations involving children arose during the study of the first cases of HPS in El Bolsón in 1995. 1) A woman belonging to the family of patient 1 contracted HPS during the first quarter of pregnancy. She had a febrile syndrome, without respiratory failure; chest X-rays showed bilateral interstitial infiltrates. Serologic tests showed both SNV IgM (≥ 1:6400) and IgG (≥ 1:6400) on April 22, 1995, 8 days after the onset of symptoms. She delivered a healthy infant in October 1995. A sample of the newborn's cord blood was positive for SNV IgG (≥ 1:6400) and negative for SNV IgM. A serum sample drawn from the mother at the same time had a SNV IgG titer ≥ 1:6400 and was negative for SNV IgM. A second serum sample, taken from the baby a year later during November 1996, had no detectable SNV IgG or IgM. 2) During a retrospective search for cases fulfilling the HPS case definition, a woman who died of ARDS in September 1994 was considered to have a possible case. No serum samples or autopsy tissues were available to make an etiologic diagnosis. Before dying, this woman breast-fed a 7-month-old baby; when tested for antibodies 8 months later in May 1995 at 15 months of age, the baby had SNV IgG (≥ 1:6400) and no detectable SNV IgM antibodies. A second serologic sample, collected 18 months later in November 1996, still had SNV IgG antibodies, with a similar titer. Both babies have continued to develop normally as of October 1997.

A case similar to that of patient 1 was detected in New Mexico in June 1993 (7) in the course of the investigation of a fatal HPS case. In this case, the patient also had a mild clinical course that did not meet the surveillance case definition for HPS. This case definition (revised 9/96) is as follows: "a febrile illness characterized by bilateral diffuse interstitial edema that may radiographically resemble ARDS, with respiratory compromise requiring supplemental oxygen, developing within 72 hours of hospitalization, and occurring in a previously healthy person; or an unexplained respiratory illness resulting in death, with an autopsy examination demonstrating noncardiogenic pulmonary edema without an identifiable cause" (8).

Our remaining four cases were sporadic, in persons without previous contact with other HPS patients, and were suspected because their clinical symptoms were typical of HPS. Results of serologic testing with SNV antigens of the household contacts in cases 2 and 5 (five persons

Table 2. Laboratory results and clinical features of children with hantavirus infection, Argentina, 1995–1997

Tests and features	Case 1	2	3	4	5
Leukocytes (/mm^3)	9,200	27,000	12,800	10,600	69,200
Hematocrit (%)	44	66	43	55	53
Thrombocytes (/mm^3)	ND[a]	266,000	200,000	97,000	ND
Sedimentation rate (mm/hr)	ND	4	28	8	1
GOT/GPT[b]	ND	Increased	Increased	Increased	Increased (mild)
Chest X-ray	HI[c]	DII[d]	DII	DII	DII
Respiratory symptoms	None	Distress	Slight dyspnea	Tachypnea, clinical and X-ray disassociation, hypoventilation	Acute respiratory insufficiency

[a]ND: Not done.
[b]GOT/GPT: Glutamic oxalacetic transaminase/Glutamic pyruvic transaminase.
[c]HI: Hilar indistinctness.
[d]DII: Diffuse interstitial infiltrate.

each) were negative. The clinical, radiologic, and laboratory findings were similar in children and in adults; severely ill patients had greater variation in laboratory values than mild cases, and in fatal cases, only SNV IgM was present.

The case-fatality rate in this series was 60%, but the small number of cases does not permit conclusions. In previously reported cases in adolescents 13 to 19 years of age, the case-fatality rate was 30%.

These cases originated in the three areas where the illness is endemic in Argentina. This is an important point because an unusual case of HPS in southern Argentina, with the possibility of person-to-person transmission, had been reported (9,10). Patient 1 and the baby that was breast-feeding when the mother died of suspected HPS could be further instances of person-to-person transmission.

A case of hemorrhagic fever with renal syndrome and pregnancy was reported in 1992 (11); the dynamics of serum antibody persistence were similar to those found in the one instance where we believe antibody was passively transferred from mother to baby. These results indicate that HPS should be considered in the differential diagnosis of respiratory distress or atypical bilateral pneumonia in children, at least in areas where these diseases have been confirmed. Mild disease should be considered too, especially in contacts of HPS patients and in younger age groups.

Our findings also suggest the transfer of passive antibodies from mother to fetus (without fetal infection) and the possibility of transmission of infection by maternal breast feeding.

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Reemergence of Dengue in Cuba: A 1997 Epidemic in Santiago de Cuba

After 15 years of absence, dengue reemerged in the municipality of Santiago de Cuba because of increasing migration to the area by people from disease-endemic regions, a high level of vector infestation, and the breakdown of eradication measures. The 1997 epidemic was detected early through an active surveillance system. Of 2,946 laboratory-confirmed cases, 205 were dengue hemorrhagic fever, and 12 were fatal. No deaths were reported in persons under 16 years of age. Now the epidemic is fully controlled.

Cuba had its first dengue epidemic of modern times in 1977; transmission continued probably until 1981, and more than 500,000 mild cases were reported. A 1978 serologic survey for flavivirus antibody indicated that 44.6% of the Cuban population had been infected with dengue-1 virus, whereas before 1977 only 2.6% had antibodies (1,2).

A second dengue epidemic in 1981, caused by dengue-2 virus (2), was unusually severe and widespread. Of 344,203 cases, 10,312 were clinically classified as dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), and 158 persons (101 children and 57 adults) died (3). Before 1981, only 60 suspected or confirmed DHF sporadic cases had been reported in the region (4). Dengue-2 virus isolated during the 1981 epidemic was classified in the same genotype as New Guinea 1944 (5). Not previously known to circulate in the Americas, this genotype was not isolated again in the region until 1994 in Venezuela and in 1995 in Mexico (6). Retrospective studies show that although the 1981 epidemic was detected in May, the first cases occurred in December 1980. After the epidemic ended on October 10, 1981, a campaign to improve mosquito control and eradicate *Aedes aegypti* was immediately launched. Eradication was not achieved, but most of the 169 Cuban municipalities were free of the vector.

Passive Surveillance—1981

A passive dengue surveillance system was established at the end of the 1981 epidemic. Of 9,543 paired sera (acute- and convalescent-phase) from all suspected dengue patients, only 14 showed seroconversion to immunoglobulin G (IgG) by enzyme-linked immunosorbent assay (ELISA) (7); none developed IgM antibodies to dengue virus by capture IgM ELISA (8). Dengue virus infection was excluded on the basis of clinical and epidemiologic investigation. No *Ae. aegypti* mosquitoes were found in the residence localities of these patients. The surveillance system detected cases, imported from other Latin American countries, that had no evidence of indigenous transmission. Since 1987, 4,983 samples received through the surveillance system for measles and rubella, as well as paired sera of patients with rash, were studied for dengue antibodies [María Guzmán, World Health Organization (WHO)/Pan American Health Organization (PAHO) Collaborating Center for the Study of Viral Diseases, unpub. info.]. No dengue cases were identified. The low *Ae. aegypti* premise indexes and the results of the passive surveillance system indicate no dengue transmission in Cuba between 1981 and the end of 1996. However, reinfestation has occurred in some areas; the municipality of Santiago de Cuba was reinfested in 1992 by *Ae. aegypti* transported in imported tires (9).

Active Surveillance—1997

In January 1997, the Institute of Tropical Medicine "Pedro Kourí" of the Cuban Ministry of Health (a WHO/PAHO Collaborating Center for the Study of Viral Diseases) established an active surveillance system for dengue in Santiago de Cuba municipality. The municipality is located in Santiago de Cuba province, in the eastern part of the country, and has several risk factors for the reemergence of dengue: limited water supply, inadequate eradication efforts, high vector infestation, and increasing migration of people from Latin American and Caribbean disease-endemic countries to the municipality. Following the Guidelines for the Prevention and Control of Dengue and Dengue Hemorrhagic Fever in the Americas (4), this surveillance system actively

searched for febrile patients in the primary health-care subsystem whose clinical picture was compatible with dengue fever and whose sera collected 5 to 6 days after onset of the disease contained dengue IgM antibodies. As a result of this system, dengue cases were detected on January 28, 1997, in one area of the municipality. In three of the first seven cases, dengue-2 virus was detected by polymerase chain reaction (10) and was confirmed by viral isolation and identification using C6/36 cell line and monoclonal antibodies to the four dengue serotypes.

Although retrospective seroepidemiologic studies indicated that the initial transmission occurred during the second half of December 1996, it is highly probable that the cases detected on January 28 were the first. Of 60,000 cases reported from the emergency rooms of Santiago de Cuba hospitals from November 1 to January 28, 592 were clinically compatible with dengue fever. Home interviews of these 592 patients reduced the figure to 154. Blood samples from 143 of 154 patients were examined for IgM antibodies, but no positive cases were detected.

The breakdown of the vector control campaign in this municipality interfered with our efforts to abort the epidemic, despite the early detection of the first dengue cases; however, the partial vector control measures implemented once the outbreak was detected prevented its extension to the other 30 Cuban municipalities infested with the *Ae. aegypti* mosquito.

Active surveillance continued from January to July 1997. Serologic confirmation of cases was carried out by IgM capture ELISA, confirming recent infection. The serologic diagnosis was decentralized to the Provincial Laboratory in Santiago de Cuba, which used an ultramicro-ELISA for dengue IgM detection (11). The Institute of Tropical Medicine served as the national reference laboratory for serology, viral isolation, and strain identification and characterization.

During the epidemic, 17,114 febrile patients were initially considered to have dengue, but serologic testing of 10,024 of these patients confirmed dengue in only 2,946; 46 dengue-2 isolates from 160 serum samples were obtained. The nucleotide sequence of the E\NS1 gene junction of the first isolated strain (12) indicated that it belonged to the Jamaica genotype, which during recent years is being transmitted extensively throughout Latin American and Caribbean countries and is associated with DHF/DSS in some countries (6,13).

Epidemiology

After the end of the 1981 Cuban DHF epidemic, seroepidemiologic studies in Palmira, Cienfuegos, and Cerro municipalities examined dengue-1 and dengue-2 seroprevalence in these populations (14,15). Taking into consideration these data and the total population of the Santiago de Cuba municipality, we estimated the prevalence of dengue-1 and dengue-2 antibodies. The estimated total population at risk for dengue-2 infection was 301,986 adults and children susceptible to a primary infection by any dengue virus serotype (63.5% of the population) and 88,108 adults with antibodies to dengue-1 virus acquired during the epidemic of 1977 to 1980, now susceptible to a secondary infection with dengue-2 and at increased risk for DHF/DSS (18.5% of the population).

The earlier Cuban experience (3) confirms other reports of secondary infection (dengue-1 and dengue-2) as the main risk factor for DHF/DSS. During the 1997 dengue outbreak, secondary infection was again confirmed as a risk factor for DHF/DSS. Of the 2,946 confirmed cases, 205 (including 12 fatal adult cases) were classified as DHF/DSS cases according to the criteria established by PAHO (4). DHF/DSS was observed mostly in adults, the only age group in whom secondary infection was possible. DHF/DSS-compatible symptoms were seen only in one child with primary infection. Preliminary studies indicated that secondary infection was present in 100 (98%) of 102 DHF/DSS cases. In fatal cases, secondary infection could be documented in 11 (92%) of 12 cases. In Thailand the greatest risk appeared when the secondary infection occurred 6 months to 5 years after the primary one (16). For that reason, an epidemic of DHF/DSS was not expected in Santiago de Cuba, perhaps only sporadic cases. However, DHF/DSS in adults who contracted a secondary infection at least 16 years after the primary infection was not previously reported.

Because in Cuba dengue-1 circulated from 1977 to 1980-81, the youngest patients expected to contract secondary infection should be older than 16 years of age; the youngest DHF/DSS patient with confirmed secondary infection was a 17-year-old, which indicates that the "enhancing" antibodies can circulate and be effective for at least 16 years and maybe for life.

A significant number of febrile patients with suspected dengue had respiratory signs and symptoms; therefore, simultaneous circulation of

respiratory or other pathogens was considered. Serologic screening for respiratory viruses using hemagglutination-inhibition and ELISA confirmed that 29.3% of 41 nonconfirmed dengue cases were influenza A, influenza B, or adenovirus infections. Additionally, some children had fever and rash clinically compatible with herpangina, and some had diarrheal disease with fever, as is common in Cuba during the summer. These febrile syndromes contributed to the high number of patients whose infections were provisionally considered suspect dengue cases. Suspect dengue cases were broadly defined to maximize sensitivity of detection and retain all possible dengue cases. This active surveillance excluded other febrile syndromes but recorded them as suspected cases. In practice, the risk perception by the population was very high, especially when the epidemic was officially declared and deaths were noted. Both the patients and the health providers appeared to think of dengue as the first diagnostic possibility. For this reason, the figure of 17,114 cases was considered the magnitude of the epidemic from the clinical management perspective. Since most cases were tested serologically, the incidence of clinical cases was probably close to the 2,946 serologically or virologically confirmed cases. Because asymptomatic and subclinical dengue cases are frequent, especially in children, the true rate of infection may be higher. In a separate and limited study on asymptomatic contacts of dengue cases, for every clinical case, 13.9 asymptomatic or subclinical cases were produced. Serologic studies of contacts in Santiago de Cuba are planned for a more in-depth study of this question.

Clinical Management

The health authorities established a liberal policy of hospitalization that varied with the availability of beds. Hospitalization permitted vector control of the human reservoir, more precise case classification, and close clinical surveillance.

When beds were available, all patients with suspected cases were hospitalized. When the numbers of patients surpassed the availability of beds, patients were treated at home under the supervision of the family doctor. The family doctor transferred the patient to the hospital if any medical complication appeared. Wards with specialized personnel were established where the patients were protected from vectors, and observation wards were organized for patients with complications. Intensive and intermediate care units, as well as an emergency subsystem for the transfer of patients from one unit to another, were available. As in 1981, some patients rapidly developed hypovolemic shock and died within hours of admission to the hospital (17).

An ad hoc task force followed the case definitions for dengue and DHF/DSS established by PAHO (4) for classifying the cases at the closure of the medical record. The accumulated experience of the Cuban scientists and doctors and the increased international knowledge about dengue and DHF/DSS in the last 15 years permitted a much deeper and more comprehensive study of this outbreak with more accurate classification and management of cases than in 1981. Nevertheless, the case-fatality rate was three times higher, mainly because of a much better classification of DHF/DSS cases. Other countries in the region with a very accurate case classification, such as Puerto Rico (13), also have a high case-fatality rate.

Vector Control

The campaign to control the vector started before the beginning of the 1997 dengue outbreak and is well established. Although the campaign required the mobilization of scarce financial resources and experts from all over the country, early intervention prevented spread of the outbreak to other potentially vulnerable municipalities. Of 169 municipalities in Cuba, 30 had *Ae. aegypti* mosquitoes. The epidemic was limited to the municipality of Santiago de Cuba; no autochthonous transmission to other municipalities of the province or country was detected.

An active search for cases detected transmission very early, before "fever alert" signaled an outbreak. In the Provincial Center for Hygiene, Epidemiology, and Microbiology of Santiago de Cuba, a special Unit for Analysis and Trends maintains a permanent fever alert system. For several years, this system has provided a weekly tabulation of febrile patients for every population. The tabulation allows us to evaluate fever alert (4) as applied to an active surveillance system. Because the fever alert did not appear in the epidemic area until May 1997, after the epidemic was already occurring, we consider fever alert an indicator with low sensitivity for the early and timely detection of dengue transmission, at least under the conditions of this study.

As a result of the 1997 epidemic, an epidemiologic alert was established, and antivector intervention, as well as active seroepidemiologic surveillance, was reinforced in the entire country. The epidemiologic characterization of the outbreak (now fully controlled) is in the final phase. Although mosquitoes persisted at a low level after the 1981 DHF/DSS epidemic, the campaign was successful in eradicating dengue from Cuba for more than 15 years, precisely when the disease was reemerging in nearly all the other tropical regions of the Americas. According to PAHO, 250,707 cases of dengue fever and 4,440 cases of DHF/DSS were reported in 1996 alone; 29 countries reported dengue in 1996, and 10 of these reported DHF/DSS. Overall, from 1981 to 1996, 25 countries reported 41,000 cases of DHF/DSS (F. Pinheiro, pers. comm.).

The 1997 Cuban dengue outbreak demonstrated once again that dengue reappears where *Ae. aegypti* control is relaxed. Taking into account these facts, Cuba maintains its policy of vector eradication and recommends an exerted effort in the American region to prevent a recurrence of dengue similar to the one in Southeast Asia, where DHF/DSS is the leading cause of hospitalization and death among children (18).

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Hantavirus Pulmonary Syndrome in a Chilean Patient with Recent Travel in Bolivia

A case of hantavirus pulmonary syndrome (HPS) was serologically confirmed in a critically ill patient in Santiago, Chile. The patient's clinical course had many similarities to that of other HPS patients in North and South America but was complicated by acute severe renal failure. The patient's history included self-reported urban and probable rural rodent exposure during travel in Bolivia. Comparison of a viral sequence from an acute-phase serum sample with other known hantaviruses showed that the hantavirus nucleic acid sequence from the patient was very similar to a virus recently isolated from rodents associated with HPS cases in Paraguay.

Since its discovery in 1993 (1) and its association with Sin Nombre virus in North America (2), hantavirus pulmonary syndrome (HPS) has been identified in several countries in South America and is associated with Juquitiba virus in Brazil (3), Andes virus in Argentina and Chile (4-6), and Laguna Negra virus in Paraguay (7,8). Hantaviruses are rodent-borne, and each is associated with a specific primary rodent reservoir. Sigmodontine rodents are the vectors of hantaviruses associated with HPS (3).

Case Report

A 20-year-old male resident of Santiago, Chile, who had no prior history of medical problems, became ill after he backpacked (from February 4 to March 9, 1997) as a tourist in Bolivia. His travel itinerary included Barrio, Oruro, La Paz, Cochabamba, Villa Tunari, Santa Cruz, Vallegrande, Higueras, and Sucre. The tourist and a fellow traveler stayed in hotel in Santa Cruz where they saw black rats running through the bathroom they used. While in Higueras, they stayed in a rustic adobe house with no floor and joined local villagers in agricultural jobs, primarily harvesting hay. A diagnosis of HPS was considered.

On March 26, 3 weeks after returning to Chile, the young man became ill with fever and cough. On March 28, he was admitted to the emergency room of a private hospital in Santiago with high fever (39°C), cough, and chest pain. Chest X-rays showed interstitial infiltrates in the left lower lobe. Laboratory results were as follows: hemoglobin 15.3 gm/dl, white blood cells 5,900, platelets 130,000, erythrocyte sedimentation rate 6, and a C-reactive protein 2.8 mg/dl. He was sent home on clarithromycin 250 mg, twice a day. Three days later (March 31), he returned to the emergency room with persistent high fever, myalgia, and shortness of breath; distal cyanosis was noted. Vital signs were as follows: blood pressure 115/80, pulse 120, temperature 38.5°C, and respiratory rate 32. Physical examination found few petechiae on the forearms, diffuse bilateral rales, regular cardiac rhythm, no murmurs, and no hepatomegaly or splenomegaly. Chest X-rays showed diffuse bilateral alveolar infiltrates. Oxygen saturation was 90%. Laboratory values included white blood cells 11,700 with a left shift, platelets 150,000, hemoglobin 19.4, erythrocyte sedimentation rate 2, C-reactive protein 8.18, INR 1.6, activated partial thromboplastin 43s, blood urea nitrogen 38.9 mg/dl, and liver function tests normal limits.

The patient was transferred to the intensive care unit with a diagnosis of bilateral pneumonia of unknown etiology and secondary respiratory failure. In 3 to 4 hours, acute respiratory distress developed; arterial blood gases (on 50% oxygen) were PaO_2 61, $PaCO_2$ 22.7, pH 7.49, and HCO_3 17.1. The patient was connected to a ventilator and was started on imipenen, erythromycin, amantadine, dopamine, dobutamine, fluids, and nitric oxide. Two 500-mg doses of methylprednisolone were administered, and a Swan-Ganz catheter was installed. The pulse wedge pressure was 7, and the cardiac output 6.1. During the first 24 hours, acute renal failure developed, and hemodialysis was started.

Hypotension was quite refractory to vasoactive drugs (mean BP 30 to 40), and critical

hypoxemia (O_2 saturation 70% to 80%) and hypotension persisted for 48 hours. Echocardiography showed a mild pericardial effusion. Blood cultures were negative for bacteria, as well as for influenza, adenovirus, respiratory syncytial virus, and parainfluenza virus. Serologic results for *Mycoplasma, Legionella*, and HIV were also negative. Blood gases started to improve on day 3, and the patient's clinical condition also slowly improved. On day 10, hemolytic anemia (Coombs negative) developed, and a bone marrow aspirate showed hemophagocytosis. On day 11, an episode of bleeding from the respiratory tract occurred. However, the patient's ventilatory function continued to improve. On day 15 after admission, his chest X-ray was clearing, but he was still on a mechanical ventilator (O_2 saturation 98% on 40% O_2). However, he was anuric (BUN 100). On day 17, he had a second episode of bronchial bleeding, and bronchoscopy showed only a 4-mm tracheal ulcer. Platelets were 72,000, and hematocrit fell to 24%. Hantavirus serology was reported positive [Centers for Disease Control and Prevention (CDC), April 18], and intravenous ribavirin was started on April 22. A central venous catheter-related infection with *Pseudomonas* and *Staphylococcus aureus* was documented. The patient's condition deteriorated progressively, with further bronchial bleeding and markedly unstable ventilatory function, and required constant administration of vasoactive drugs; he died of massive pulmonary hemorrhage and shock on April 28. A postmortem lung sample was taken with a needle.

The patient's serum, collected on April 1, was tested for immunoglobulin G (IgG) and IgM antibodies to Sin Nombre virus at CDC. Both IgG and IgM antibodies were found, which suggested recent infection with a hantavirus associated with Sigmodontine rodents. Subsequent testing of sera collected on April 21 and April 25 confirmed the initial findings. Hematoxylin and eosin stained sections of the lung sample showed diffuse alveolar damage with extensive hyaline membrane formation, proliferation of type II pneumocytes, and fibroblastic and edematous thickening of alveolar walls. Abundant fibrin, necrotic debris, and acute inflammatory cellular infiltrates were also observed in the alveolar spaces. Rare endothelial cells and macrophages were hantavirus-antigen positive by previously described immunohistochemical procedures (9). The destructive changes seen by histopathology and a small amount of antigen found in the patient's tissues are compatible with the long course of the patient's illness (9).

Viral RNA was extracted from the earliest serum sample, and reverse transcriptase-polymerase chain reaction amplification with primers designed specifically for hantaviruses associated with Sigmodontine rodents (8) yielded polymerase chain reaction fragments for the S and M segments. These cDNA fragments were sequenced and compared with those of other American hantaviruses. These comparisons show that the virus is closely related to other South American hantaviruses, and most closely related to Laguna Negra virus detected in patients and *Calomys laucha* rodents (vesper mice) in the Chaco region of Paraguay (7,8). The nucleotide sequence identity was 84% for the G1 protein encoding fragment and 87% for the N protein encoding fragment. However, the deduced amino acid sequences for both fragments were identical to Laguna Negra virus. This shows that the virus associated with this HPS case is a Laguna Negra virus variant and suggests that the virus is probably associated with the same or a very closely related species of rodent host.

C. laucha, the apparent primary rodent reservoir for Laguna Negra virus, is common in savanna and grassland areas as far north as southern Brazil and Bolivia, throughout much of Paraguay and Uruguay, and in Argentina as far south as Rio Negro province, but not in Chile (10). A number of the lowland rural locations that the patient visited in Bolivia are within the range of *C. laucha*.

The clinical course and travel history of the patient and the laboratory serology and molecular characterization of the viral RNA are compatible with infection in Bolivia with a Laguna Negra virus variant. This report and evolving information concerning hantaviruses associated with clinical HPS in Argentina and Paraguay strongly suggest that a diagnosis of HPS should be considered in patients with febrile respiratory distress syndrome throughout Latin America.

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Prevalence of Tick-Borne Pathogens in *Ixodes scapularis* in a Rural New Jersey County

To assess the potential risk for other tick-borne diseases, we collected 100 adult *Ixodes scapularis* in Hunterdon County, a rapidly developing rural county in Lyme disease–endemic western New Jersey. We tested the ticks by polymerase chain reaction for *Borrelia burgdorferi*, *Babesia microti*, and the rickettsial agent of human granulocytic ehrlichiosis (HGE). Fifty-five ticks were infected with at least one of the three pathogens: 43 with *B. burgdorferi*, five with *B. microti, and* 17 with the HGE agent. Ten ticks were coinfected with two of the pathogens. The results suggest that county residents are at considerable risk for infection by a tick-borne pathogen after an *I. scapularis* bite.

The vector-borne diseases Lyme disease, human babesiosis, and human granulocytic ehrlichiosis (HGE) are emerging in the Northeast and upper Midwest regions of the United States (1,2). The etiologic agents for these diseases (*Borrelia burgdorferi*, *Babesia microti*, and the HGE agent, respectively) appear to share the same vertebrate reservoir host (*Peromyscus leucopus*) and tick vector (*Ixodes scapularis*) (3-6). Immunologic evidence of human coinfection with these pathogens has been reported (7-10), and a culture-confirmed case of coinfection with *B. burgdorferi* and the HGE agent has recently been described (11). Thus, in Lyme disease–endemic areas, there may be a substantial risk for coinfection with *B. burgdorferi* and either *B. microti* or the HGE-causing rickettsia.

Hunterdon County (population 118,000) is a rural, but rapidly developing, county in western New Jersey (Figure), with many homes in wooded settings. In 1996, the county had the third highest case rate of Lyme disease (524 per 100,000) of all counties in the United States (Centers for Disease Control and Prevention case reports). The county has also had many suspected, but no serologically confirmed, cases of HGE.

The risk of acquiring a tick-borne pathogen in a specific geographic location depends largely on tick density and the prevalence rate of the agent in the tick population. Although cultivation of a microbial agent is considered optimal, molecular detection is more practical since it permits the rapid assay of large numbers of individual specimens. We report here the prevalence rate of *B. burgdorferi*, *B. microti,* and the agent of HGE in *I. scapularis* in Hunterdon County by species-specific polymerase chain reaction (PCR).

One hundred adult *I. scapularis* were collected by drag cloth or from personal clothing at 10 sites in the county during the fall of 1996 (Figure). Ticks were stored at room temperature in 70% ethanol until analysis. DNA was isolated from individual ticks with the Isoquick DNA extraction kit (ORCA Research, Bothell, WA)

Figure. Map of New Jersey showing Hunterdon County. Black dots indicate tick collection sites.

(12). The final DNA pellets were resuspended in 50 µl of sterile water, and a 10-µl aliquot was used for each PCR test. *B. burgdorferi*–specific PCR used primers IS1 and IS2 (13). The HGE agent was detected by PCR using primers GER3 and GER4, as described by Munderloh et al. (14), and *B. microti* DNA was detected according to methods used by Persing et al. (15). PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide. For *B. burgdorferi* and *B. microti,* hybridization with specific probes was carried out after transfer to nylon membranes (13). In each PCR experiment appropriate negative controls were used; these included master mix controls lacking template and reactions containing *B. burgdorferi* DNA, HGE agent DNA, or *Escherichia coli* DNA as additional controls for HGE agent, *B. burgdorferi,* or *B. microti* PCR, respectively. To prevent cross-contamination, specimen preparation, PCR amplification, and post-PCR analysis were performed in separate laboratories.

Results of PCR analysis for each of the pathogens on individual ticks are presented in the Table. At least one of the three pathogens was present in 55% of the ticks. The highest prevalence rate was that for *B. burgdorferi* (43/100), followed by the agent of HGE (17/100) and *B. microti* (5/100). Furthermore, these pathogens were widely distributed. At least one tick at each site was infected with *B. burgdorferi*, nine of 10 sites had HGE agent–positive ticks, and *B. microti*–positive ticks were found at five different collection sites. Ten ticks were coinfected with two of the three agents; no ticks were infected with all three pathogens. The *B. microti* PCR amplification products were gel purified and subjected to automated DNA sequencing. The 238 bp products yielded sequences identical to that reported for *B. microti* 18S ribosomal DNA (15).

Table. Prevalence rate of *Borrelia burgdorferi*, human granulocytic ehrlichiosis (HGE) agent, and *Babesia microti* in 100 adult *Ixodes scapularis*

Pathogen	No. infected ticks
B. burgdorferi	43
HGE agent	17
B. microti	5
B. burgdorferi and HGE agent	6
B. burgdorferi and *B. microti*	2
HGE agent and *B. microti*	2
B. burgdorferi, HGE agent, and *B. microti*	0

The prevalence rates for the three pathogens reported here are consistent with earlier studies at selected sites in the northeastern United States. Telford et al. reported prevalence rates of 36%, 11%, and 9% for *B. burgdorferi*, the HGE agent, and *B. microti*, respectively, in adult *I. scapularis* on Nantucket Island, Massachusetts; coprevalence of *B. burgdorferi* and the HGE agent was 4%, and no simultaneous infection with *B. burgdorferi* and *B. microti* was observed (4). We have reported prevalence rates of 52% and 53% for *B. burgdorferi* and the HGE pathogen, and coprevalence of 26% at a site in Westchester County, New York (5). Given the simultaneous infection of *I. scapularis* with these pathogens, it is not surprising that numerous serosurveys of Lyme disease patients indicate the coincident presence of antibodies to *B. burgdorferi* and *B. microti* and human granulocytic ehrlichia (7-10). These findings do not establish concurrent, active infection, nor do they demonstrate simultaneous transmission by a single tick bite; however, they suggest that persons living in disease-endemic areas are exposed to these agents, presumably as a result of tick bites.

Human infection by any of the three tick-borne agents alone generally results in similar acute manifestations, including fever, headache, and myalgia (1,10,16). Reported cases of Lyme disease greatly outnumber those of babesiosis or HGE. This, in conjunction with seroepidemiologic data, suggests that many infections with *B. microti* and granulocytic ehrlichia are subclinical. Dual infection with *B. burgdorferi* and *B. microti* often results in more severe illness (10); whether this is also true for concurrent infection by the agents of Lyme disease and HGE remains to be established. Human coinfection by multiple tick-borne agents may account for the variable nature of the clinical manifestations of Lyme disease.

This study demonstrates that the agents of Lyme disease, human babesiosis, and HGE coexist in the tick population of a previously identified Lyme disease–endemic region of New Jersey. The study provides further evidence that these three pathogens may be prevalent throughout the range of *I. scapularis.* Infection with any of these three tick-borne pathogens should be considered for residents or visitors of a disease-endemic area who have flulike symptoms and a history of a tick bite.

Acknowledgments

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Plague, a Reemerging Disease in Madagascar

Human cases of plague, which had virtually disappeared in Madagascar after the 1930s, reappeared in 1990 with more than 200 confirmed or presumptive cases reported each year since. In the port of Mahajanga, plague has been reintroduced, and epidemics occur every year. In Antananarivo, the capital, the number of new cases has increased, and many rodents are infected with *Yersinia pestis.* Despite surveillance for the sensitivity of *Y. pestis* and fleas to drugs and insecticides and control measures to prevent the spread of sporadic cases, the elimination of plague has been difficult because the host and reservoir of the bacillus, *Rattus rattus*, is both a domestic and a sylvatic rat.

In the last 15 years, Madagascar (population 13 million) has accounted for 45% of the cases of plague in Africa (1).

Epidemiology

Plague was brought to the island of Madagascar in 1898 by steamboats from India and has never disappeared. As a result of vaccination campaigns, improved housing and public hygiene, and the discovery of streptomycin and insecticides, plague was controlled in the 1950s. During the next 30 years, only 20 to 50 cases per year were reported in the entire country. However, since 1989, the number of suspected cases has increased steadily (Figure 1). Since 1990, 800 to 1,500 cases of suspected plague have been reported, of which 150 to 230 were smear-positive (presumptive cases) or confirmed by the isolation of *Y. pestis* (2,3). The population exposed to plague in the plateaus is approximately 5 million; the mean annual rate of known human cases (except in Mahajanga) is 3 to 4 per 100,000 inhabitants. The mean annual death rate is 20% of the confirmed or presumptive cases. Bubonic plague is the main clinical form of the disease (approximately 95% of cases).

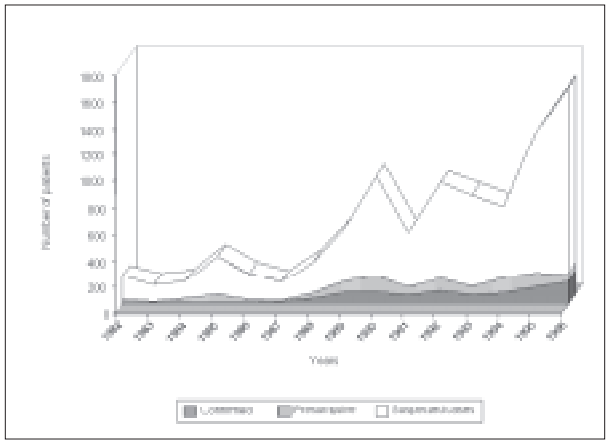

Figure 1. Human plague, Madagascar, 1982–1996.

With the exception of the west coast port of Mahajanga, plague is endemic in areas more than 800 m high. The major focus area is a central triangle, and the minor focus is a northern diamond (Figure 2). The main plague foci (in order of importance) are Mahajanga, the district of Ambositra, the town of Antananarivo, and the districts of Fianarantsoa II, Miarinarivo, Betafo, and Soavinandriana. In the plateaus, the human plague season is September to April, while in Mahajanga it is July to November.

In 1996, 1,644 suspected plague cases were reported; biologic samples were available for 1,316. A total of 173 confirmed and 56 presumptive cases were officially reported. In 1997, 2,127 suspected cases were reported from January to October, and more than 2,500 total cases were expected by the end of 1997. So far, 260 are confirmed and 33 are presumptive cases. The number of confirmed or presumptive plague cases is underestimated because of underreporting in remote areas and the lack of sensitivity of the bacteriologic techniques used for diagnosis. Our preliminary results, obtained by immunodiagnostic tests, such as anti-F1 antibody detection (4) and F1 antigen tests (tests supplied by the Naval Medical Research Institute, Bethesda, MD), suggest a number of plague cases two to three times higher than that obtained by conventional methods (S. Chanteau and J. Burans, unpub. data). Whether the significant increase between 1996 and 1997

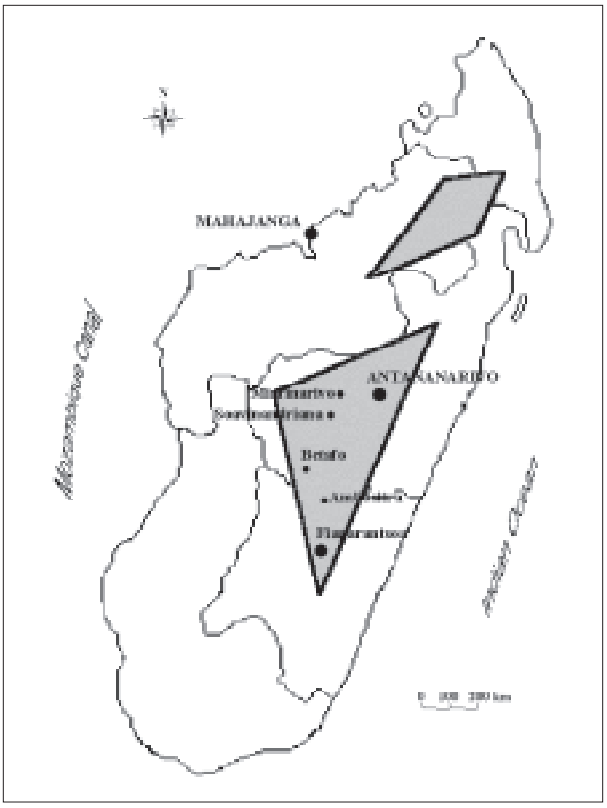

Figure 2. Plague foci in Madagascar. The plague-endemic zones are in the gray areas and in the port of Mahajanga.

results from better notification of the cases following educational campaigns or a real increase in disease incidence is unknown.

In Madagascar, the reservoirs of *Y. pestis* are two species of rats, *Rattus norvegicus* and *R. rattus*. The sylvatic reservoir of *Y. pestis* is not well documented. The urban domestic rat *R. norvegicus,* introduced to Madagascar during the 20th century, can only be found (in larger numbers than *R. rattus)* in coastal cities and Antananarivo. In contrast, *R. rattus*, probably imported into Madagascar with the first human migrations, has invaded every ecosystem of the island. Thus, it is both a domestic and a wild rodent. It is generally the only type of rodent found in small towns, villages, rice fields, and grasslands. The reservoirs of *Y. pestis* during the interseason period are the *R. rattus* and *R. norvegicus* that have resisted infection; therefore, it is almost impossible to eliminate plague from the island (2).

Two species of rat fleas are vectors of plague in Madagascar. The most effective are the classic oriental rat flea, *Xenopsylla cheopis,* and the endemic flea, *Synopsyllus fonquerniei* (2); both are found on *R. rattus*, although *X. cheopis* is almost exclusively on urban and indoor rodents, while *S. fonquerniei* is found on outdoor and wild rodents (S. Laventure and J.M. Duplantier, unpub. data). Ongoing research is examining the interrelationships between the two species of rats and the two species of fleas in the epidemiologic cycle of plague in Madagascar.

The reemergence of plague in Madagascar probably reflects the general breakdown of traditional measures of plague control. Prolonged maintenance of the domestic rat-to-flea cycle that accounted for the historic plague pandemics will almost surely provide an opportunity for selection of new traits that could make the organism even more virulent. In fact, three new variants of *Y. pestis* have recently emerged in the region of Ambositra and Ambohimahasoa, one of the most active plague foci of the island. These new ribotypes tend to spread to new geographic areas; whether or not they have acquired selective advantages is being explored (5). Furthermore, the opportunity for gene exchange with enteric bacteria is greatly enhanced. It may be important that the first naturally occurring antibiotic-resistant strain of *Y. pestis* was recently isolated in Madagascar (6,7).

Plague in Mahajanga

In Mahajanga (population 150,000), after two epidemics in the beginning of the 20th century, plague remained under control between 1928 and 1990 (2). In July 1991, the disease suddenly reappeared in a shantytown near the market-place of Marolaka; of the 170 suspected cases, 41 were confirmed or presumptive. After 3 years without reported cases, three successive out-breaks occurred: July 1995 (8,9), July 1996, and July 1997. During the 1995 and 1996 outbreaks, a total of 1,058 suspected cases were reported; only 109 cases were confirmed, and 30 cases were smear-positive. However, serologic testing, mainly F1 antibody detection by enzyme-linked immunosorbent assay (ELISA) (4), allowed the diagnosis of 93 additional cases (10). Thus, the mean annual rate of known human cases is 77 per 100,000 inhabitants. During the ongoing epidemic, from July to October 1997, 376 suspected cases (120 bacteriologically confirmed) have been reported and tended to spread in new quarters of the town.

Every human outbreak has been preceded by a high number of rat deaths. During and after

epidemics, all shrews captured were infected with many *X. cheopis.* Ongoing studies are examining the role of the shrew *Suncus murinus* in the maintenance of plague between epidemics.

Plague in Antananarivo

Since plague was introduced in the highlands in 1921, it has never disappeared from remote villages; however, in Antananarivo, it has been controlled; no human cases were reported between 1953 and 1978. In 1979, the first confirmed case was found in one of the ancient foci of the town (11). In the 1990s, a growing number of cases have been reported; 10 to 25 each year have been confirmed or are presumptive (Figure 3). In 1996, confirmed plague cases were found in 17 quarters of the capital. The mean annual rate of human cases is 1.4 per 100,000 inhabitants in 1995 and 1996.

In 1995, 10% of 625 rats trapped near a marketplace were infected with *Y. pestis;* 80% were anti-F1 seropositive (S. Chanteau, J.A. Drominy, and B. Rasoamanana, unpub. data). The monthly flea index (mean number of *X. cheopis* per rat) in this quarter was more than 4 year-round. Such a high index represents a serious threat, especially since most of these fleas are resistant to deltamethrin (12). Other families of insecticides, such as the carbamates, can be proposed. Since 1996, the Ministry of Health and local government have made serious efforts to educate the population and improve public hygiene.

A preliminary study of rats trapped in eight other quarters of the capital in 1997 showed a mean seroprevalence of 14% by ELISA (J.A. Drominy and S. Chanteau, unpub. data). The circulation of *Y. pestis* in the rat population was significantly larger than it was in 1965 (4.5%) (2).

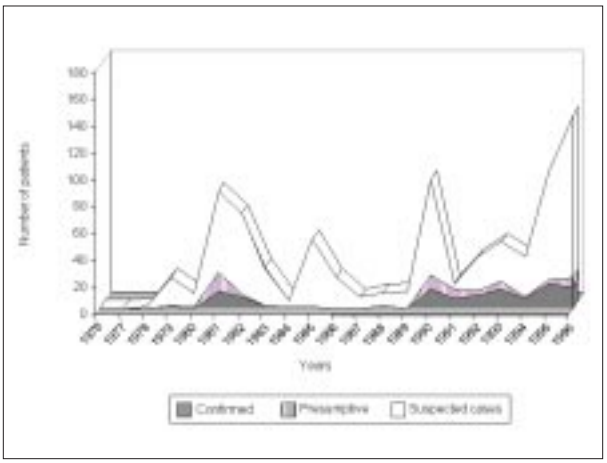

Figure 3. Human plague, Antananarivo, 1976–1996.

National Plague Control Program

The national plague control program is financially supported by the World Bank and the French Ministry of Cooperation. The surveillance system used is based on immediate compulsory notification of every suspected case of plague and its biologic confirmation by the Central Laboratory. All patients with suspected cases are treated with streptomycin, and their contacts are treated with sulfonamides to prevent disease spread. Insecticides are used for flea control. Every strain of *Y. pestis* isolated from humans, rats, and fleas is tested for drug sensitivity, and the susceptibility of fleas to insecticides is determined. The isolation of the first multidrug-resistant strain of *Y. pestis* in 1995 (6,7) and the increasing resistance of fleas to insecticides have caused much concern (12).

This national program, implemented in Madagascar for several decades, has been hampered by economic and operational difficulties and urgently needs to be strengthened.

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Bayou Virus-Associated Hantavirus Pulmonary Syndrome in Eastern Texas: Identification of the Rice Rat, *Oryzomys palustris*, as Reservoir Host

We describe the third known case of hantavirus pulmonary syndrome (HPS) due to Bayou virus, from Jefferson County, Texas. By using molecular epidemiologic methods, we show that rice rats (*Oryzomys palustris*) are frequently infected with Bayou virus and that viral RNA sequences from HPS patients are similar to those from nearby rice rats. Bayou virus is associated with *O. palustris;* this rodent appears to be its predominant reservoir host.

The 1993 discovery of a clinically distinct form of hantavirus disease now known as hantavirus pulmonary syndrome (HPS) highlighted the existence of a previously unrecognized complex of New World hantaviruses, each of which is associated with a specific rodent of the subfamily Sigmodontinae, family Muridae. The prototype of this complex is the Sin Nombre (SN) virus of the deer mouse, *Peromyscus maniculatus*. SN virus, which occurs most frequently in the western United States and Canada, is responsible for more than 95% of cases of HPS in North America. The case-fatality rate of HPS is nearly 50% (1).

Three viruses other than SN have been associated with HPS in North America. All cases associated with viruses other than SN have occurred outside the range of the deer mouse. Two cases have been linked to New York virus in the northeastern United States; the white-footed mouse, *P. leucopus*, was the only hantavirus carrier in the area where the infections were contracted. The cotton rat (*Sigmodon hispidus*)–associated Black Creek Canal virus is believed to have caused a single case in southern Florida, albeit without molecular confirmation. Bayou (BAY) virus has been identified from cDNA sequences amplified from the necropsied tissues or the blood of patients from Louisiana and eastern Texas (1-6).

Evidence implicates the rice rat, *Oryzomys palustris*, as the carrier of BAY virus. In a survey of archived rodent tissue samples obtained from species indigenous to Louisiana, only *O. palustris* samples were positive for hantavirus antibodies, and cDNAs of a BAY-like virus were amplified from two of those samples (7). A few additional BAY virus–RNA-positive rice rat samples have appeared in subsequent studies (6). However, no substantial rodent collections have been conducted in association with BAY virus HPS cases, and no direct molecular association has been found between human BAY virus genomic sequences and those obtained from samples from rodents trapped at possible sites of human infection. The purpose of this study was to identify the carrier rodents associated with two human cases of BAY virus infections and to further characterize the clinical consequences of human infection with BAY virus.

Diagnostic Studies

Initial serologic investigations to detect antibodies to hantavirus used a sandwich μ-capture enzyme-linked immunosorbent assay (ELISA) as well as an immunoglobulin (Ig) G ELISA, with recombinant SN virus nucleocapsid (N) protein as the target antigen (8). Confirmatory testing with a larger array of recombinant-expressed viral N antigens was conducted by using strip immunoblot and Western blot formats (9-11). The strip immunoblot assay incorporates five membrane-bound antigens (recombinant-expressed SN virus N and G1, recombinant-expressed Seoul virus N, and synthetic peptides of SN N and G1)(11). Antibodies reactive to these antigens are detected with an anti-human immunoglobulin heavy+light chain conjugate, and the assay thus has some sensitivity for IgM but greater sensitivity for IgG. The Western blot uses both anti-IgG and anti-IgM conjugates in separate assays (9).

Western blot studies used a panel of affinity-purified, full-length N antigens produced in *Escherichia coli* in fusion with the phage T7 gene 10 protein in the pET23b vector (10). Recombinant N antigens were purified over metal chelation affinity columns through a polyhistidine tag on the C-terminus of each protein. The recombinant proteins were produced in an isogenic background and loaded at 500ng/lane before sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation and electrophoretic transfer. The antigens were derived from the following hantaviruses: SN (3H226); BAY (OP-LA-475); Rio Mamoré (OM-556), Muleshoe (SH-Tx-339), Puumala (P360), and Seoul (80/39).

Rodent Collection and Processing

Since the two BAY virus-HPS cases from Texas occurred in neighboring cities, this study includes rodent samples collected in the investigation of both case P/Tx (6) and the case described here, T/Tx. Sherman live-traps were used to collect rodents in Jefferson County and neighboring Orange County. Heart blood samples were screened by a recombinant SN virus N antigen ELISA (8).

Viral Sequencing and Phylogenetic Analyses

Human peripheral blood mononuclear cells (2 x 10^6) and rodent lung, kidney, and spleen samples (~200 mg total) were used to make RNA (6,10). A standard set of partially nested primers in the viral S segment was used in reverse transcription-polymerase chain reaction (RT-PCR) analyses of the RNA (6,10). This procedure produces a 442-nucleotide (nt) amplification product; 397 nt of that sequence is internal to the primers. The PCR products were subjected to direct DNA sequencing with an ABI 377 automatic sequencer. Phylogenetic trees were constructed from the 397 nt of informative sequence by using maximum parsimony with PAUP 3.1 software (12).

A Third Case of HPS Due to BAY Virus

Patient T/Tx is a 54-year-old African-American man from Jefferson County, Texas. A heavy tobacco user, he has a more than 100 pack-years* smoking history and chronic shortness of breath. Approximately 2 weeks before admission, his spouse noted that he had increased fatigue and somnolence. By August 20, 1996, his symptoms worsened, and he sought medical care. He visited a local emergency room with complaints of increasing shortness of breath and low-grade fever for 2 days. Hospital personnel believed that he had probable chronic obstructive pulmonary disease and chronic bronchitis. Results of a physical examination of the patient at that time were unremarkable, and a chest radiograph was interpreted as hyperinflated but without infiltrates (Figure 1A). He was afebrile (37°C) in the emergency room. Laboratory analysis showed a white blood cell count of 4,080/µl and a platelet count of 122,000/µl. He was given amoxicillin and bronchodilators and was discharged.

On August 21, the patient returned to the emergency room with persistent and worsening dyspnea. He also had flulike symptoms, including myalgias, and had had a temperature of 39.4°C. Physical examination revealed bilateral rhonchi. He had a blood pressure of 90/60, pulse of 96/min, respiratory rate of 16/min, and an oral temperature of 37°C. Laboratory analysis noted an increase in his white blood cell count to 7,700/µl with a left shift and a decrease in platelet count to 65,000/µl. The patient was admitted to the hospital with a preliminary diagnosis of chronic obstructive pulmonary disease exacerbation and possible pneumonia. He was given broad-spectrum antibiotics, methylprednisolone, aminophylline, and ipatronium nebulizer treatments.

The patient's condition deteriorated rapidly over the next 24 hours, and he was severely short of breath by the second hospital day. He was also febrile and was transferred to the intensive care unit to manage his worsening shortness of breath. His oxygen demand increased, and on the third hospital day, the patient required endotracheal intubation. A physical examination found bilateral expiratory wheezing and bibasilar rales. A chest radiograph showed increasing bilateral interstitial and alveolar infiltrates. By the fourth hospital day, the patient required 100% oxygen and positive end-expiratory pressure support. A chest radiograph again showed interstitial and alveolar infiltrates with peribronchial cuffing and pleural effusion. His fluid intake was restricted to manage his pulmonary edema; he briefly required dopamine for hypotension. Blood and urine cultures remained negative. Sputum Gram stain showed a moderate number

*Pack-year = no. packs/day x no. years

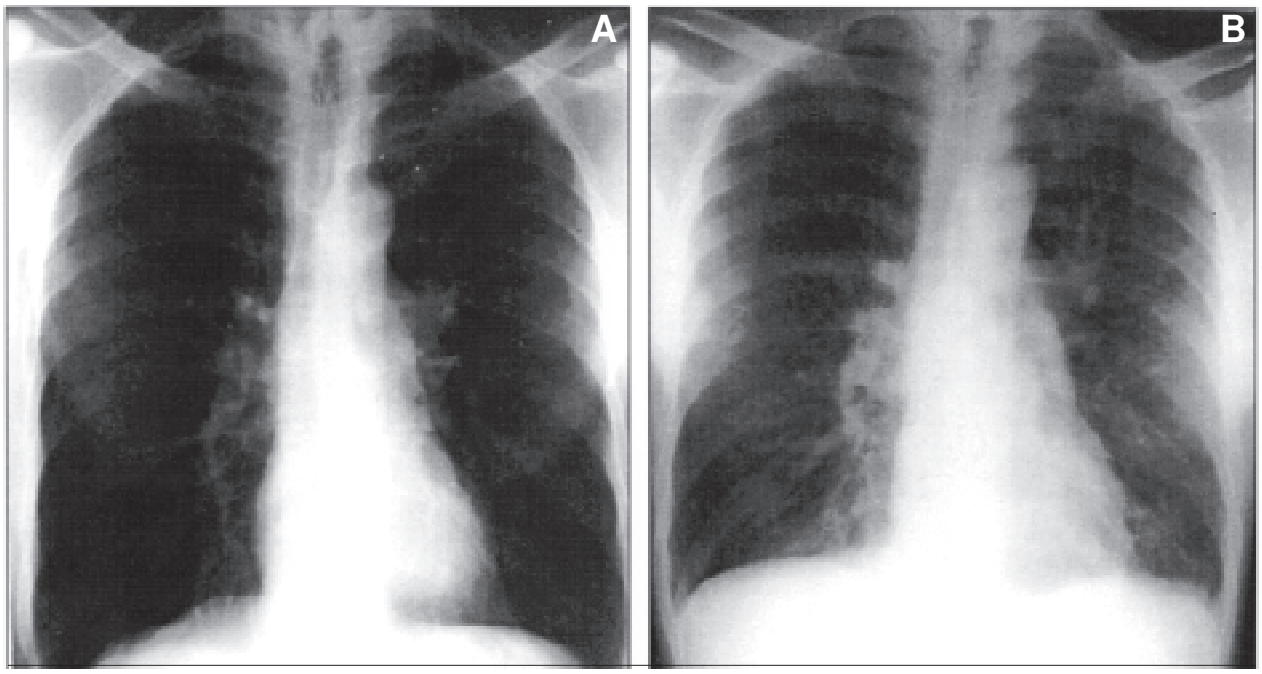

Figure 1. Chest radiographs of patient T/Tx at hospital admission on August 20, 1996 (Panel A), and during a period of increasing respiratory distress on August 22, 1996 (Panel B). Note the diffuse interstitial infiltrate and peribronchial cuffing that developed over that interval.

of white blood cells and few budding yeast; culture showed no pathogens. Serologic tests for mycoplasma and *Legionella* were negative.

By the fifth hospital day, the patient's condition had begun to improve. Over the next 5 days the fever diminished, the pulmonary edema improved, and requirement for supplemental oxygen decreased. The endotracheal tube was removed on the 10th day of hospitalization; by the 14th day, the patient was discharged—ambulatory, afebrile, and no longer requiring supplemental oxygen.

The hematologic laboratory findings were generally most abnormal when the patient was most acutely ill clinically; the peak serum chemistry abnormalities trailed the clinical illness by several days. The maximum white blood cell count was 31,700/µl on August 23, with a differential count of 7% lymphocytes (including the characteristic plasmacytoid forms; [13]), 44% mature neutrophils, 35% band neutrophils, 3% metamyelocytes, and 2% myelocytes. The platelet count reached a nadir of 19,000/µl on that day, and the hemoglobin reached a maximum of 17.2 g/dL on August 22. The serum creatinine was 0.6 mg/dL on August 24 but peaked at 1.9 mg/dL on August 25. This mild azotemia was associated with proteinuria (300 mg/dL in a spot sample obtained on August 22). The serum enzymes creatine kinase and lactic dehydrogenase peaked on August 27 at 917 units/L and 933 units/L, respectively. Fractionation of the creatine kinase and lactic dehydrogenase isoenzyme forms were not supportive of myocardial injury as a cause for their elevation. Serum levels of the liver transaminases were modestly elevated.

Environmental Assessment and Rodent Collection

The patient worked as a laborer at a railroad construction company. For 2 months before his illness, he replaced old rail lines at three industrial plants (Beaumont, Neches River, and Orange) in Orange and Jefferson Counties and cleaned the yard and garage at work headquarters. He manually removed and replaced tracks and ties serving warehouses, transport pipes, and scrap metal piles.

The Beaumont and Orange work sites were chemical plants. All rail work occurred within the confines of the mowed plant complex. Some of the track sites were next to drainage ditches and canals; others coursed by warehouses reported by

plant employees to have rats. The patient had not seen any rodents in the months before becoming ill, but he remembered rodent droppings at a wooden crew trailer at the Beaumont plant. All rodents trapped at these plants tested negative for hantavirus antibodies.

The Neches River work site was a recycling plant situated for commerce by rail and ship. The rails were within several meters of permanent bayous and swamps, piles of scrap metal, and the Neches River. The vegetation near the water was dense with trees and undergrowth. Truck and train traffic at the plant created a substantial dust problem. The site contained stray dogs and cats as well as a variety of wildlife, including snakes and alligators. Although trapped heavily, few rodents were collected from near the tracks and adjacent swamps. All rodents trapped at this facility were negative for hantavirus antibodies.

Although the patient lived in an older pier-and-beam home, he and his wife saw no evidence of rodent infestation. A Texas Department of Health investigator verified the absence of rodents and rodent excreta. Although traps were set, no rodents were collected at the residence.

During the 2 months before he became ill, the patient visited a casino boat in Louisiana and fished three times from the Pleasure Island jetty in southern Jefferson County. The jetty had large rock boulders at the water's edge and an asphalt road along its length. Sand and dense 6-foot high grasses covered the remainder of the jetty. Household trash and fishing debris were scattered throughout the area. Most rodents collected by the Texas Department of Health investigation team during this investigation were from this jetty; and most were *O. palustris*, including three seropositive specimens (Area 1, Figure 2).

Other sites were chosen between the patient's work sites and home because their habitats were likely to support rodents. Rice rat habitats (permanent water and grasses) were among those targeted, because the first Jefferson County hantavirus investigation documented the Bayou strain of hantavirus in *O. palustris* (6). The Table and Figure 2 show the location and species of each rodent collected and the number of seropositive specimens in each of four targeted areas. Four hantavirus antibody-positive rodents were trapped at these miscellaneous locations during the investigation of the current HPS case. Two of the seropositive samples were from Area 1 of Figure 2 (not the jetty), and two were in Area 2. Area 2 yielded two seropositive rodents during the first Texas BAY virus case investigation (6).

Diagnostic Studies

The IgG ELISA assay for SN virus antibodies in patient T/Tx's serum was negative, but the IgM test was positive at a 1:6400 dilution (8). The strip immunoblot assay showed antibody reactivities typically seen with HPS caused by a hantavirus other than SN virus, with 2+ reactivity to the immunodominant SN virus N peptide and 4+ reactivity to the full-length recombinant N protein (11). There was no reactivity to the SN virus G1 antigen, either in peptide or recombinant form, or to Seoul virus recombinant N protein (data not shown). Antibody reactivity to the SN virus G1 antigen is specific for infection with SN virus.

Western blot analysis of patient T/Tx's serum showed IgG antibodies to hantavirus N proteins

Figure 2. Location of rodents trapped during investigations related to patient P/Tx (March 1996; [6]) and patient T/Tx (October 1996; this report). The locations in which rodents were collected are broadly classified into four areas, designated 1 through 4.

Table. Rodents collected in association with two cases of Bayou virus-hantavirus pulmonary syndrome in Jefferson and Orange Counties, Texas, March and October 1996

Species	No. specimens collected (no. seropositive) Area 1[a]	Area 2	Area 3	Area 4	Tot.	No. PCR[b] tested (no. pos.)
Baiomys taylori	0	0	1 (0)	3 (0)	4 (0)	0
Mus musculus	30 (0)	21 (0)	12 (1)	26 (0)	88 (1)	1 (0)
Ochrotomys nuttali	0	0	0	1 (0)	1 (0)	0
Oryzomys palustris	45 (7)	20 (5)	3 (0)	8 (3)	76 (15)	15 (14)
Peromyscus leucopus	0	3 (0)	0	0	3 (0)	0
Rattus norvegicus	1 (0)	1 (0)	0	1 (0)	3 (0)	0
Rattus rattus	21 (0)	8 (0)	0	1 (0)	30 (0)	0
Reithrodontomys fulvescens	0	7 (0)	0	7 (0)	14 (0)	0
Reithrodontomys humulis	1 (0)	0	0	2 (0)	3 (0)	0
Sigmodon hispidus	95 (0)	5 (0)	11 (0)	4 (1)	114 (1)	1 (1)

[a]Refer to Figure 2 for map areas.
[b]PCR=polymerase chain reaction.

(Figure 3, Panel A). Although the most intense staining was observed with the homologous (BAY virus) antigen, varying cross-reactivity was present against the N antigens of Muleshoe, SN, Rio Mamoré, and Puumala viruses but not of Seoul virus. A similar pattern was observed when another blot membrane was probed with an anti-human IgM conjugate, except that reactivities were somewhat more intense (data not shown). Serum samples of seropositive rice rats and deer mice showed similar cross-reactivities, although the intensity of reactivity to a particular N antigen was related to the sequence similarity between the membrane-bound antigen and the virus against which the antibodies were directed (Figure 3, Panels B and C).

RT-PCR analyses and sequencing of the cDNA product of the patient's blood sample identified definitively BAY virus as the etiologic agent. When the 442 nt viral S segment amplification product from patient T/Tx was compared with the homologous sequence of other hantaviruses, it was closely similar to previously described BAY viruses from Louisiana and Texas (Figure 4). Nine viral sequences from seropositive *O. palustris* and one from a seropositive *S. hispidus*

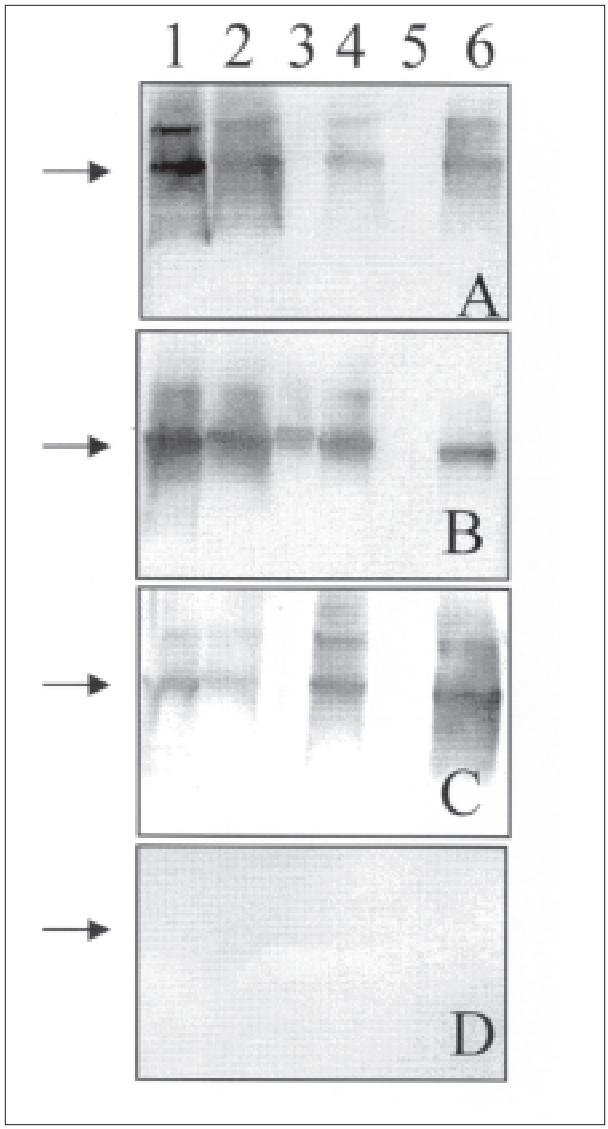

Figure 3. Western blot assay for detecting IgG antibodies in patient and rodent blood samples. A 1:500 dilution of serum was used to probe Western blots containing equimolar amounts of recombinant-expressed N antigens of various hantaviruses: 1) Bayou; 2) Muleshoe; 3) Puumala; 4) Rio Mamoré; 5) Seoul; and 6) Sin Nombre. Serum samples are directed against specific hantaviruses as verified by reverse transcription-PCR and sequence analyses: (A) patient T/Tx, Bayou virus; (B) Bayou virus-seropositive *Oryzomys palustris* specimen #505, collected in Jefferson County, Texas; (C) a Sin Nombre–seropositive deer mouse, collected in California; (D), a negative control (hantavirus-seronegative) deer mouse. Antibodies bound to the solid-phase antigens were detected with alkaline phosphatase-conjugated anti-human IgG (A) or with anti-*Peromyscus leucopus* IgG (B-D). The arrow indicates the migration of the full-length T7-viral N antigen, ~55kDa.

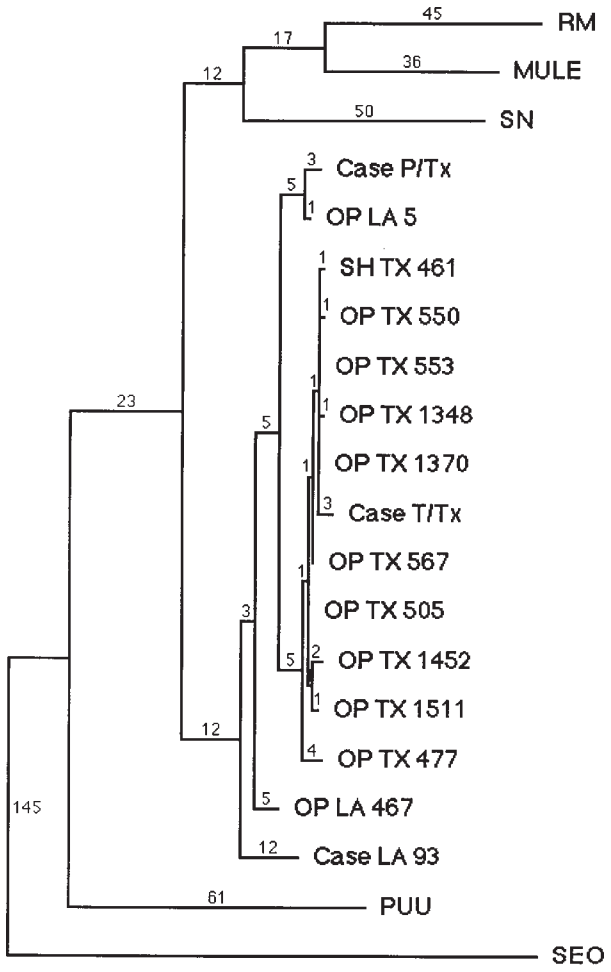

Figure 4. Unweighted maximum parsimony tree produced by PAUP 3.1 software comparing a 397 nt portion of the hantavirus S genomic segments (residues 207 to 603) of patients and rodents infected with Bayou virus. A selection of other prototypical hantavirus sequences, each of which was compared antigenically in Figure 2, was included for comparison. Other hantaviruses are abbreviated as follows: RM=Rio Mamoré; MULE=Muleshoe; SN= Sin Nombre; PUU=Puumala; and SEO=Seoul. Human-derived Bayou virus sequences are indicated by Case, and rodent sequences by OP (*Oryzomys palustris*) or SH (*Sigmodon hispidus*). TX=Texas, LA=Louisiana.

from Jefferson County, Texas, and Cameron and Terrebonne Parishes, Louisiana, were compared with those of patients T/Tx and P/Tx and with that of a 1993 case-patient from northern Louisiana (Case-LA-93, Figure 4).

The sequences of all the Jefferson County rodents and patient T/Tx were closely related to each other, differing by 1 to 10 nt (0.25% to 2.5% of 397 evaluable residues) in pairwise comparisons. Four rodent-derived viral sequences differed from that of the patient T/Tx virus by 4 nt (1%), and all were from rodents collected in Jefferson County.

Except for a brief visit to a Louisiana casino, patient T/Tx reported that he had not traveled outside east Texas in the 2 months before his illness. The generally close genetic similarity among the viral sequences from east Texas supports the hypothesis that patient T/Tx became infected in that region (14). Potential exposure sites, as defined by his travel history, were too numerous to allow us to identify a more precise site of infection. No rodent sequence was completely identical to that of patient T/Tx.

The sequence obtained from patient P/Tx (6), who was thought to have been infected in Jefferson County, was not closely aligned with any eastern Texas rodent sequence or with that of patient T/Tx. The closest of the 11 eastern Texas viral sequences differed from that of patient P/Tx at 13 residues (3.3%). One sequence available from previous studies was far closer to that of patient P/Tx, differing at only four residues (1%). This sequence was obtained from a rice rat (OP-LA-5) collected in Cameron Parish, Louisiana, approximately 30 km from the former residence of patient P/Tx. Although patient P/Tx initially reported no travel back to his former residence (where his mother still lived) in the 6 weeks before his illness (6), he later was not certain about his travel history in the weeks before his illness, stating that he might have visited his mother in Louisiana in the weeks before his illness.

In previous studies, *O. palustris* was tentatively implicated as the predominant rodent reservoir for BAY virus, but the sample sizes were small, and specific cases of HPS were not linked to the occurrence of BAY virus-infected rodents at the presumed sites of infection (6,7). For this study, analysis of a substantial collection of rodents collected in the vicinity of sites of human infection showed that *O. palustris* is the species with the highest hantavirus seroprevalence. Furthermore, the hantavirus genotypes in circulation in the eastern Texas/western Louisiana area were related to those of two human case-patients from the area, and all but one of those rodent-derived sequences came from *O. palustris*.

Rice rats are found in wetlands and marshes from Texas throughout the southeastern United States, extending north as far as New Jersey on the eastern seaboard. Like all carriers of viruses associated with HPS, *O. palustris* is a member of

the subfamily Sigmodontinae, family Muridae. Although only a single species exists in the United States, the tribe Oryzomyini has scores of species in Latin America, where HPS is increasingly recognized as an important zoonotic disease. Viruses similar to BAY virus have been recognized recently in members of the Oryzomyini from Bolivia and Argentina, and in some cases these rodent-borne viruses have been linked to HPS in humans (1,15).

The possibility that the genetically distinct clade of viruses associated with oryzomine and *Sigmodon* rodents produces a disease that is qualitatively different from SN virus-associated HPS (16) has been raised repeatedly as new cases have been identified (5,6,17). Although patient T/Tx had a relatively mild course of HPS, his serum creatinine, urine protein concentration, and creatine kinase were nevertheless slightly elevated. Both the elevation of creatine kinase and chemical evidence of myositis have been observed in HPS caused by viruses of the oryzomine and *Sigmodon* clade, but much less commonly in SN virus infection (1). Further studies are needed to determine the basis for the variant clinical symptoms of HPS in these patients.

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Laboratory Survey of Drug-Resistant *Streptococcus pneumoniae* in New York City, 1993–1995

Wide geographic variation in the prevalence of drug-resistant *Streptococcus pneumoniae* demonstrates the importance of tracking antimicrobial resistance locally. This survey of hospital microbiology laboratories in New York City found that penicillin resistance (MIC ≥ 2.0 μg/ml) increased from 1.5% of *S. pneumoniae* isolates in 1993 to 6.3% in 1995 and that in 1995, one-third of isolates nonsusceptible to penicillin (MIC ≥ 0.1 μg/ml) were also nonsusceptible to an extended-spectrum cephalosporin (MIC ≥ 1 μg/ml).

The emergence of drug-resistant *Streptococcus pneumoniae* underscores the need for timely, local, population-based surveillance of antimicrobial resistance. The prevalence of resistance in U.S. communities varies widely, with 2% to 53% of *S. pneumoniae* isolates found to have reduced susceptibility to penicillin (1-4). The Centers for Disease Control and Prevention recommends that empiric antibiotic therapy for pneumococcal infections be based upon local susceptibility patterns (2,5). However, few communities track drug-resistant *S. pneumoniae*.

The Survey

To estimate the prevalence of drug-resistant *S. pneumoniae* in New York City, we surveyed hospital-based clinical microbiology laboratories from 1993 to 1995. A standardized questionnaire was mailed annually to each laboratory, and those that did not respond were contacted by telephone or were visited. To evaluate compliance with *S. pneumoniae* penicillin susceptibility testing guidelines established by the National Committee for Clinical Laboratory Standards (NCCLS) (6), we asked about criteria for selecting specimens and techniques for oxacillin disk diffusion screening and determination of penicillin MICs.

To determine the prevalence of penicillin resistance, we asked for the number of *S. pneumoniae* isolates identified during the year, the number tested for susceptibility to penicillin, and the number found to be possibly resistant by the oxacillin disk diffusion test and penicillin-intermediate or -resistant by MIC testing. We also asked that information be provided separately for isolates from normally sterile sites (e.g., blood, cerebrospinal fluid) and from nonsterile sites (e.g., sputum, nasopharyngeal swab). In 1995, we added questions regarding the MIC test results for extended-spectrum cephalosporins (ESCs), including the number of penicillin-nonsusceptible isolates that were also nonsusceptible to an ESC. No individual patient information was obtained. A report summarizing the results of the survey and describing NCCLS guidelines was mailed annually to microbiology laboratories, hospital infection control departments, and local infectious disease physicians and pediatricians.

Analysis

Of 67 hospital-based clinical microbiology laboratories in New York City, 100% completed the survey in 1993, 98% in 1994, and 100% in 1995. Overall, more than 5,000 *S. pneumoniae* isolates were reported annually.

Data were analyzed by using EpiInfo Version 6.0 (CDC, Atlanta, GA, USA). Drug-susceptibility results are presented for laboratories that conformed with NCCLS guidelines and provided complete data on all *S. pneumoniae* isolates identified (Table 1).

Susceptibility Criteria

The NCCLS recommends routine screening by the oxacillin disk diffusion test of clinically important *S. pneumoniae* isolates for susceptibility to penicillin. Isolates with a zone size ≤ 19 mm, or any isolate from the blood or cerebrospinal fluid, should be tested with an approved MIC method such as broth dilution or antibiotic gradient strips (e.g., E-test). Isolates whose penicillin MICs are either intermediate (MIC ≥ 0.1 and ≤ 1 μg/ml) or resistant (MIC ≥ 2 μg/ml) should also have MICs determined for susceptibility to an

Table 1. Penicillin resistance among *Streptococcus pneumoniae* isolates at New York City hospital laboratories

No. of isolates	1993 No. (%)	1994 No. (%)	1995 No. (%)
Screened with oxacillin disk[a]	3,227	4,133	4,912
zone size ≤ 19 mm[b]	273 (9)	549 (13)	995 (20)
Screened and confirmed with approved MIC[c]	1,229	2,491	3,535
zone size ≤ 19 mm	154 (13)	350 (14)	704 (20)
I[d]	70 (6)	209 (8)	310 (9)
R[d]	19 (2)	115 (5)	222 (6)
Sterile-site isolates screened and confirmed with MIC[e]	462	649	1,321
I	16 (3)	66 (10)	83 (6)
R	5 (1)	20 (3)	65 (5)
Nonsterile isolates screened and confirmed with MIC[f]	456	662	1,596
I	12 (3)	83 (13)	200 (13)
R	5 (1)	31 (5)	110 (7)

[a]Number of laboratories reporting oxacillin disk diffusion test results in 1993, 1994, 1995 was 33, 40, 51, respectively.
[b]Oxacillin disk diffusion test zone size.
[c]Approved penicillin MIC tests include antibiotic gradient strips and broth dilution or micro-dilution using Mueller-Hinton broth supplemented with 2-5% lysed horse blood. Number of laboratories reporting MIC test results in 1993, 1994, 1995 was 10, 22, 35, respectively.
[d]I=Penicillin-intermediate (MIC ≥ 0.1 and ≤ 1.0 µg/ml); R=Penicillin-resistant (MIC ≥ 2.0 µg/ml).
[e]Number of laboratories reporting sterile site isolate results in 1993, 1994, 1995 was 8, 12, 32, respectively.
[f]Number of laboratories reporting nonsterile site isolate results in 1993, 1994, 1995 was 5, 9, 28, respectively.

Table 2. Penicillin susceptibility testing protocols for *Strepococcus pneumoniae* in New York City hospital laboratories

No. of laboratories[a]	1993 No. (%)	1994 No. (%)	1995 No. (%)
Screened with oxacillin disk	55 (82)	63 (97)	65 (97)
Screened all isolates	38 (57)	56 (86)	59 (88)
Screened only sterile	9 (13)	6 (9)	6 (9)
Other	8 (12)	1 (2)	0
Performed penicillin MIC test	39 (58)	51 (78)	56 (84)
MIC-tested			
All isolates	10 (15)	9 (14)	7 (10)
Oxacillin-resistant only	18 (27)	39 (60)	42 (63)
Sterile isolates only	5 (7)	1 (2)	0 (0)
By request only	4 (6)	1 (2)	5 (7)
Other	2 (3)	1 (2)	2 (3)
Penicillin MIC technique[b]			
Antibiotic gradient strip[c]	11(16)	41 (63)	50 (75)
Automated tests[c]	21 (31)	9 (14)	2 (3)
Broth dilution	8 (12)	11 (17)	5 (7)
Unknown	2 (3)	3 (5)	0
Conformed with NCCLS guidelines[d]	15 (22)	38 (58)	46 (69)

[a]Total number of laboratories responding to survey in 1993, 1994, 1995 was 67, 65, 67, respectively.
[b]13 laboratories used more than one MIC technique.
[c]Antibiotic gradient strip: e.g., E-test. Automated tests: e.g., Microscan, Vitek.
[d]NCCLS=National Committee for Clinical Laboratory Standards. Screened all *S. pneumoniae* isolates with oxacillin disk diffusion test and confirmed resistance with approved penicillin MIC test.

ESC such as cefotaxime or ceftriaxone (ESC-intermediate MIC = 1 µg/ml; ESC-resistant MIC ≥ 2 µg/ml) (6). We will use the term "nonsusceptible" to refer to both intermediate and resistant isolates.

Findings

The proportion of laboratories conforming with NCCLS guidelines for penicillin susceptibility testing of *S. pneumoniae* increased from 22% in 1993 to 69% in 1995. This was due to an increase in the number of laboratories that screened all isolates, a sharp decrease in the use of automated MIC tests, and a fourfold rise in the use of antibiotic gradient strips for determining MICs (Table 2). Overall, the proportion of isolates with oxacillin disk diffusion test zone size ≤ 19 mm, suggesting possible resistance, increased from 8.5% of isolates in 1993 to 20.2% in 1995 (Table 1). MIC test results showed that 5.7% of isolates were penicillin-intermediate and 1.5% penicillin-resistant in 1993, compared with 8.8% and 6.3%, respectively, in 1995. The prevalence of resistant organisms increased among isolates from both sterile and nonsterile sites and was somewhat higher among nonsterile-site isolates than among sterile-site isolates in 1995 (6.9% vs. 4.9%, Chi-square p = 0.03).

Seven of the laboratories followed NCCLS-recommended methods and provided complete penicillin susceptibility results for both 1993 and 1995. These seven reported that among 779 isolates in 1993, 25 (3.2%) were intermediate and six (0.8%) were resistant to penicillin. Among 896 isolates in 1995, 83 (9.2%) were intermediate, and 47 (5.2%) were resistant.

The prevalence of penicillin resistance varied between laboratories and by geographic area. At

35 laboratories in 1995, 0% to 80% (median 13.1%) of isolates were nonsusceptible to penicillin. Laboratories in the New York City borough of Manhattan reported that 18% of isolates were nonsusceptible to penicillin, compared with 16% in Queens, 14% in the Bronx, and 10% in Brooklyn (MIC data from Staten Island were not available).

In 1995, 18 laboratories reported that of 275 *S. pneumoniae* isolates nonsusceptible to penicillin, 90 (33%) were also nonsusceptible to an ESC.

Limitations

The survey design had several limitations. First, levels of expertise varied among laboratories regarding antibiotic susceptibility testing of pneumococci. Laboratory variation may partly account for the wide range in the proportion of isolates nonsusceptible to penicillin observed at individual laboratories, which could in turn influence citywide estimates. We did not collect and test isolates to confirm laboratory results. Second, the number of laboratories included in our results increased each year as more laboratories adopted NCCLS methods and provided complete data. This improved the accuracy of the survey but made interpreting trends difficult. Third, citywide estimates inevitably mask important differences in the risk for drug-resistant *S. pneumoniae* infections in specific subpopulations; for example, day-care attendance and prior antibiotic use have been associated with drug-resistant pneumococcal infections in children (1,7). Finally, since information was collected on isolates, rather than individual patients or infections, actual disease incidence could not be calculated.

Conclusions and Recommendations

Our results document a marked improvement in penicillin susceptibility testing protocols for *S. pneumoniae* during this period. Because of an increase in the proportion of isolates tested and widespread adoption of antibiotic gradient strips for MIC testing, more than two-thirds of laboratories conformed with NCCLS guidelines in 1995, compared with fewer than one-fourth in 1993.

The survey demonstrates that drug-resistant pneumococci are prevalent and may be increasing in New York City. Penicillin resistance increased fourfold between 1993 and 1995, and data from 1996 indicate that it has remained at this level or continued to increase. Nine percent of blood isolates in 1996 were intermediate and 6% resistant to penicillin (New York City Department of Health, unpub. data), compared with 6% and 5%, respectively, among sterile-site isolates in our survey for 1995. The proportion of *S. pneumoniae* isolates resistant to penicillin in New York City was similar to U.S. national averages during this period (2-4). Our finding that 33% of penicillin-nonsusceptible *S. pneumoniae* isolates were resistant to ESCs is also similar to the 29% (4) observed nationally.

On the basis of these results, we recommended that New York City clinicians consider carefully the possibility of penicillin and ESC resistance when treating suspected *S. pneumoniae* infections. Although the response to therapy may vary by route of administration, site of infection, and age and immune status of the patient, treatment failure is increasingly common and can have serious consequences (8,9). Educational efforts are needed to promote appropriate use of antibiotics and encourage use of the 23-valent pneumococcal vaccine in populations at increased risk for pneumococcal disease (10).

This laboratory survey was a relatively low-cost method of estimating the prevalence of drug-resistant pneumococci in New York City. It may serve as a model in areas where clinical laboratories routinely perform drug-susceptibility testing but the resources to collect or test isolates centrally for surveillance are limited. Despite the survey's limitations, the estimates provided may be sufficient to guide clinicians in selecting appropriate empiric therapy for suspected pneumococcal infections. Considerable effort was spent each year disseminating these data to laboratories, hospital infection control departments, infectious disease physicians, pediatricians, and other primary care physicians.

Our ability to expand this survey approach beyond *S. pneumoniae*, penicillin, and ESCs is limited by constraints on hospital laboratory staff. As laboratories computerize and standard formats are developed for the electronic transmission of laboratory test results, collection and dissemination of susceptibility data will be possible on a wider range of pathogens and antimicrobial drugs.

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B-virus from Pet Macaque Monkeys: An Emerging Threat in the United States?

Of primary concern when evaluating macaque bites are bacterial and B-virus infections. B-virus infection is highly prevalent (80% to 90%) in adult macaques and may cause a potentially fatal meningoencephalitis in humans. We examined seven nonoccupational exposure incidents involving 24 persons and eight macaques. Six macaques were tested for herpes B; four (67%) were seropositive. A common observation was that children were more than three times as likely to be bitten than adults. The virus must be assumed to be a potential health hazard in macaque bite wounds; this risk makes macaques unsuitable as pets.

B-Virus in Nonhuman Primates

Cercopithecine herpesvirus 1 (*Herpesvirus simiae* or B-virus) frequently infects Old World primates of the genus *Macaca*. Of at least 19 species of macaques, rhesus, Japanese, cynomolgus, pig-tailed, and stump-tailed macaques are the species most commonly used in biomedical research (1). Seroprevalence of neutralizing antibodies to B-virus in captive adult macaque populations is 73% to 100% (1-3). Like *Herpesvirus simplex* virus infection in humans, B-virus infection in monkeys is characterized by lifelong infection with intermittent reactivation and shedding of the virus in saliva or genital secretions, particularly during periods of stress or immunosuppression (4). B-virus infection is transmitted among free-ranging or group-housed animals, primarily through sexual activity and bites. In captivity, as well as in the wild, mature macaques are more likely than immature animals to have been infected with, and shed, the virus. Antibody titer to B-virus indicates infection but can neither confirm nor eliminate actual viral shedding at the time of the bite (4).

B-Virus in Humans

B-virus disease in humans usually results from macaque bites or scratches (4). Incubation periods may be as short as 2 days, but more commonly are 2 to 5 weeks (1,3,5-7; Centers for Disease Control and Prevention [CDC], unpub. data). Most documented infections have occurred among biomedical research employees who had occupational exposure to macaques, although transmission has also been documented among laboratory workers handling infected central nervous system and kidney tissues (1,5).

From 1990 to 1992, 28 U.S. residents reported nonoccupational macaque bites to CDC (L. Chapman, pers. comm.). Since 1993, additional nonoccupational exposure cases have been reported, seven of which (involving 24 persons and eight macaques) are listed in Table 1. Of the six macaques for which herpes B serologic results were available, four (67%) were positive. Two owners refused requests for testing. Four (44%) of nine exposed children were bitten, versus only three (20%) of 12 adults. Children were 3.2 times more likely to be bitten than adults; although a common observation, this association is not statistically significant for this case series.

Most free-ranging monkey populations are thought to be part of the exotic fauna of distant tourist destinations and wild animal parks; however, macaque species have established free-ranging feral populations in Texas and Florida. In such settings, contact between humans and macaques cannot be safely controlled (8-10), and workers and visitors are at risk. Guidelines for B-virus prevention and diagnosis have recently been published (9-12).

Symptomatic human infection with B-virus is rare; fewer than 40 cases were reported from 1933 to 1994 (1,4-7,13-15; CDC, unpub. data). However, the consequences of symptomatic infection may be severe. Viral infection rapidly progresses to central loci in the spinal cord and, eventually, the brain. Of 24 known symptomatic patients whose cases were reviewed in 1992, 19 (79%) died (CDC; unpub. data).

Before 1987, most surviving human patients had moderate to severe neurologic impairment, sometimes requiring lifelong institutionaliza-

Table 1. Selected pet macaque bite cases[a,b]

Location	Primate species, age, B-virus status	Nature of exposure	Comments
Illinois	Rhesus, 20+ yrs, B-virus positive Cynomolgus, 2-4 yrs, B-virus negative	Household contact (2 adults/3 children), bites, scratches (2 adults)	Bought at auction, wife bitten multiple sites, children hand-fed monkey
Florida	Cynomolgus, 2 yrs, B-virus positive	Household contact (1 adult), bite (1 child)	Kissed on lips, ate off owner's plate, shared bed
Arizona	Cynomolgus, 2 yrs, B-virus negative	Bites on toe and buttock (child)	Unprovoked attack on neighbor, declared vicious animal by judge, no. of household contacts (owner) unreported
	Cynomolgus, 7 weeks, B-virus positive	Household contact (6 adults), bite on face (1 adult)	Diapered, shared chewed gum, oral ulcers noted by veterinarian, bite incident at neighborhood bar
	Macaque, (species undetermined), 2 yrs, B-virus status unknown	Bite on thigh (1 child)	Unprovoked attack (climbed fence to bite child)
	Macaque, (species and age undetermined), B-virus status unknown	Severe bite (1 child)	Injured child attended an unlicensed day-care facility run by monkey owner, 7 other monkeys on premises
Minnesota	Rhesus, 2 yrs, B-virus positive	Household contact, owners' friend bitten	Acquired as "child-substitute" (full-time baby-sitters hired)

[a]Cases referred to Centers for Disease Control and Prevention since 1993.
[b]As of November 1997, no confirmed transmission of B-virus in these persons has been documented.

tion (1). Recently, acyclovir has prevented progression of the disease in a limited number of patients. In at least three patients, this treatment reversed the neurologic symptoms and was life-saving (7,14,15). Rapid diagnosis and initiation of therapy are of paramount importance in preventing death or permanent disability in surviving patients.

Human and Macaque Interactions

Most owners form an emotional bond with infant primates. This bond is probably strengthened by the neonatal monkey's physical and behavioral resemblance to a human infant. Although physically and emotionally dependent on their mothers (or human substitutes) for up to 2 years of age, most macaques exhibit unpredictable behavior as they mature. Males tend to become aggressive, and both male and female macaques bite to defend themselves and to establish dominance. Dominance within the social hierarchy of macaques is established by aggression toward other monkeys, generally the younger and smaller members of the group. Both veterinary specialists and breeders of nonhuman primates agree that as a rule, all these animals bite (16,17). Biting incidents eventually bring the animals to the attention of animal control authorities. Most state health departments can require that any biting nondomestic animal be euthanized and the brain be submitted for rabies testing.

Regulations, Guidelines, and Policies Regarding Nonhuman Primates

Table 2 lists the principal federal regulations affecting the possession, distribution, and uses of nonhuman primates. The United States is obligated under the Convention in International Trade in Endangered Species (CITES) to restrict and control trafficking in exotic and endangered species.

Since October 10, 1975, U.S. Public Health regulation 42 CFR 71.53(c) has prohibited the importation of nonhuman primates into the United States as pets, and neither nonhuman primates imported since that date nor their offspring may be legally bred or distributed for any uses other than bona fide science, university-

Table 2. Federal regulations regarding nonhuman primates

Agency	Statute	Regulation	Subjects
Departments of Health, Centers for Disease Control and Prevention	Public Health Services Act, 42 USCS 201	42 CFR 71.53	Importation, distribution, bona fide uses in the U.S., breeding colony requirements, pet/avocationist uses
Department of Agriculture	Animal Welfare Act, 7 USCS 2131-2159	9 CFR Subchapter A	Licenses (breeders, dealers, laboratories, exhibitors, auctions), interstate health certificates, humane care and transport
Department of the Interior, U.S. Fish and Wildlife Service	Endangered Species Act, 16 USCS 1540 Lacey Act, 18 USCS 42	50 CFR 10, 11, 13, 14, 16	Endangered species, smuggling, interstate sales

level educational programs, or full-time zoologic exhibition. Furthermore, the regulation states, "the maintenance of nonhuman primates as pets, hobby, or an avocation with occasional display to others is not a permissible use" (18).

All states require their citizens to comply with applicable federal regulations. Many state officials, however, may be unaware of regulatory restrictions on the uses and distribution of nonhuman primates and may be confused by the distinctions among federal agencies regarding regulatory restrictions on captive-bred animals. State wildlife authorities may not know that a federal public health regulation prohibits the keeping ("maintenance") of nonhuman primates imported after October 10, 1975, as pets, for a hobby, or as an avocation; likewise, many do not know the compelling public health and safety reasons for enforcement.

Captive-bred offspring of animals purported to have been imported before October 10, 1975, are frequently offered for sale. Without documentation it is very difficult to determine whether this is the case. Depending on the specific circumstances, it is possible for undocumented animals to be considered deliberately misclassified (i.e., intentionally mislabeled), a violation under the Lacey Act (18 USC 42) and under 16 USC 3373 (19).

In 1987 and 1988, occupational safety guidelines were published based on evidence that all macaque species are inherently dangerous to humans because of the risk for B-virus transmission, as well as the likelihood of serious physical injury from bite wounds (9-12,14,15). Several recent reviews of monkey-bite injuries worldwide indicate that severe lacerations, wound infections, and permanent sequelae (e.g., flexure contractures, osteomyelitis) were present in 33% of cases (20,21).

In 1990, the American Veterinary Medical Association issued a general policy statement opposing the keeping of wild animals (especially those inherently dangerous to humans) as pets and advising veterinarians to exert their influence to discourage this practice (22). In 1995, updated guidelines for the prevention and treatment of B-virus infections in exposed persons were published (12). Despite these continuing public health educational efforts, nonhuman primates (including macaques) continue to be marketed and kept as pets in many states (16,17,23).

The Frequency of Exposure Resulting in Infection

Much remains to be learned about the pathogenesis of B-virus infection in humans. In this very limited case series (Table 1), one family (two adults and two of three children) exposed to a B-virus positive macaque had flulike symptoms. One of the adults had additional symptoms related to the injury site, which suggested B-virus infection. In the other six cases, no suspect clinical symptoms were noted, and disease-specific antiviral postexposure prophylaxis was not given. B-virus is still rare, and diagnostic evaluation of clinical cases of aseptic meningitis does not routinely include B-virus testing.

Owners of pet macaques are often reluctant to report bite injuries from their pets, even to their medical care providers, and may fail to appreciate that the premonitory headache and flulike symptoms (which may lead them to seek medical attention) could be associated with healed, often minor, bite wounds dating back more than a month (23). The Southwest Foundation for

Biomedical Research, which is the designated National Institutes of Health B-virus resource laboratory, reports processing 2,000 to 3,000 human diagnostic specimens per year between 1990 and 1994, or approximately 200 per month, most of which reflect occupational exposure (8).

Some Public Health Consequences of the Nonhuman Primate Pet Trade

The pet trade in a variety of nonhuman primate species, and particularly the apparent increase in macaque species as part of this trade, may constitute an emerging infectious disease threat in the United States. Although the U.S. Fish and Wildlife Service indicates that illegal traffic in nonhuman primates is a significant aspect of the estimated $3 billion worth of wildlife illegally traded in the United States annually, more data are needed on the actual number of macaques in the private sector and on trends in the population (24; U.S. Fish and Wildlife special agents, pers. comm.).

The public resources deployed when a monkey-bite case is referred to public health authorities are similar to those required for rabies investigations (M. Leslie and T. Parrott, unpub. obs.). Persons bitten by pet and feral macaques are more likely than persons bitten in the workplace to require public resources, delay seeking medical care, and have an initial medical evaluation by care givers who are largely unfamiliar with the potentially serious consequences of B-virus exposure (23). In contrast, occupational exposure generally occurs within highly structured workplace settings, where health professionals are prepared to provide prompt, appropriate, and specific care at no public cost.

Ongoing efforts to establish B-virus–free macaque colonies illustrate the difficulties of ascertaining B-virus–negative status, even with a battery of sophisticated laboratory tests and extended longitudinal follow-up of individual macaques (25). The high percentage of death in known cases of human B-virus disease underscores the potential seriousness of all bite or scratch exposures from macaques.

The extremely high prevalence of B-virus along with their behavioral characteristics make the macaque species unsuitable as pets.

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Infectious Diseases and Mental Illness: Is There a Link?

To the Editor: The report by Hatalski et al. (1) on Borna virus as a probable human pathogen provides yet another example of an infectious agent being tentatively associated with neuropsychiatric disorders. Earlier this year, researchers at Rockefeller University and the National Institute of Mental Health suggested that after streptococcal infection, some children may be at increased risk for obsessive-compulsive disorders and Tourette syndrome (2). The human B-cell antigen D8/17, believed to be a marker for increased susceptibility to poststreptococcal rheumatic heart disease, has been tentatively linked to this increased risk for psychiatric illness in children. Other reports of patients with complicated Lyme borreliosis, including some whose infections have progressed to encephalopathies, describe persistent verbal and memory deficits among these patients (3). In a few Lyme disease patients, the only overt symptoms of disease at the time of initial diagnosis and treatment were classified as mental confusion (4). Two newly emergent infectious diseases in the United States, leptospirosis and neurocysticercosis, have been found among inner city residents and poor immigrants, respectively. Occasionally leptospirosis has been associated with a variety of postinfectious psychiatric symptoms, including depression, dementia, and psychosis (5). Neurocysticercosis, a tropical parasitic infection, is increasingly associated with emergency room admissions for seizures and epilepsy (6). Still other infectious diseases are being examined for links with cognitive symptoms and emotional disorders.

The primary cause of many common psychiatric disorders, including depression, manic depression, anxiety, and schizophrenia, remains a mystery. The World Health Organization estimates that 1.5 billion people worldwide suffer from a neuropsychiatric disorder. Of the 10 leading causes of disability in 1990, four were psychiatric disorders: unipolar depression, manic depression, schizophrenia, and obsessive-compulsive disorders (7). The National Institute of Mental Health recently estimated that as many as 20% of young Americans ages 7 to 14—approximately 10 million children—have mental health problems severe enough to compromise their ability to function (8). Infectious agents may play a role in some of these diseases to some unknown degree. A better understanding of the role of infection may speed treatment and prevention efforts and reduce the degree of disability and stigma associated with mental illness.

Vaccines and antimicrobial agents might enhance current therapeutic options for mental illnesses. Even if infectious diseases were a primary factor in only 1% of neuropsychiatric illnesses, some 10 million persons might benefit from antimicrobial therapies. Identifying those susceptible to neuropsychiatric illnesses (because of environmental factors or genetic predisposition) may also permit vaccination or antimicrobial prophylaxis and a subsequent lowering of disease incidence.

Physicians and federal agencies addressing the problems of emerging infectious diseases should examine the possibility of infection as a cause of mental illness. Better communication among infectious disease and mental health experts, as well as additional training, will be needed to shed light on the growing phenomenon of infectious diseases manifesting themselves as neuropsychiatric disorders.

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Mycobacterium nonchromogenicum Bacteremia in an AIDS Patient

To the Editor: *Mycobacterium avium* complex is the most common nontuberculous mycobacterium that causes disseminated infection in HIV-positive patients (1). Other less common nontuberculous mycobacteria responsible for disseminated disease in these patients are *M. fortuitum* (2), *M. genavense* (3), *M. gordonae* (4), *M. haemophilum* (5), *M. kansasii* (6), *M. malmoense* (7), *M. marinum* (8), *M. scrofulaceum* (9), *M. simiae* (10), *M. szulgai* (11), *M. terrae* (9), and *M. xenopi* (2,9). Although only *M. genavense*, *M. kansasii*, and *M. xenopi* are significantly more frequent in these patients (2,3,9), HIV infection is likely a predisposing condition for all nontuberculous mycobacterial infections. We report the first case of disseminated infection caused by *M. nonchromogenicum* in an HIV-infected patient.

A 28-year-old man with HIV infection acquired by sharing injection tools was seen in our outpatient clinic because of intermittent fever, drenching nocturnal sweats, and cough with purulent sputum of 4 months' duration. He also reported a weight loss of 10 kg in the previous 2 months. He had been diagnosed with bronchial infection in another hospital and had been treated with an unknown antibiotic. After this treatment, respiratory symptoms had improved somewhat, but fever and constitutional symptoms continued. His only previous opportunistic infection had been recurrent oral and esophageal candidiasis. The last CD4-cell count had been 16/µL 1 year earlier, and he was receiving didanosine and prophylactic therapy with cotrimoxazole and fluconazole. On physical examination the patient appeared ill; he was febrile, cachectic, and had thrush and oral hairy leukoplakia. Neither lymphadenopathy nor abnormal cardiopulmonary symptoms were found. The liver, which had enlarged since the last examination, was palpated 6 cm below the right costal margin. Abnormal laboratory values included aspartate aminotransferase 61 U/L, gamma-glutamyl transferase 209 U/L, lactate dehydrogenase 516 U/L, hemoglobin 12.3 g/dL, leukocyte count 4,300/µL (66% neutrophils, 19% band forms, 1% metamyelocytes, 3% lymphocytes, 11% monocytes), platelet count 130,000/µL, and erythrocyte sedimentation rate 72 mm/h. Chest X-rays were unremarkable, and a set of blood cultures was sterile. A sputum culture yielded *Haemophilus influenzae* sensitive to ampicillin, and smears and cultures for mycobacteria in one stool and three sputum samples were negative.

The patient was treated with oral amoxicillin for 2 weeks without improvement. Empirical therapy against *M. avium* complex with clarithromycin, ciprofloxacin, and ethambutol was started; the patient's condition improved dramatically within the next few days, and the fever and diaphoresis disappeared, although cough and sputum production remained unchanged. Three weeks later, a slow-growing nonphotochromogenic mycobacterium, identified as *M. nonchromogenicum* in a reference laboratory (Centro Nacional de Microbiología, Majadahonda, Madrid, Spain) by biochemical tests and confirmed by polymerase chain reaction-restriction enzyme pattern analysis, was isolated from a blood sample obtained on admission. This microorganism was sensitive to the three drugs administered, and the treatment was continued. Two months later the patient had gained 10 kg, hemoglobin had increased to 13.8 g/dL, the erythrocyte sedimentation rate had decreased to 52 mm/h, and the differential leukocyte count had returned to normal. Antimycobacterial drugs were withheld after 1 year of treatment. Twenty-two months after the diagnosis, the patient is doing well. He is receiving combination antiretroviral therapy, and his CD4-cell count is 128/µL.

M. nonchromogenicum, a slow-growing nonpigmented (Runyon's group III) mycobacterium, belongs to the *M. terrae* complex, together with *M. triviale*; it is traditionally considered nonpathogenic. However, it has been involved in a few cases of pulmonary infection (12) and chronic tenosynovitis secondary to puncture wounds (13), like the related organism *M. terrae*. In fact, some authors think that *M. nonchromogenicum* is the true pathogen in the *M. terrae* complex (13), and it is possible that some reports have misidentified this organism. This complex was first isolated in soil washings

from radishes, but it has been found to be ubiquitous in the aquatic environment, including a hospital potable water supply (14).

Unlike osteoarticular infections, which commonly occur in previously healthy people, the scanty reports on pulmonary and disseminated infection by *M. terrae* complex suggest that either immunosuppression or local predisposing conditions (e.g., tuberculous cavities) are necessary pathogenetic cofactors (15). To our knowledge, *M. nonchromogenicum* bacteremia has never been reported before.

No specific DNA probes exist for *M. terrae* complex, but false-positive reactions with *M. tuberculosis* complex DNA probes have been described (16). Isolates are usually resistant to most antituberculosis drugs, with the exception of ethambutol and streptomycin, and susceptible to erythromycin, ciprofloxacin, and sulfonamides.

Only one case of disseminated infection by *M. terrae* has been described in a patient with advanced HIV infection and positive cultures in blood and bronchoalveolar lavage fluid, but no additional data were provided (9). Although the isolate we recovered might represent a laboratory contaminant, several pieces of evidence make this possibility very unlikely: lack of alternative explanation for a persistent and progressive clinical picture of 4 months' duration, absence of response to standard antibiotic therapy, negative results in the search for other pathogens, rapid and sustained clinical and laboratory response to drugs active against this strain, clear improvement despite the lack of treatment for other conditions, and absence of other isolates of this pathogen in our hospital despite the large number of samples examined for mycobacteria.

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Escherichia coli O157:H7 Infection in Colombia

To the Editor: The prevalence of *Escherichia coli* O157:H7 in Colombia is not known; we conducted a study to determine its prevalence in children with infectious diarrhea, in adult cattle, and in ground beef.

Between March 1996 and March 1997, we examined 538 children under 5 years of age with infectious diarrhea who had been admitted to either of the two children's hospitals in Bogotá. Diarrhea was defined as three or more loose stools within the previous 24 hours. One hundred and sixty-one children under 10 years of age admitted to the hospital for a medical reason other than infectious diarrhea served as controls.

Stool samples from children with and without diarrhea were placed in Stuart transport medium and sent to the laboratory within 24 hours; the samples were injected into sorbitol MacConkey agar (Oxoid Basingstoke, United Kingdom). After 24 hours of incubation at 37°C, sorbitol nonfermenting colonies were tested with 4-methylumbelliferyl-ß-glucuronide (MUG); all typical colonies of *E. coli* O157:H7 that were sorbitol-negative were confirmed as *E. coli* by biochemical tests (1,2) and were tested for agglutination with a latex test kit (Oxoid Basingstoke, United Kingdom) for detecting *E. coli* O157 and *E. coli* H antiserum H7 (Difco, Detroit, MI, USA). All human isolates were confirmed as Shiga-toxin producers by latex agglutination (Oxoid Basingstoke, United Kingdom). We used as a control strain *E. coli* O157:H7 provided by M. Karmali.

Rectal swabs from 307 healthy adult cattle from farms in Cundinamarca and Meta Departments were placed in Stuart transport medium, stored at 4°C, and transported to the laboratory within 6 hours. Swabs were injected into sorbitol MacConkey agar, and colonies that did not ferment sorbitol were characterized by standard techniques (3).

One hundred and fifty beef patties (31 cooked, 119 raw), collected in Bogotá, were examined by direct plating and enrichment culturing. Samples (1.0 g each) were serially diluted (1:10) in 0.85% NaCl solution, and 0.1 ml portions were plated in duplicate onto sorbitol MacConkey agar. Serologic and biochemical confirmation was done as mentioned above.

E. coli O157:H7 prevalence among children was 7.2%, with an age range of 0–60 months (average 21 months); diarrheal illness lasted an average of 2.5 days. Of 39 patients, eight were 6 months old or younger, five were 6 to 12 months old, 17 were 12 to 24 months old, and nine were older than 24 months. Renal failure associated with hemolytic uremic syndrome (HUS) developed in three (7.7%). Epidemiologic data were not collected regarding contaminated foods as a possible source of *E. coli* O157:H7 infection in the patients. *E. coli* O157:H7 was isolated from five (3.1%) of the 161 controls; the prevalence of *E. coli* O157:H7 was substantially higher in patients with infantile diarrhea than in controls.

All 39 strains from human cases were sorbitol-negative; five did not display MUG activity. Overall, 39 strains agglutinated strongly with antiserum O157:H7. Antimicrobial susceptibility tests were performed by the Bauer method (4). All *E. coli* O157:H7 isolated were susceptible to ciprofloxacin; 92% were resistant to ampicillin; 76% were resistant to furazolidone; and 76% were resistant to trimethoprim-sulfamethoxazole (TMP-SMZ).

E. coli O157:H7 was isolated from 20 (6.5%) of 307 rectal swabs from cattle. The strains isolated were sorbitol-negative and agglutinated strongly with antisera; five did not present activity. All strains were susceptible to ciprofloxacin; 90% were resistant to ampicillin; and 26% were resistant to TMP-SMZ. *E. coli* O157:H7 was isolated from 13 (87%) of 150 beef patties, six from raw beef, and seven from cooked beef.

Stool cultures of all patients with acute bloody diarrhea should be tested for *E. coli* O157:H7 to identify those at risk for HUS (5); however, serotyping, cytotoxicity assays, or DNA probing for *E. coli* O157:H7 are not routinely performed in Colombia.

This preliminary report suggests that *E. coli* O157:H7 is emerging as an important cause of endemic childhood diarrhea in Colombia and that the chain of contamination is present. The incidence is greatly underestimated because of limited surveillance and reporting. Further studies are needed to identify the pathogenic mechanisms of these *E. coli* O157:H7 strains and to determine the fecal carriage rate in healthy children. Data obtained will help elucidate the role of *E. coli* O157:H7 in childhood diarrhea. In addition, molecular analysis should be performed

to establish the connection between the strains isolated from different sources in Colombia.

Our findings suggest that the risk for *E. coli* O157:H7 infection in Colombia is high; therefore, more active screening and surveillance would enhance case detection, epidemiologic understanding of *E. coli* O157:H7 infection and HUS, and could lead to more specific therapeutic interventions.

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Autofluorescence and the Detection of Cyclospora Oocysts

To the Editor: From May through July 1997, we searched for the seasonally occurring *Cyclospora cayetanensis,* along with other coccidia and microsporidia, in fecal samples from 385 patients. The samples, in 10% formalin for evaluation of coccidia and microsporidia, were initially processed by a routine formalin-ethyl acetate concentration method; the parasite was detected in 18 patients (1,2). The resulting sediment was examined as follows. A drop of sediment was placed on a slide, cover-slipped, and examined microscopically as a wet mount at 200x and 400x magnification and subsequently at 200x magnification by epifluorescence with a 330 to 380 nm UV filter. Four smears were also prepared and stained by routine trichrome (2), modified trichrome (3), auramine-rhodamine (4), and Kinyoun acid-fast (5) procedures. All wet mount and stained preparations were evaluated by at least two trained persons.

Of the 385 fecal samples examined, 18 were positive for *C. cayetanensis*. The positive samples were from eight states, which encompassed northeastern (Rhode Island, New York, Massachusetts, Pennsylvania), midwestern (Wisconsin), western (Oregon, California), and southern (Florida) sections of the United States.

In 12 of 18 patients, the organisms were detected without much difficulty in wet mounts as round or partially collapsed nonrefractile bodies; however, in the other six, repeated wet preparations were needed to detect the organisms. When the same wet mounts were examined with epifluorescence microscopy, oocysts were easily discerned in all samples, even the six in which repeated wet preparations and stains were needed. While the trichrome procedures were ineffective, the auramine-rhodamine and Kinyoun stains gave varied results. The autofluorescence technique, however, was distinctly superior to the wet mount and staining procedures.

Extensive outbreaks of diarrhea caused by *C. cayetanensis* were reported in 1997 from different parts of the United States (6-8), and several procedures have been used to confirm the diagnosis in clinical samples. While the organisms are large enough to be seen in direct wet mounts, they are frequently caught up in mucus or covered by debris, so they are difficult to detect. Autofluorescence in *C. cayetanensis* oocysts makes them easily visible in clinical samples (1,9) with the use of a 330 to 380 nm UV filter; this feature enhanced their detection at least twofold over the direct wet mount, especially when the wet mount and stained slides contained few oocysts. (The same wet mount preparation can be used for the epifluorescence procedure.)

The 18 patients with cyclosporiasis were ages 2 to 71 years, which indicates that the infection was not specific to any age group. Twelve of the 18 cases were in women. Massachusetts had 11, the largest number of *C. cayetanenis*-positive patients. Of the 18, 16 were adults; the other two were children with a coexisting parasite (*Dientamoeba fragilis*). In one instance, three members of the same family were infected, the parents with only *C. cayetanensis*, the son with *D. fragilis* and *Blastocystis hominis*.

Because *C. cayetanensis* is a seasonal diarrheal agent, fecal samples from persons with persistent unexplained explosive diarrhea during the summer should be carefully evaluated for this infection. Stool specimens should be fixed in 10% formalin and examined with autofluorescence microscopy for enhanced detection.

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Partnerships for Detecting Emerging Infectious Diseases: Nepal and Global Influenza Surveillance

To the Editor: With new influenza strains emerging each year, identification of circulating strains by coordinated global surveillance is crucial to vaccine development for the coming year (1-3). Approximately 110 laboratories in 80 countries voluntarily participate in the World Health Organization (WHO) influenza surveillance network (4). Comprehensive surveillance is especially important in Asia, since new influenza strains often originate there. To participate in influenza global surveillance, countries need not rely on their own laboratory capability. Clinical specimens from patients thought to have influenza can be sent to designated laboratories around the world for analysis. A unique partnership has led to the expansion of the WHO global influenza surveillance network to Nepal.

The U.S. Army Medical Component - Armed Forces Research Institute for Medical Sciences (AFRIMS) (5) in Bangkok, Thailand, is well situated to assist with surveillance in Asia. Scientists at AFRIMS have conducted medical research in collaboration with Nepali colleagues for more than 20 years. Several studies have been conducted in collaboration with the CIWEC Clinic Travel Medicine Center (a travel medicine clinic that serves the diplomatic, aid, and tourist communities in Nepal). The clinic has approximately 5,000 patient visits per year, of which half are drawn from the 2,500 expatriates in Nepal and half from the 200,000 non-Indian tourists who visit Nepal annually.

A protocol was developed for a pilot influenza surveillance program. The staff of the CIWEC Clinic was responsible for volunteer recruitment, clinical evaluation, and specimen collection. Febrile upper respiratory infections were defined as temperature ≥ 100°F (37.8°C, oral or equivalent) and cough or sore throat of ≤ 72 hours duration. Other symptoms, such as streptococcal pharyngitis, were excluded. No age or gender restrictions were included. Volunteers had to have been in Nepal for the 5 days preceding illness. Only the first patient in any single household with similar symptoms within days of other household members was asked to participate.

The AFRIMS field station in Kathmandu (locally known as the Walter Reed/AFRIMS Research Unit - Nepal or WARUN) was responsible for shipping specimens collected by the CIWEC Clinic to AFRIMS, Thailand. Since dry ice was not available in Kathmandu, dry ice and shipping containers were sent by AFRIMS, Thailand for use by WARUN. Shipments from WARUN were then sent back to AFRIMS, where specimens were repacked in dry ice and sent for testing at the central laboratory of the U.S. Air Force's Project Gargle (6) in San Antonio, Texas. Project Gargle has been testing viral respiratory

specimens from distant Air Force installations for more than 20 years. Each specimen was tested for influenza A and B; parainfluenza virus 1, 2, and 3; adenovirus; enterovirus; and herpesvirus. Characterization of selected influenza A and B isolates by hemagglutination-inhibition testing was performed by the Centers for Disease Control and Prevention (CDC).

Between December 1996 and February 1997, the CIWEC staff collected specimens from 18 patients. Samples were collected from 11 (61%) residents and seven (39%) tourists, who were evenly distributed by gender and had a median age of 35 years. Influenza B/Beijing/184/93-like viruses were isolated from five (28%) of the 18 specimens. All patients from whom influenza viruses were obtained had mild illnesses with fever and upper respiratory syndromes. Herpes virus type 1 and adenovirus type 6 were each identified in one other specimen. No respiratory viruses were identified in the remaining 11 specimens.

Because of the importance of China in the emergence of new strains of influenza, CDC's WHO Collaborating Center for Surveillance, Epidemiology, and Control of Influenza has worked with colleagues in China to establish a national Chinese network of influenza surveillance sites. Analysis of viruses isolated in China between 1988 and 1997 in comparison with other viruses obtained through WHO's global influenza surveillance network has shown that influenza variants are frequently identified in China before becoming prevalent in other regions of the world. Nepal is another especially valuable surveillance site, given its location between China and India (at the crossroads between northern and southern Asia) and its historic importance as a trans-Himalayan trade route.

Especially relevant are data from China demonstrating that the two antigenically and genetically distinct lineages of influenza B viruses represented by B/Victoria/02/87 and B/Yamagata/16/88 (7) have continued to circulate and evolve in China, while only viruses related to B/Yamagata have been detected elsewhere in the world and are represented in the current trivalent vaccine by the B/Bejing/184/93-like component. Virologic surveillance in surrounding countries (8) such as Nepal is necessary to detect geographic spread of B/Victoria-like virus in the region. Our data suggest that these viruses have not yet spread to Kathmandu.

Our unique international partnership between several civilian and military organizations (e.g., CIWEC Clinic, CDC, U.S. Air Force, and U.S. Army) demonstrates the feasibility of such partnerships as well as the usefulness of influenza surveillance data at both the local and global levels. Despite the small number of isolates obtained during this study, we were able to determine that the influenza B component of the trivalent vaccine prepared for the 1996-1997 influenza season would likely have offered protection for travelers and the local population against the influenza B strains isolated in Kathmandu. Ongoing surveillance data will establish geographic and temporal patterns of circulation of influenza viruses and thus provide valuable information for guiding public health policies for influenza vaccination. On a global level, these data are useful for annual vaccine strain selection.

Advances in communication, laboratory, and specimen transport technologies contributed greatly to the identification of viral pathogens from a new sentinel surveillance site in Nepal. In evaluating future collaborative sites, prior surveillance experience and reliable specimen shipping should be prime considerations. Approaches that use existing resources might foster greater international cooperation toward improved global detection and reporting of infectious diseases.

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HIV-2 Infection and HIV-1/HIV-2 Dual Reactivity in Patients With and Without AIDS-Related Symptoms in Gabon

To the Editor: Between 1996 and 1997, we evaluated the incidence of HIV-2 infection at the Fondation Jeanne Ebori, the second largest hospital in Libreville, capital of Gabon; we found an unexpected high prevalence of HIV-2–infected or HIV-1/HIV-2–dually reactive patients.

During a 10-month period, 147 (14.3%) of 1,029 sera from inpatients and outpatients were found HIV-positive by the type III method recommended by the World Health Organization (two enzyme-linked immunosorbent assays are used to screen anti-HIV antibodies) (1). Further discrimination between HIV-1 and HIV-2 infections was assessed by using synthetic peptides specific for the gp41 and the gp120 of HIV-1 and the gp36 of HIV-2 (ImmunoComb II, PBS Orgenics, Illkirch, France). Of the 147 HIV-positive sera, 141 (96.0%) were exclusively HIV-1–positive; four were exclusively HIV-2–positive; and two were both HIV-1– and HIV-2–positive. Of the six sera with anti-gp36/HIV-2 reactivities, two (from patients A and B) were positive on HIV-2 Western blot, with marked anti-gag HIV-1 cross-reactivity and a discrimination assay positive only for HIV-2; two (from patients D and E) were positive on HIV-2 Western blot, with anti-gag and pol reactivities markedly lower than anti-env reactivities and a discrimination assay positive only for HIV-2; the two remaining sera (from patients C and F) showed typical dual reactivities for HIV-1 and HIV-2 infections, with positive patterns of HIV-1 and HIV-2 Western blots and a discrimination test positive for both viruses. As a whole, six (4.1%) of 147 HIV-positive sera showed either HIV-2 infection alone (n = 4) or dual reactivity. Of those, four were from Gabonese patients B, C, D, and E, and two were from immigrants from West Africa (patient A from Mali and patient F from Nigeria); two were female patients B and E. Among Gabonese patients, only one (patient E) had traveled to West Africa; the remaining three had never visited any neighboring country. However, one Gabonese man (patient C) lived in Port-Gentil, which has many West African immigrants. For all patients, the most likely risk factor for HIV was a heterosexual relationship with an unknown HIV-infected person. In three asymptomatic patients (A, B, and C) the HIV-2–serostatus was unexpected; in contrast, the three other patients had AIDS-related symptoms. Patients D and E had an HIV-2 Western blot pattern showing a marked decrease in anti-gag and pol reactivities compatible with their advanced stage of HIV-2 disease.

The case of a 55-year-old exclusively heterosexual asymptomatic woman (patient B) suggests the possibility of a specific variant of HIV-2 in Central Africa (2). The high frequency in primates in Gabon of natural infection with simian immunodeficiency retroviruses, which show a high degree of genetic relatedness to HIV-2 (3), could support such a hypothesis.

Two patients had typical dual reactivities to HIV-1 and HIV-2 antigens. To our knowledge, such dual reactivities have never been reported in Gabon (4). In the patient from Nigeria (patient F), the serologic pattern was typical of that usually observed in West Africa (5). Dual reactivity can result from genuine mixed infections and from serologic cross-reactivity in HIV-1 and HIV-2 infection alone; theoretically, it could also represent infection with a different, cross-reacting recombinant strain (5).

HIV-2 infection in Gabon is epidemiologically related to West Africa, because of cultural and, above all, economic ties. However, HIV-2 is not limited to immigrant populations from West Africa or to Gabonese citizens traveling in this area; it has also reached the indigenous Gabonese

population. The possibility of rare cases of HIV-1 and HIV-2 coinfections, recombinant HIV-1 and HIV-2 strains, and also peculiar HIV-2 variants from Central Africa, should be considered in Gabon. A possible entry of HIV-2 infection into Central Africa from Gabon in the near future could have major public health implications.

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Q Fever in French Guiana: New Trends

To the Editor: Q fever, the endemic disease caused by the rickettsial organism *Coxiella burnetii*, was first described in French Guiana in 1955 (1). Only sporadic cases were reported until 1996 when three patients were hospitalized in the intensive care unit of the Cayenne Hospital for acute respiratory distress syndrome. One of the patients died. Many cases of Q fever were diagnosed in the general population at the same time. A seroepidemiologic study was performed to determine whether the increase in cases was due to an increase in incidence or to an improvement in diagnosis. All paired samples of sera (acute-phase and convalescent-phase) from patients sent to the arbovirus laboratory for diagnosis of dengue infection from January 1, 1992, to December 31, 1996, were tested for antibodies to *C. burnetii* by immunofluorescence. All positive samples were also tested for immunoglobulin (IgM) by the same method; the IgG and IgM titers were determined by using a serial twofold dilution. A diagnosis of Q fever was made when there was a seroconversion from negative to positive or a twofold increase in IgG titer associated with the presence of IgM in the second sample.

One hundred and fifty-one of 426 paired sera collected between 1992 and 1996 were from patients recently infected with dengue fever. Twenty-five (9.1%) of 275 remaining sera were from Q fever patients. Significant differences were observed in the rates of Q fever in different years ($p < 0.01$); one (1.9%) of 53 was positive in 1992, five (9.1%) of 55 in 1993, five (8.6%) of 58 in 1994, three (4.8%) of 63 in 1995; a large increase was observed in 1996 (11 [23.9%] of 46). Differences by residence were also assessed. Rates of infection were higher in Cayenne (21 [13.0%] of 161) than in rural areas (4 [3.5%] of 114) ($p < 0.01$).

This study shows that cases of Q fever have occurred in French Guiana in recent years and that a significant increase in the incidence rate occurred in 1996. The reasons for this increase are unclear, and further studies of the epidemiology of Q fever in French Guiana are necessary. The epidemiology of Q fever is unusual in French Guiana because the rates of infection are much higher in Cayenne, the capital city, than in rural areas. No link with classical sources of infection (cattle, sheep, or goat birth products, or work in a slaughterhouse) was found. Indeed, Cayenne, with 80,000 inhabitants, is located near the Atlantic Ocean, and the prevailing winds blow from the sea. Airborne contamination from rural areas is therefore impossible. Furthermore, no large farm is in the immediate vicinity of the city. For identical reasons, contamination from the abattoirs is not likely; they are located on the west side of the city, near the Cayenne River, and the winds blow from the east. In our study, cases were almost equally distributed

throughout the city, although many patients came from the same area.

A seroepidemiologic study to determine possible new sources of infection (e.g., dogs, cats) and estimate rates of seropositivity in cattle and sheep and a case-control study on new cases are being conducted.

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Ixodes dammini: A Junior Synonym for *Ixodes scapularis*

To the Editor: The authors of "A new tick-borne encephalitis-like virus infecting New England deer ticks, *Ixodes dammini*" (1) provide useful information regarding a possibly new tick-borne encephalitis-like virus. However, the use of the name *Ixodes dammini* is not accurate for describing this species. *I. dammini* (Spielman, Clifford, Piesman, and Corwin) was synonymized with *Ixodes scapularis* (Say) in 1993 by Oliver et al. (2) and was redescribed in 1996 (3) to reduce confusion regarding identification. Keirans and colleagues summarize a wide array of rigorous studies involving hybridization, assortative mating, isozymes, and morphometrics, all of which provide evidence supporting the synonymization of the two tick species (3).

The synonymization of *I. dammini* with *I. scapularis* has been widely accepted. "*I. scapularis* (= *I. dammini*)" is still often used, but the use of *I. scapularis* as the sole nomen for this species is becoming more common (4). Oliver et al. (2) have established *I. dammini* as a junior subjective synonym of *I. scapularis.* If scientifically rigorous evidence exists justifying the reestablishment of the species name *I. dammini,* it must be published according to proper procedure. The proper nomenclature of any species, let alone one of such widespread notoriety and public health importance, is too important to be relegated to a footnote. Until such evidence is presented, the continued misuse of *I. dammini* serves only to confuse health-care providers, public health professionals, and lay persons.

On a secondary matter, on page 167 of the dispatch, the authors state that "*I.* (Pholeoixodes) *cookei* is a one-host tick that is only distantly related to *I. dammini* and only rarely feeds on humans or mice" (1). *I. cookei* is a three-host tick (D.E. Sonenshine, pers. comm.), as are all the members of the genus Ixodes.

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The Name *Ixodes dammini* Epidemiologically Justified

To the Editor: Although a large body of evidence has been interpreted as supporting conspecificity of the deer tick (*Ixodes dammini*) and the blacklegged tick (*Ixodes scapularis*), according to Chapter VI, Article 23L of the International Code of Zoological Nomenclature (1), "A name that has been treated as a junior synonym may be used as the valid name of a taxon by an author who considers the synonymy to be erroneous...."

Current use of *I. scapularis* to refer to the vector of Lyme disease obscures important epidemiologic issues. One of the reasons for "sinking" *I. dammini* was to make it easier to diagnose Lyme disease in areas where the disease was thought to be nonendemic: "The belief that *I. dammini* does not occur south of Maryland and that *I. scapularis* is a separate and

distinct species yet unproven as a natural vector of Lyme disease has caused delays in Lyme disease surveillance in the South. The general attitude among physicians and veterinarians has been that Lyme disease is not a problem in that area, although patients present clinical symptoms of it" (2). Recognizing and reporting Lyme disease in southern and southcentral states should not, however, depend on whether the two ticks are conspecific. Only peer-reviewed descriptions of human cases of Lyme disease, with appropriate documentation of the diagnoses, should be accepted as evidence. Few such reports exist, and the evidence does not convincingly support a conclusion that Lyme disease exists as an epidemic zoonosis in southern states (3). This is not to say that residents outside the well-established eastern United States zoonotic sites (the Northeast and upper Midwest) do not have symptoms that fit one or more aspects of the current Centers for Disease Control and Prevention/Council of State and Territorial Epidemiologists/Association of State and Territorial Public Health Laboratory Directors surveillance definition for Lyme disease. Lyme disease-like infections, mainly manifesting as erythema migrans and strongly associated with Lone Star tick (*Amblyomma americanum*) bites are commonly seen in southern and southcentral states, but *Borrelia burgdorferi* does not seem to be the etiologic agent (4).

Enzootic transmission of Lyme disease spirochetes among rodents and ticks had been documented in southern and southcentral states by the late 1980s (5-7). The question, however, is whether there is frequent zoonotic transmission. There are widespread southern U.S. enzootic cycles of *Trypanosoma cruzi*, but few autochthonous human Chagas disease cases seem to occur because the vectors (such as *Triatoma sanguisuga*) have behavioral traits that reduce their capacity to serve as zoonotic vectors (8). Nymphal *I. scapularis* apparently do not frequently bite humans (7,9), although adult ticks do. The major feature of Lyme disease epidemiology in the Northeast and in the upper Midwest, however, is transmission by nymphal *I. dammini* (10).

Whether the predilection of nymphal *I. dammini* to feed on humans is environmentally determined or is a heritable trait with undescribed genetic markers remains unexplored. Particular mitochondrial DNA haplotypes seem to be more characteristic of *I. dammini* (11,12), and the use of such typing methods may enhance future analyses of the vectorial capacity of these ticks. For example, one might test the hypothesis that nymphal ticks removed from residents of sites in coastal North Carolina through Georgia, where both kinds of ticks have been collected, represent only *I. dammini*. But, if it is "widely accepted" that no differences exist between the two ticks, such studies may never be done. Similarly, many may wrongly assume that Lyme disease, human babesiosis, and human granulocytic ehrlichiosis are, or will become, epidemic throughout virtually all of the eastern United States. An equally likely scenario is that these zoonoses may never become public health problems for more southerly states. For the moment, then, distinguishing tick populations that frequently bite humans from those that rarely do seems to be a rational use of nomenclature, particularly for public health officials.

Dr. Sanders is correct in pointing out that all *Ixodes* spp. are "three-host" ticks, although my intent in using the term "one-host" was to indicate that all stages of *I. cookei* tend to feed on the same kind of animal (sometimes a single animal, within burrows), usually woodchucks, skunks, or raccoons. I regret the confusion from my use of the acarologic term in a descriptive context.

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Ebola/Athens Revisited

To the Editor: After our hypothesis that the plague of Athens (430 B.C.–425 B.C.) could have been caused by Ebola virus was published in this journal (1996;2:155-6), it was brought to our attention that this hypothesis had been previously entertained.

Gayle D. Scarrow had published a paper entitled "The Athenian Plague: A Possible Diagnosis" in The Ancient History Bulletin 2.1 (1988). Unfortunately, this had not come to our attention in our literature search, and therefore we assumed that we were the first to recognize the possibility. Clearly, Ms. Scarrow deserves credit for suggesting this first. Her arguments are compelling, even without the support of more recently available information and the observations advanced in our publication.

We believe an evolving knowledge base (e.g., the information about the Côte d'Ivoire outbreak where a protracted epidemic has been meticulously documented) will serve to enhance the credibility of the Ebola/Athens hypothesis.

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Meeting Summaries

The Hot Zone–1997: Conference on Emerging Infectious Diseases

On June 27-28, 1997, the University of Kentucky College of Medicine, University of Cincinnati College of Medicine, and Kentucky AIDS Consortium held a conference for clinicians and researchers in Lexington, Kentucky. Participants presented the latest findings from worldwide epidemiologic studies, basic science, and clinical research on emerging infectious diseases. The findings indicate that the war against infectious diseases is far from over. In the United States, between 1980 and 1992, deaths from infectious diseases increased by 58%, making serious infections the third leading cause of death; HIV infection is the leading cause of death among 25- to 44-year-olds; and antibiotic resistance costs the health-care system an estimated $100 million to $30 billion each year.

With the global population growing from 2.5 to 5.8 billion over the last 25 years, large urban centers throughout the developing world are overcrowded and have inadequate sanitation, ideal for the emergence of infectious diseases. By 2025, the global population will reach 8.6 billion. In developing countries, this represents an 84% increase, which will intensify overcrowding in these areas. In industrialized countries, an aging population base, the advent of immunosuppressive medications, and the emergence of HIV are combining to increase the risk for opportunistic infection. Moreover, with increased travel, clinicians see increasing numbers of patients with exotic diseases acquired abroad. Recent migration of epidemic diphtheria from the former Soviet Union to Europe and the emergence of multidrug-resistant tuberculosis (TB) in the United States and elsewhere are but two examples of infections resulting from international travel; in addition, nearly 70% of the fruits and vegetables consumed in the United States originate in developing countries; disease outbreaks related to imported food frequently go unreported.

Extensive cross-species contact among humans and certain domestic animals can dictate antigenic shifts in influenza viruses. The likelihood of the emergence of a new influenza virus in the near future increases with the growth of the hog population in China. The emergence of new viruses, such as HIV and filoviruses, indicates the virtually unlimited capacity of pathogenic organisms to mutate and rapidly adapt to environmental changes and selective pressures.

HIV/AIDS Research

Recent data from long-term survivors support the concept that HIV replication occurs when the number of CD4+ cells drops below the minimum level required to maintain CD8+ cell control of HIV. CD4+ cell production of IL-2 is needed for strong cell-mediated immunity. Without CD8+ cell responses, a more virulent, highly cytotoxic viral strain emerges, killing greater numbers of CD4+ cells and leading to AIDS. Because CD8+ cell loss appears to be related to a shift from a TH-1- to a TH-2-type cytokine response, therapeutic approaches that maintain TH-1 cell response, or enhance CD8+ cell anti-HIV activity through factors yet to be fully defined, are being actively investigated. Vaccines relying exclusively on antibody responses will almost certainly prove to be of limited value, while those using CD8+ cell antiviral activities hold substantially greater promise.

A basic science research forum described the use of a murine model of immunodeficiency induced by a type C retrovirus. Because models of this type reproduce a number of the clinical pathophysiologic manifestations associated with human AIDS, they can significantly enhance our understanding of retrovirus-induced immunodeficiency.

The results of recent research involving host immune responses to *Pneumocystis carinii* infection indicated that, compared with healthy controls, HIV patients with less than 200 CD4+ cells have similar IL-4 levels but significantly lower peripheral blood mononuclear cell proliferative responses and IFN-γ levels to *P. carinii* major surface class glycoprotein (MSG). Centers for Disease Control and Prevention (CDC) Class 3 patients with previous *P. carinii* pneumonia (PcP) have significantly higher IL-4 (but not IFN-γ) levels than Class 3 patients with no history of PcP. HIV+ patients who have recovered from PcP have sufficient memory cells to recognize MSG, but demonstrate a shift from a TH-1- to a TH-2-type antigen recall response.

HIV Therapy

The current state of CD4 and viral load testing and the 11 drugs available for HIV treatment were reviewed. Although combination

drug therapy has consistently proved more effective than monotherapy in maintaining reduced viral load, the results of a recently completed follow-up study confirmed the effectiveness of zidovudine (AZT) in preventing neonatal HIV transmission; in a large cohort of pregnant women, AZT plus high titer immunoglobulin G was no more effective than AZT plus placebo, with both regimens producing results comparable with those achieved with AZT alone. A number of issues in HIV treatment remain unresolved, including when treatment should begin, how to manage inadequate response to therapy, whether to use HIV resistance genotyping to direct therapy, and how to best deal with prophylaxis for opportunistic infections in patients showing dramatic reductions in viral load.

Dengue and Dengue Hemorrhagic Fever (DHF)

Tens of millions of cases of dengue and hundreds of thousands of cases of DHF are reported annually, and more than 2.5 billion people are at risk for infection. Factors contributing to the emergence of dengue include unplanned and uncontrolled population growth associated with urbanization in tropical regions, lack of effective mosquito control, deteriorating water systems that increase densities of *Aedes aegypti*, and viral migration among tropical urban centers due to increasing international air travel. More than 50% of all air travel from the United States is to tropical destinations, and from 1977 to 1994, 2,248 suspected cases of imported dengue were reported in the United States. Because their clinical symptoms are initially nonspecific, dengue and other arboviral infections can be difficult to distinguish from other viral, bacterial, and parasitic infections. Correct diagnosis requires a detailed clinical summary, thorough epidemiologic information (including recent travel history), and a diagnostic laboratory test.

The severe hemorrhagic form, DHF/dengue shock syndrome (DSS), has an average incubation period of 4 to 6 days before sudden onset of fever and nonspecific signs and symptoms. Because the major pathophysiologic abnormality observed in DHF/DSS is increased vascular permeability and leakage of plasma from the vascular compartment, early fluid replacement is effective. The geographic distribution of DHF/DSS has been expanding and now can be found in tropical areas of Asia, the Pacific, and the Americas, including Central America and Mexico. This expansion is associated with increased movement of dengue viruses by airplane travelers and the development of hyperendemicity in the Pacific Region and the Americas. A similar scenario, generated largely from human encroachment into new environments, may also emerge for other *Aedes*-transmitted illnesses such as yellow fever. The most cost-effective approach to control dengue and DHF is larval source reduction in disease-endemic areas. Programs should use both government and community resources to integrate environmental sanitation with the use of insecticides and biologic controls, targeted to breeding grounds such as tire dumps.

Other Viral Diseases

The exponential increase in ecologic change, both environmental and behavioral, was cited as the major driving force for the increasing human risk for viral infection. Microbial variability can play a causal role in disease emergence, but it more often enables viruses to adapt to new circumstances. Travel of infected humans and international transport of microbes and vectors help provide the maximum possible microbial evolutionary opportunities in the minimum amount of time.

Viruses have emerged in the past, with measles providing a good example of the worrisome potential for future emerging RNA viruses. The emergence of cities in the Mesopotamian basin, resulting largely from the advent of irrigated agriculture, provided a populated substrate for interhuman transmission of short-incubation, nonlatent viruses. Domestication of livestock likely brought measles progenitors into close proximity with humans; a precursor of rinderpest/peste de petit ruminants then made an interspecies leap; much later measles spread to Europe, and, in the post-Columbian interchange, to the Americas.

Viral hemorrhagic fevers include those caused by Ebola filovirus and hantaviruses. Ebola hemorrhagic fever is characterized by extensive and disseminated infection and necrosis in major organs, and lymphoid depletion. Aerosol transmission of Ebola virus has occurred between nonhuman primates and guinea pigs, but no evidence exists for interhuman transmission by airborne infection. Barrier nursing precautions generally prevent the spread to humans, but in areas having

inadequate medical care facilities, the virus can amplify in humans and cause epidemics. Hantaviruses belong to a single genus in the family Bunyaviridae, and each virus infects a limited or unique rodent species with no apparent disease. Although hemorrhagic fever with renal syndrome has rarely been diagnosed in the Americas, hantaviruses from sigmodontine rodents cause hantavirus pulmonary syndrome, characterized by large bilateral pleural effusions and heavy, edematous lungs, interstitial pneumonitis, and extensive infection of endothelial cells in the pulmonary microvasculature. The first documented interhuman transmission of a hantavirus was an outbreak of 20 cases in Patagonia, with evidence overwhelmingly indicating spread between patients and physicians. The reasons for, and mechanism of, the spread are unknown, but the registry of U.S. cases was revised to ensure that this phenomenon was adequately monitored. Although early diagnosis and supportive care are potentially lifesaving in cases of hantavirus pulmonary syndrome, such efforts are of limited value in Ebola hemorrhagic fever.

Prion Illnesses

In prion illnesses such as Creutzfeldt-Jakob disease (CJD), risk factors (family history of CJD or dementia, history of poliomyelitis, exposure to sheep or cows) and iatrogenic factors (dura or corneal grafts and exposure to pooled human growth hormone) are important. At onset, CJD is commonly misdiagnosed as psychotic illness, Alzheimer's dementia, paraneoplastic syndrome, vascular brain disease, parkinsonism, or even drug-induced delirium. A variant of CJD, caused by a prion with an altered protein configuration, is bovine spongiform encephalopathy (BSE or mad cow disease). Although the precise mechanism of infection originally reported in U.K. cattle is unclear, BSE has been exported to other countries by feeding cattle inadequately processed bone meal. The potential for the emergence of BSE in the United States exists because similar reservoirs of infection are present. Casual handling of beef and beef products in processing plants and uncontrolled disposal of sick cattle may increase that risk. Several cases of CJD have been reported in Kentucky patients who consumed squirrel brains; however, a causal link has not been established. The most recently identified prion illness is a new variant of CJD reported in England that, unlike sporadic CJD, occurs in much younger patients (16 to 50 years of age), can last longer than 1 year, and is characterized by the presence of psychiatric and sensory symptoms and the absence of kuru plaques and electroencephalogram periodic complexes. The illness is thought to be linked to BSE. Mathematical modeling suggests that 75,000 to 80,000 cases will occur in the foreseeable future.

Tick-Borne Diseases

As with many other emerging infectious diseases, concern about tick-borne zoonotic diseases in the United States has increased because of environmental changes brought on by human settlement and socioeconomic factors that place humans at greater risk for tick exposure, such as the development of suburban housing in disturbed natural settings. Current conditions appear favorable for continued increases in vector tick populations and their geographic expansion and for increasing interaction between ticks and humans. In the United States, the prevalence of Rocky Mountain spotted fever may continue to increase as the urbanization of the western and southern regions expands opportunities for human exposure to tick-borne pathogens.

The epidemiology of Lyme disease and tularemia was reviewed, and the concept of a southern (U.S.) tick-associated rash illness (STARI), the epidemiology of which is still being defined, was introduced. STARI is characterized by an expanding erythematous rash resembling that of Lyme disease, mild or absent constitutional symptoms, and no well-described sequelae. The rash responds well to antibiotic treatment. STARI is seen in the range of the human-biting Lone Star tick (*Amblyomma americanum*) and is frequently associated with a Lone Star tick bite. Studies indicate that this infection is not caused by *Borrelia burgdorferi* or other known tick-borne agents. Amplified segments of the genome of this spirochete have recently been described, and some investigators have proposed that it represents a new species, *B. lonestari*. It has been suggested that STARI may be closely related or identical to a commensal spirochete of deer, *B. theileri*.

The need for early diagnosis and treatment of Rocky Mountain spotted fever was stressed by presenting data from 94 infected patients in North Carolina. Death rates were significantly lower (6.5% vs. 22.9%, $p < 0.03$) in patients receiving appropriate treatment within 5 days of

onset of illness than in patients who received delayed or no treatment. Predictors of death included renal failure; elevated serum creatinine, aspartate transaminase, and bilirubin; decreased serum sodium; thrombocytopenia; neurologic involvement; and male gender.

Parasitic Diseases

The role of the cell surface glycoconjugate lipophosphoglycan (LPG) in the survival of *Leishmania* parasites whose life cycle alternates between intracellular parasitism and extracellular life in sand fly vectors was explored. Data indicating that LPG is a multifactorial molecule may lead to a biochemical rationale for LPG-targeted chemotherapeutic regimens.

Isolation of a full-length genomic clone of the acid α-mannosidase from an epimastigote genomic library of *Trypanosoma cruzi* was reported. Sequence analysis showed a single open reading frame encoding the α-mannosidase gene. Because the size of this frame is consistent with that of lysosomal mannosidases in humans, these results could lead to the exploration of new chemotherapeutic options in Chagas disease.

A bank of 5,200 insertion mutants has been used to characterize the parasitic mechanisms used by *Legionella pneumophila*. Results from transmission electronmicroscopy indicate that *L. pneumophila* has acquired genetic loci specific for survival and replication within mammalian cells, allowing evolution from a protozoan parasite into its present disease-causing agent. Alternatively, ecologic coevolution of *L. pneumophila* to parasite protozoa has led to the development of multiple redundant mechanisms, some of which do not function within mammalian macrophages.

TB

Poverty, changing immigration patterns, and the emergence of HIV disease were cited as factors contributing to an attenuated rate of decrease of TB incidence in the United States since the late 1980s and its increase as a major global problem. Poorly managed TB control programs, suboptimal access to health care, an inadequate physician knowledge base, and poor patient compliance have combined to increase the incidence of TB and, especially, multidrug-resistant TB; sensitivity testing is critical in the management of resistant TB. Updated CDC guidelines for interpreting the purified protein derivative skin test call for responses to be considered positive if induration is greater than 5 mm in those with HIV or who have recent TB contact, and greater than 10 mm in foreign-born patients, intravenous drug users, the homeless, and immunocompromised patients. Because of the "boosting" phenomenon, two-step purified protein derivative skin testing is now recommended for health-care workers and nursing home patients who are retested periodically. Treatment of active TB should use multiple drugs, avoid adding single agents, and include compliance monitoring, preferably directly observed therapy. Workplace prevention measures should be driven by a consistently high index of suspicion and should include appropriate isolation of suspected cases, work-ups following exposure, rigorous reporting to health departments, and locating recalcitrant patients. Effective control of TB will require social, political, and cultural changes, as well as medical innovation.

Plague

The epidemiology, pathophysiology, and treatment of plague, as well as the improvement of diagnostic techniques for infections caused by *Yersinia pestis,* were reviewed. Such new tests will permit the public health laboratory to quickly identify a plague outbreak and apply the appropriate control measures to limit its spread.

The pathogenesis of plague was explored, and two separate, but essential, iron transport systems were identified in *Y. pestis*. The first, the yersinabactin (Ybt) system, enables the organism to proliferate from the site of an open wound or bite, while the second, identified as Yfe, is used by Ybt mutants to obtain iron during infection of internal organs. The Ybt iron transport system appears to be essential for growth in the early stages of bubonic plague infection, while the Yfe system functions to allow growth during, or after, infection of internal organs. Both systems are required for *Y. pestis* to be fully virulent.

A novel mechanism by which a *Y. pestis* virulence protein is sequestered in, and transported within, host cells was described. When yersiniae contact a eukaryotic cell, a signaling event activates the expression and secretion of a set of four toxins (Yops) and causes their vectorial translocation into the cell cytoplasm. Three of the Yops derange cell signaling and cytoskeletal functions by kinase, tyrosine phosphatase, and actin depolymerization activities. Although no activity or intracellular target has

been identified for the fourth known translocated Yop, YopM, immunoblot analysis and laser scanning confocal microscopy demonstrated that most YopM is vectorially translocated into HeLa cells or the macrophagelike cell line J774 by adherent *Y. pestis* and travels to the eukaryotic cell nucleus. Because a growing number of important human pathogens (e.g., *Salmonella, Shigella*, and *Pseudomonas*) have similar, but less well-studied, secretion/translocation mechanisms and putative secreted toxins, these findings will facilitate studies that ultimately could lead to novel therapies for these agents.

Antibiotic Resistance

The current development of staphylococci resistant to methicillin or fluoroquinolones and gram-negative bacilli resistant to extended-spectrum beta-lactams are but the most recently recognized patterns of antibiotic resistance. General approaches for modifying these trends include 1) source control, particularly handwashing, and the need to wear gloves during contacts with all patients, 2) improved antibiotic use and control, 3) improved infection control devices, and 4) better use of pathophysiology and immunologic modulation.

The growing problem of vancomycin-resistant enterococci (VRE), with an incidence of 20% to 40% in some groups of U.S. hospitalized patients, necessitates maximal use of all these approaches. Skin proliferation of VRE produces extensive environmental contamination that may require universal use of gloves to control outbreaks or hyperendemic disease. In addition, vancomycin should be limited to treatment of beta-lactam resistant gram-positive bacteria (such as methicillin-resistant *Staphylococcus aureus* and gram-positive bacteria in beta-lactam allergic patients) and *Clostridium difficile* (only after metronidazole failure) and to endocarditis prophylaxis. Vancomycin should be avoided in routine surgical prophylaxis, empiric treatment of febrile neutropenia with negative cultures, and pneumonia prophylaxis in the intensive care unit. A number of experimental peptides and other agents (such as quinupristin-dalfopristin) under investigation as treatments for VRE infections were identified.

Future trends in resistance may include further spread of vancomycin-resistant staphylococci (already reported in Japan), quinolone- or carbapenem-resistant gram-negative bacilli, and treatment-resistant viruses. Seventeen isolates of methicillin-resistant *S. aureus* with unique genotypes were studied to determine rates of resistance to the fluoroquinolones ciprofloxacin and levofloxacin. The mean single-step resistance to 4 x MIC ciprofloxacin was 1.05×10^{-5} and to levofloxacin was 4.03×10^{-6}. When serially passaged in increasing antibiotic concentrations, the geometric mean MICs for ciprofloxacin and levofloxacin increased 3.0 ± 1.5 times and 1.8 ± 1.4 times, respectively ($p < 0.0005$). Only four strains became resistant to levofloxacin, but eight became resistant to ciprofloxacin, indicating that ciprofloxacin selects methicillin-resistant *S. aureus* more frequently than levofloxacin.

Other Topics

The laboratory evidence supporting the role of *Chlamydia pneumoniae* in the development of atherosclerosis was reviewed. Recent data indicate that infection of vascular endothelial cells with *C. pneumoniae* is associated with the production of chemokines and adhesion molecules that promote transendothelial migration of neutrophils and monocytes. These findings suggest that immunopathogenic responses to *C. pneumoniae* infection may contribute to the development of clogging deposits.

Data were presented demonstrating the proliferation of human CD4+ T cells from unexposed persons in response to in vitro exposure to *Toxoplasma gondii*. Further studies showed that this proliferative response depends on HLA-DR molecules and requires processing of *Tg* antigens. In contrast to typical exogenous superantigens, analysis of TCR Vß expression after stimulation with *Tg* did not show a pattern of preferential increase of a specific TCR Vß-bearing subpopulation. αßT cells secreted significant amounts of IFN-γ after incubation with *Tg*-infected monocytes. This process may play an important role in the early events of the immune response to *T. gondii*.

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International Meeting on Borreliosis, Prague, Czech Republic

Approximately 150 participants from 10 countries gathered in Prague, the Czech Republic, August 27-29, 1997, to discuss research topics related to the theme of the meeting, "Lyme Borreliosis—Basic Science and Clinical Approaches." The meeting was organized by the National Institute of Public Health (Centre of Epidemiology and Microbiology); the World Health Organization Collaborating Center for Reference and Research on Borreliosis; the Second School of Medicine, Charles University (Prague); and the Czech Medical Association J.E. Purkyně. Meeting sessions focused on topics including epidemiology, clinical treatment, dermatology, diagnosis and treatment, neurology, and laboratory diagnosis.

The session on epidemiology presented surveillance data on the incidence of Lyme borreliosis (LB) in the Czech Republic (incidence rates were 61.8/100,000 in 1995 and 41.2/100,000 in 1996) and in Slovakia, Austria, and Slovenia. Data underscored the high risk for transmission of LB in central and eastern Europe. The results of vaccine trials using the recombinant outer surface protein (Osp)A antigen of *Borrelia burgdorferi* were also presented; more detailed studies are needed to examine intraspecies variability of OspA antigens in Europe.

The session on clinical approaches and treatment reviewed research conducted in the United States and discussed the diagnostic importance of organism-specific biologic markers, e.g., Borrelia-specific antigens or DNA, as well as pleocytosis in cerebrospinal or synovial fluid. Experience with the diagnosis and treatment of LB in the hyperendemic-disease regions of west Bohemia underscored the importance of accurate diagnosis in avoiding overtreatment.

The use of nonhuman primates as models for studying neuroborreliosis was examined in the session on neurology. Problems related to the diagnosis and treatment of chronic disease, and their economic consequences, were identified. Several methods to assist clinicians in making a correct diagnosis were presented and discussed. The persistence of *B. burgdorferi* DNA in patients with Lyme arthritis was considered in the rheumatology session. Ultrastructural evidence for the intracellular location of *B. burgdorferi* in synovium also was presented.

The session on laboratory diagnosis focused on the genomic sequence of the linear chromosome of *B. burgdorferi* (B31 strain) and the crystal structure of OspA; both apply to the laboratory diagnosis of LB. Other studies affirmed the importance of standardizing diagnostic methods to ensure reproducibility and uniformity of the results from different laboratories. The influence of certain in vivo–expressed antigens (virulence antigens) on invasiveness and the ability of *B. burgdorferi* to adapt to the host environment were noted. Other topics were the sensitivity and reproducibility of polymerase chain reaction and the importance of the primers selected for the assay.

The studies presented in the poster session addressed a wide array of themes: among them, epidemiology and population awareness, reactivity of *B. burgdorferi* antigens in immunoblot procedures when specimens derived from humans or animals are used, and incidence of ticks and their association with disease in different regions.

The importance of apoptosis in the morphology of LB, the role of Langerhans cells in the skin reactions, and the role of integrin CR3 in the interaction of *B. burgdorferi* with host cells were discussed. The sensitivity and the selection of the primers used for polymerase chain reaction to detect *B. burgdorferi* in ticks were considered. Aspects of vector biology and ecology were investigated (e.g., habitats, the tick as LB's major vector, vector capacity). Other diseases transmitted by *Ixodes ricinus* ticks in Europe (e.g., tick-borne encephalitis, babesiosis, ehrlichiosis) as well as human ehrlichiosis in Europe were reviewed.

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Workshop on Climate Change and Vector-Borne and Other Infectious Diseases

Climate changes may affect human health through a myriad of pathways; of particular interest are pathways affecting the geographic ranges and incidence of vector- and water-borne diseases. As society chooses how to deal with projections of long-term climate change, decisions must be based on scientific knowledge. A 2-day

workshop[1] was convened in September 1997 to discuss what is known about the relationship between projected climate changes and the incidence of water-borne diseases (e.g., cholera) and vector-borne diseases, including those typically considered tropical (malaria, dengue fever, yellow fever, and schistosomiasis), plus subtropical or temperate-zone diseases whose vectors are likely to be affected by projected climate changes.

The workshop participants discussed the systems involved in potential climate changes, from the global ocean-atmosphere-landmass system that drives climate to the regional ecologic and human socioeconomic systems where disease dynamics occur. These systems are extremely complex, as are the interactions among them, which underscores the need for more research before accurate projections can be made. Major research gaps were identified, and an agenda was framed for a sound scientific basis for public policy debates and decisions. The proposed agenda included the following items: climate modeling; ecosystem and habitat dynamics; disease surveillance; technologies for disease prevention and mitigation; disease transmission dynamics; data sets for empirical studies; integrated assessments; and detecting, understanding, and responding to unexpected events. Further discussion and implementation of this research agenda is encouraged. A summary of the workshop is available from the Electric Power Research Institute, TR-109516, EPRI Distribution Center, 207 Coggins Drive, P.O. Box 23205, Pleasant Hill, CA 94523; Telephone: 510-934-4212.

[1]The workshop was commissioned by the Electric Power Research Institute, with additional sponsorship from the Department of Energy, the National Institute of Allergy and Infectious Disease, the National Institute of Environmental Health Sciences, and the National Aeronautics and Space Administration. The workshop was organized and conducted by the Washington Advisory Group. The 28 participants included representatives from agencies and institutions that conduct or fund research and experts in the fields of climatology and global climate modeling, public health, and the biology and ecology of vectors, pathogens, and the ecosystems they inhabit.

The Fourth International Conference on HFRS and Hantaviruses

Atlanta, Georgia, USA, March 5–7, 1998

The Centers for Disease Control and Prevention and other cosponsors will host the Fourth International Conference on HFRS and Hantaviruses to facilitate the exchange of scientific information in the following areas: 1) clinical aspects, 2) laboratory diagnostics, 3) pathogenesis and immune response, 4) hantavirus ecology, 5) hantavirus epidemiology, 6) molecular biology and cell interactions, 7) health education and prevention, and 8) antiviral and vaccine development. The meeting will offer plenary sessions with invited speakers, as well as oral and poster sessions based on accepted abstracts.

For further information, contact Amy Corneli, Centers for Disease Control and Prevention, 1600 Clifton Road, MS A26, Atlanta, GA 30333, USA; fax: 404-639-1509; e-mail: akc8@cdc.gov; URL: http://www.cdc.gov/ncidod/diseases/hanta/hantconf.htm.

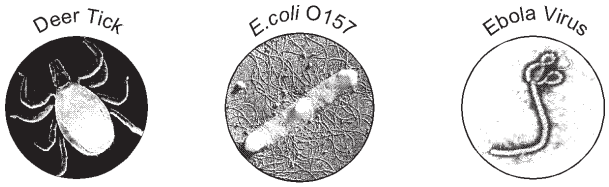
Third International Congress on Tropical Neurology

November 30–December 2, 1998

Organized by the Groupe Francophone d'Etude et de Recherche en Neurologie Tropicale, the Third International Congress on Tropical Neurology will convene in Fort de France, Martinique, from November 30 to December 2, 1998. The four main themes of the congress are central nervous system inflammatory, neurodegenerative, epileptic, and cerebrovascular disorders in tropical environments; however, presentations on other themes are welcome.

A symposium on epilepsy in tropical zones will be held during the congress.

For additional information, contact Professor M. Dumas (phone: 33-5-55-43-58-20, fax: 33-5-55-43-58-21) or Professor J.C. Vernant (phone: 33-5-96-55-22-61, fax: 33-5-96-75-45-90).



International Conference on Emerging Infectious Diseases

March 8-11, 1998
Atlanta, Georgia

International Conference on Emerging Infectious Diseases
March 8–11, 1998
Atlanta Marriott Marquis Hotel

Late-breaker abstract submission deadline: January 30, 1998

Information on abstract submission, conference registration, and exhibits can be obtained at www.asmusa.org, by sending an e-mail message to meetinginfo@asmusa.org, or by calling 202-942-9248. Proceedings of the conference will be published in the journal Emerging Infectious Diseases.

Registration: limited to 2,500 - register NOW!

Preliminary program information is available at http://www.cdc.gov/ncidod/EID/98conf.htm.

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Emerging Infectious Diseases thanks the following reviewers for their support through thoughtful, thorough, and timely reviews from the journal's inception to the present. We apologize for any names we may have omitted inadvertently.

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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 has an international scope and 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, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-639-3967 (tel), 404-639-3075 (fax), or eideditor@cdc.gov (e-mail).

Emerging Infectious Diseases is published in English and features three types of articles: Perspectives, Synopses, and Dispatches. The purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

Spanish translations of some articles can be accessed at ftp://fcv.medvet.unlp.edu.ar/pub/EID. Articles by authors from non-English-speaking countries can be made simultaneously available in English and in the author's native language (electronic version of the journal only).

Instructions to Authors

Manuscript Preparation

The journal complies with the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (Ann Int Med 1997:126[1]36-47) (http://www.acponline.org/journals/resource/unifreqr.htm).

Begin each of the following sections on a new page and in this order: title page, abstract, text, acknowledgments, references, tables, figure legends, and figures.

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). Also provide address for correspondence (include fax number and e-mail address).

Abstract and key words. Avoid citing references in the abstract. Include up to 10 key words; use terms listed in the Medical Subject Headings from Index Medicus (http://www.nlm.nih.gov/tsd/serials/lji.html).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Type only on one side of the paper and number all pages, beginning with the title page. Indent paragraphs 5 spaces; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use Courier font size 10 and ragged right margins. Italicize (rather than underline) scientific names when needed.

Electronic formats. For word processing, use WordPerfect or MS Word. For graphics, use Corel Draw, Harvard Graphics, Freelance, PowerPoint, .TIF (TIFF), .GIF (CompuServe), .WMF (Windows Metafile), .EPS (Encapsulated Postscript), or .CGM (Computer Graphics Metafile). The preferred font for graphics files is Helvetica. Convert Macintosh files into one of the suggested formats. Submit slides or photographs in glossy, camera-ready photographic prints.

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Tables and figures. Create tables within the word processing program's table feature (not columns and tabs within the word processing program). For figures, use color as needed; send slides, photographs, or prints. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced. Place figure keys within the figure.

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Include a cover letter verifying that the final manuscript has been seen and approved by all authors.

Submit three copies of the original manuscript with three sets of original figures and an electronic copy (on diskette or by e-mail) to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS C-12, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov.

Types of Articles

Perspectives: Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases or related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaption 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. Articles should be approximately 3,500 words and should include references, not to exceed 40. Use of additional subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Synopses: 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. Synopses should be approximately 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Dispatches: These brief articles 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. Dispatches (1,000 to 1,500 words of text) need not be divided into sections. Provide a short abstract (50 words); references, not to exceed 10; figures or illustrations, not to exceed two; and a brief biographical sketch.

Letters: This section includes letters that give preliminary data or comment on published articles. Letters (500 to 1,000 words of text) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.