JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1998, p. 1117–1121 Vol. 36, No. 4
0095-1137/98/$04.00+0
Copyright © 1998, American Society for Microbiology

Intravitam Diagnosis of Human Rabies by PCR Using Saliva and Cerebrospinal Fluid

P. CREPIN,[1] L. AUDRY,[1] Y. ROTIVEL,[1] A. GACOIN,[2] C. CAROFF,[2] AND H. BOURHY[1]*

Rabies Unit, Institut Pasteur, Paris,[1] and Regional and University Hospital, Rennes,[2] France

Received 3 September 1997/Returned for modification 18 December 1997/Accepted 15 January 1998

An optimized reverse transcription (RT)-PCR protocol for the intravitam detection of rabies virus genomic RNA was tested with clinical samples obtained from 28 patients suspected of having rabies, 9 of whom were confirmed to have had rabies by postmortem examination. RT-PCR using saliva combined with an immunofluorescence assay performed with skin biopsy samples allowed detection of rabies in the nine patients.

According to results of global surveillance by the World Health Organization, about 50,000 cases of human rabies occur each year (45), the majority of them in developing countries (63). Human rabies can be prevented through the combination of stringent animal vaccination, quarantine programs, and the availability of expensive vaccines and specific immunoglobulins (32). In spite of the fact that most developed countries have good public health measures in place to prevent human rabies, these countries are dealing with an increasing number of human rabies cases. This is clearly the case in the United States, where bat rabies virus variants as well as some imported cases of human rabies have recently achieved an increased public health significance (41, 51, 53, 56). This is also the case in France, where seven human cases were recorded from 1988 to 1997; only eight cases had been recorded in the preceding 20 years. All of the human rabies cases in France were imported. A similar situation has also been observed throughout the whole European Union. Of the 15 cases reported in the last 10 years, 12 were imported from Asia, Africa, and Latin America (46; unpublished data from France). These cases underscore the importance of alerting travellers of the risk of rabies contamination and of the prophylactic methods to prevent the disease (42). They also indicate the importance of maintaining a good system of surveillance, of keeping medical staffs well informed on clinical presentation of a disease which rarely occurs in developed countries, and of the necessity to develop diagnostic tools for the identification of rabies in these patients.

The clinical diagnosis of rabies is sometimes suggested by epidemiological (history of exposure) and clinical (e.g., paresthesia, hydrophobia) findings (36). However, the disease is often mistaken for other disorders (30). Differentiation from other neurologic diseases may require extensive investigations. Therefore, diagnosis is often confirmed late in the course of the disease or postmortem (31). Delays in diagnosis greatly increase the number of contacts that require postexposure prophylaxis. The average number of contacts (hospital personnel, family) receiving postexposure treatments (PET) is approximately 50 per case ($n = 19$) in France and between 41 and 55 per case in the United States (29, 35). In the United States, one case resulted in 209 PET (50), and 290 PET for one case were reported recently in France. The early diagnosis of rabies is also essential to eliminate the expense and discomfort of unnecessary diagnostic tests and inappropriate therapy.

A wide variety of viruses, bacteria, and parasites, all of which are capable of causing aseptic meningitis and encephalitis, have been detected by PCR (39, 49, 52, 58). The objective of the present study was to establish a reverse transcription (RT)-PCR protocol for use in evaluating diagnostic specimens, including saliva and cerebrospinal fluid (CSF). CSF samples were centrifuged at $11,000 \times g$ for 20 min at 4°C. Total RNA was extracted from specimens of saliva and pellets of CSF by four different techniques, including the following: (i) proteinase K (34), (ii) guanidinium thiocyanate together with silica particles (5, 6), (iii) cationic surfactant (Catrimox-14; Iowa Biotechnology Corporation) (43), and (iv) chelating resin (Chelex 100; Bio-Rad) (60). The proteinase K method was performed as follows. Briefly, 200 μl of biological fluid (saliva or CSF including the pellet) was incubated for 2 h at 37°C with 400 μl of proteinase K buffer containing 40 μg of proteinase K (Gibco BRL). The RNA was then purified by a phenol-chloroform extraction and precipitated in absolute ethanol. The pellets were resuspended in 100 μl of pyrolyzed water. Considering that the N gene is the most conserved in the lyssa viruses (except some domains of the L protein gene) and that the sequence data concerning this gene are the most exhaustive, we used primers in the N gene that were shown to allow amplification of a wide range of genetically diverse lyssa viruses (7, 40). One microliter of primer N12 (5′ GTAACACCTCTA CAATGG 3′, positions 57 to 74; all the positions of the primers are given based on the PV strain sequence [59]) (100 ng/μl) was incubated with 2 μl of RNA (1 μg) at 65°C for 3 min and chilled on ice. Each tube was then incubated at 37°C for 90 min with 4 μl of a solution containing each nucleotide triphosphate (10 mM), 0.6 μl of RNasin (15 U), 1 μl of dithiothreitol, 2 μl of Super Script reverse transcriptase buffer (Gibco BRL), and 0.5 μl of Super Script reverse transcriptase (100 U; Gibco BRL). The RNA-cDNA hybrids were diluted 10 times in Tris-EDTA buffer. Five microliters of diluted cDNA was mixed with 50 μl containing a 1 μM concentration of N12 and N40 (5′ GCTTGATGATTGGAACTG 3′, positions 1368 to 1349), 20 mM each nucleotide triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, and 2 U of *Taq* polymerase (Gibco BRL). PCR was performed with a GeneAmp PCR System 9600 (Perkin-Elmer) by using the following program: 1 cycle of denaturation at 94°C for 60 s, annealing at 50°C for 90 s, and elongation at 72°C for 90 s; 30 cycles of denaturation for 50 s;

* Corresponding author. Mailing address: National Reference Center for Rabies, Institut Pasteur, 75724 Paris Cedex 15, France. Phone: 01 45 68 87 85. Fax: 01 40 61 30 20. E-mail: hbourhy@pasteur.fr.

TABLE 1. Patients and specimens analyzed

Patient	No. of specimens from:			Total no. of specimens	Diagnosis for rabies[a]		
	CSF	Saliva	Bronchoalveolar washings		Postmortem	Intravitam by IF assay of skin biopsy sample	Intravitam by RT-PCR using saliva sample
P1	1			1	−	−	−
P2	1			1	−	−	−
P3	1			1	−	−	−
P4		1		1	+	−	+
P5	2	2		4	+	−	+
P6		1		1	−	−	−
P7	9	11	1	21	+	−	+
P8	2			2	−	−	−
P9	1			1	−	−	−
P10	1			1	−	−	−
P11	2	1		3	−	−	−
P12		1		1	−	−	−
P13	1	1		2	+	+	−
P14		1		1	−	−	−
P15	1			1	−	−	−
P16	1			1	−	−	−
P17		2		2	−	−	−
P18	1	1		2	+	+	−
P19	2	3		5	+	+	−
P20	3	4		7	+	+	−
P21	1	1		2	+	−	+
P22	1			1	−	−	−
P23	1			1	−	−	−
P24	1			1	−	−	−
P25	2			2	−	−	−
P26	1			1	−	−	−
P27	2	1		3	−	−	−
P28	3	13		16	+	+	+
Total	41	44	1	86			

[a] +, positive; −, negative.

and a final cycle of elongation for 5 min. The optimum temperature for annealing and magnesium ion concentration for the PCR was determined by standard titration experiments of a cDNA of the N gene of strain 9147FRA. Five microliters of PCR products was diluted into 95 μl of pyrolyzed water, denatured for 3 min at 95°C, and chilled on ice. The samples were then distributed onto a Hybond nylon transfer membrane (Amersham) with a Bio-Dot microfiltration unit (Bio-Rad) and fixed by irradiation on a UV transilluminator. Primer set N3 (5′ GTCTCTTTGAAGCCTGAG 3′, positions 113 to 130)-N23 (5′ GGTCTCTCGTCAGTTCCAT 3′, positions 464 to 446) and primer set N17 (5′ TTCTTCCACAAGAACTTTG 3′, positions 848 to 866)-N2 (5′ CCCATATAGCATCCTAC 3′, positions 1030 to 1013) were used to generate two digoxigenin-labelled probes by PCR. Hybridization with the two complementary digoxigenin-labelled probes and detection by chemiluminescence were performed by using a nonradioactive DNA labelling and detection kit (Boehringer).

The four different methods of RNA extraction were tested in parallel, each on two independent series of experimentally infected samples, to determine the threshold of detection of the RT-PCR. Briefly, negative specimens of saliva and CSF were pooled and infected with serial dilutions of a fox rabies isolate (isolate 9147FRA) (40). This virus was produced on BHK-21 cells infected at a multiplicity of infection of 0.1 and harvested 48 h after infection to limit the number of defective viral particles. The Catrimox-14 and Chelex 100 techniques produced negative results. In our hands, poor results were achieved when guanidinium thiocyanate was used together with silica particles (threshold of detection, around 10^4 focus-forming units [FFU]/ml). However, the results obtained with the proteinase K method were reproducible with a threshold of detection of 10^2 FFU/ml in the saliva and 10 FFU/ml in the CSF. These results are similar to the theoretical viral infectivity obtained by rapid tissue culture infection test (RTCIT) (20 FFU/ml) (9). The difference in the results for saliva and CSF can be explained by the presence of RNase in the saliva.

Forty-one specimens of CSF and 44 specimens of saliva collected daily by aspiration or by use of cotton swabs were obtained from 28 patients with manifestations of aseptic meningitis, encephalitis, and suspected rabies infection (Table 1). Rabies was confirmed in the brain tissue of nine of these patients by postmortem examination (8, 28). One patient each was infected in the Middle East, Latin America (38), and India, and six patients were infected in Africa (18). One of these patients (patient P13) was known to be infected with human immunodeficiency virus (1). The day of onset of illness was defined as the day when the first symptom attributed to rabies could be noted. These data are missing for two patients. The average duration of illness was 12.4 days for the seven remaining rabies-infected patients. The specificity of RT-PCR was one for the samples of saliva and CSF. Eleven of the 37 putative positive specimens of saliva (sensitivity, 0.30) and only 2 of 22 specimens of CSF (sensitivity, 0.09) were confirmed positive (Table 2). The bronchoalveolar washing was negative. Intravitam diagnosis performed by RT-PCR identified the presence of rabies virus in the saliva of five patients (sensitivity for the patient, 0.56) and in the CSF of only two patients (sensitivity for the patient, 0.22). RT-PCR performed with saliva sam-

TABLE 2. Frequency of intravitam diagnosis of rabies in nine patients in France (1986–1997)

Specimen type	Method(s)[a]	Frequency of rabies diagnosis[b]						Sensitivity[d]
		0–4 days[c]	5–8 days	9–12 days	13–16 days	>16 days	Unknown date	
Serum	Serology (ELISA, RFFIT)	0/4	1/2	0/2		1/1	0/3	0.17 (n = 12)
CSF	Serology (ELISA, RFFIT)	0/4	0/1	0/3		1/1	0/3	0.08 (n = 12)
	RT-PCR	0/5	1/3	0/2	0/1	0/2	1/9	0.09 (n = 22)
Saliva	RREID	0/1	0/5	0/4	0/4	1/4	0/1	0.05 (n = 19)
	RT-PCR	1/5	3/8	1/5	0/4	3/6	3/9	0.30 (n = 37)
Skin biopsy	IF	3/3	2/2			0/1	1/1	0.86 (n = 7)

[a] RFFIT, rapid fluorescent focus inhibition test; RREID, rapid rabies enzyme immunodiagnosis for rabies antigen detection.
[b] The number of patients diagnosed with rabies relative to the total number is given.
[c] Days after onset of symptoms.
[d] n = number of samples.

ples is of particular help in early diagnosis of the disease (Table 2).

Patient P7, who experienced a long clinical period, was confirmed positive for rabies at the time of death. Specimens of saliva from patient P7 were confirmed positive consecutively on days 21 and 22 of the disease, became negative on day 23, and became positive again on day 24. To investigate the relationship between the intermittence of rabies virus excretion and the time of day of sample collection, 11 specimens of saliva were collected every 2 or 4 h from one patient (patient P28) during a 5-day period midway through the course of the disease. This period starts 2 days after the last collection of saliva which was found positive, 9 days after the onset of the symptoms, and 12 days before death. None of these specimens were confirmed positive. The question of titer and duration of virus shedding is of particular importance for rabies diagnosis because saliva remains the major source of exposure in contacts for whom rabies prophylaxis is recommended (35). The signal obtained after RT-PCR using some saliva samples indicated that the level of rabies virus in the saliva could reach more than 10^3 FFU/ml (data not shown). This evidence confirms that any contact with saliva from a patient suspected of having rabies should be taken seriously.

Most of the conventional techniques used for postmortem analysis of the brain are of limited value to support the intravitam diagnosis of rabies (9, 37, 61). We reviewed records of 39 cases of intravitam diagnosis of rabies in the United States during the period 1960 to 1996 (3, 10–27) and of 16 cases of intravitam diagnosis performed in France during the period 1970 to 1997 (Table 3). This confirms that the corneal smear first developed with mice by Schneider (54) was too insensitive for accurate clinical diagnosis (3, 4, 44, 62). The only test that has demonstrated reliable results is the immunofluorescence (IF) test on skin biopsy samples (62). In our study, the IF test (33), performed on frozen sections of the skin biopsy samples, exhibited the highest sensitivity (sensitivity, 0.86; n = 7). It detected the presence of rabies virus very early in the course of the disease and could be considered one of the most important tests for intravitam diagnosis. In routine laboratory testing, we noticed that the examination of a minimum of 20 sections was needed to ensure the observation of several hair follicules. Typical rabies nucleocapsid inclusions can be observed in the nerve endings around the base of some of these hair follicules (data not shown). RT-PCR assay of saliva and IF assay of skin sections together allowed a positive intravitam diagnosis in all of the nine laboratory-confirmed cases of human rabies presented in this study. The average delay between the onset of clinical symptoms and the collection of specimens that confirmed the presence of rabies virus was 6.7 days (n = 7). The sensitivities of detection of rabies antibodies by enzyme-linked immunosorbent assay (ELISA) (48) and by seroneutralization on cell culture (57) were very low in serum as well as in CSF (results obtained from the sera and CSF of patients P13 and P28, who had received a PET protocol different from that recommended by the WHO Expert Committee on Rabies [64], were excluded) (Table 2). This confirms that serological testing is of limited value because seroconversion occurs late in the course of the disease (55) (Table 3). The detection of rabies antigen in the saliva by ELISA (47) also gave poor results.

On the basis of these results, we propose a simple testing protocol for intravitam diagnosis of rabies. Two different specimens should be sent to the laboratory in the early phase of the disease, a skin biopsy specimen and a saliva specimen. Skin biopsy samples should be analyzed by direct IF and saliva samples should be analyzed by the RT-PCR method described above. Then, after 1 week of illness, serum samples should also be examined for the presence of rabies-specific antibodies. RT-PCR has also been proven to be a valuable adjunct to an epidemiological investigation because sequences can be ob-

TABLE 3. Results of laboratory confirmation of rabies attempted in 55 patients during life[a]

Determination	Frequency of rabies diagnosis[b]					Sensitivity[d]
	0–4 days[c]	5–8 days	9–12 days	13–16 days	>16 days	
Antigen in skin biopsies	5/6	6/10	5/5	1/2	1/3	0.69 (n = 26)
Antibodies in serum	1/15	5/28	8/25	14/20	12/14	0.39 (n = 102)
Antibodies in CSF	0/7	0/12	1/10	3/8	6/10	0.21 (n = 47)
Antigen in corneal smears	0/4	1/16	3/16	2/7	2/16	0.14 (n = 59)

[a] These results were obtained from the analysis of 39 cases of rabies in the United States (3, 10–27) and 16 cases in France (1970–May 1997) (unpublished results).
[b] The number of patients diagnosed with rabies relative to the total number is given.
[c] Days after onset of symptoms.
[d] n = number of samples.

tained rapidly from PCR products, thus permitting rapid identification of the virus (2, 40).

We express our gratitude to those who sent us the specimens included in this evaluation, namely, P. Hubert and M. Cloud, Pediatric Intensive Care Unit, Hôpital des Enfants Malades, Paris, France; C. Laffont, Laboratory of Virology, Regional and University Hospital, Nice, France; C. Ricard, Antirabic Centre, Hôpital Bellepierre, Saint Denis, La Réunion, France; P. Hofman, Department of Pathological Anatomy and Cytology, Hôpital Pasteur, Nice, France; P. Dellamonica, Unit of Infectious Diseases, Hôpital de l'Archet, Nice, France; H. Adle-Biassette, B. Godeau, and P. Marcowicz, Hôpital Henri Mondor, Creteil, France; and H. Gallais, Unit of Infectious Diseases, Hôpital de la Conception, Marseille, France. We are grateful to Pascale Cozette and Nicole Vachet for expert technical assistance.

REFERENCES

1. **Adle-Biassette, H., H. Bourhy, M. Gisselbrecht, F. Chrétien, L. Wingertsmann, M. Beaudrimont, Y. Rotivel, B. Godeau, and F. Gray.** 1996. Rabies encephalitis in a patient with AIDS: a clinicopathological study. Acta Neuropathol. **92:**415–420.
2. **Amengual, B., J. E. Whitby, A. King, J. Serra Cobo, and H. Bourhy.** 1997. Evolution of European bat lyssavirus. J. Gen. Virol. **78:**2319–2328.
3. **Anderson, L. J., K. G. Nicholson, R. V. Tauxe, and W. G. Winkler.** 1984. Human rabies in the United States, 1960 to 1979: epidemiology, diagnosis and prevention. Ann. Intern. Med. **100:**728–735.
4. **Blenden, D. C., W. Creech, and M. J. Torres-Anjel.** 1986. Use of immunofluorescence examination to detect rabies virus antigen in the skin of humans with clinical encephalitis. J. Infect. Dis. **154:**698–701.
5. **Boom, R., C. J. A. Sol, R. Heijtink, P. M. E. Wertheim-van Dillen, and J. van der Noordaa.** 1991. Rapid purification of hepatitis B virus DNA from serum. J. Clin. Microbiol. **29:**1804–1811.
6. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
7. **Bourhy, H., B. Kissi, and N. Tordo.** 1993. Molecular diversity of the Lyssavirus genus. Virology **194:**70–81.
8. **Bourhy, H., P. E. Rollin, J. Vincent, and P. Sureau.** 1989. Comparative field evaluation of the fluorescent-antibody test, virus isolation from tissue culture, and enzyme immunodiagnosis for rapid laboratory diagnosis of rabies. J. Clin. Microbiol. **27:**519–523.
9. **Bourhy, H., and P. Sureau.** 1991. Laboratory methods for rabies diagnosis. Institut Pasteur, Paris, France.
10. **Centers for Disease Control.** 1981. Human rabies acquired outside the United States from a dog bite. Morbid. Mortal. Weekly Rep. **30:**537–540.
11. **Centers for Disease Control.** 1983. Human rabies—Michigan. Morbid. Mortal. Weekly Rep. **32:**159–160.
12. **Centers for Disease Control.** 1983. Imported human rabies. Morbid. Mortal. Weekly Rep. **32:**78–86.
13. **Centers for Disease Control.** 1984. Human rabies—Pennsylvania. Morbid. Mortal. Weekly Rep. **33:**633–635.
14. **Centers for Disease Control.** 1984. Human rabies—Texas. Morbid. Mortal. Weekly Rep. **33:**469–470.
15. **Centers for Disease Control.** 1988. Human Rabies—California, 1987. Morbid. Mortal. Weekly Rep. **37:**305–308.
16. **Centers for Disease Control.** 1991. Human rabies—Texas, 1990. Morbid. Mortal. Weekly Rep. **40:**132–133.
17. **Centers for Disease Control.** 1991. Human rabies—Texas, Arkansas, and Georgia, 1991. Morbid. Mortal. Weekly Rep. **40:**765–769.
18. **Centers for Disease Control.** 1992. Imported human rabies—France, 1992. Morbid. Mortal. Weekly Rep. **41:**953–955.
19. **Centers for Disease Control.** 1993. Human rabies—New York, 1993. Morbid. Mortal. Weekly Rep. **42:**799–806.
20. **Centers for Disease Control.** 1994. Human rabies—Texas and California, 1993. Morbid. Mortal. Weekly Rep. **43:**93–96.
21. **Centers for Disease Control.** 1995. Human rabies—Alabama, Tennessee, and Texas, 1994. Morbid. Mortal. Weekly Rep. **44:**269–272.
22. **Centers for Disease Control.** 1995. Human rabies—Washington, 1995. Morbid. Mortal. Weekly Rep. **44:**625–627.
23. **Centers for Disease Control.** 1995. Human rabies—West Virginia, 1994. Morbid. Mortal. Weekly Rep. **44:**86–93.
24. **Centers for Disease Control.** 1996. Human rabies—California, 1995. Morbid. Mortal. Weekly Rep. **45:**353–356.
25. **Centers for Disease Control.** 1996. Human rabies—Connecticut, 1995. Morbid. Mortal. Weekly Rep. **45:**207–209.
26. **Centers for Disease Control.** 1996. Human rabies—Florida, 1996. Morbid. Mortal. Weekly Rep. **45:**719–727.
27. **Centers for Disease Control.** 1997. Human rabies—New Hampshire, 1996. Morbid. Mortal. Weekly Rep. **46:**267.
28. **Dean, D. J., M. K. Abelseth, and P. Atanasiu.** 1996. The fluorescent antibody test, p. 88–95. *In* F.-X. Meslin, M. M. Kaplan, and H. Koprowski (ed.), Laboratory techniques in rabies. World Health Organization, Geneva, Switzerland.
29. **Drenzek, C. L., D. L. Noah, J. S. Smith, C. E. Rupprecht, J. W. Krebs, M. A. Fekadu, and J. E. Childs.** 1996. Human rabies in the United States, 1980 to 1986: epidemiologic and clinical features, p. 82. *In* Abstracts of the Seventh Annual International Meeting on Advances towards Rabies Control in the Americas.
30. **Emmons, R. W.** 1979. Rabies diagnosis and rabies vaccine. N. Engl. J. Med. **301:**331–332.
31. **Fishbein, D. B.** 1991. Rabies in humans, p. 519–549. *In* G. M. Baer (ed.), The natural history of rabies. CRC Press, Inc., Boca Raton, Fla.
32. **Fishbein, D. B., and L. E. Robinson.** 1993. Rabies. N. Engl. J. Med. **25:**1632–1638.
33. **Goldwasser, R. A., R. E. Kissling, T. R. Carski, and T. S. Hosty.** 1959. Fluorescent antibody staining of rabies virus antigens in the salivary glands of rabid animals. Bull. W. H. O. **20:**579–588.
34. **Grünewald, K., H. Feichtinger, O. Dietze, and J. Lyons.** 1990. DNA isolated from plastic embedded tissue is suitable for PCR. Nucleic Acids Res. **18:**6151.
35. **Helmick, C. G., R. V. Tauxe, and A. A. Vernon.** 1987. Is there a risk to contacts of patients with rabies? Rev. Infect. Dis. **9:**511–518.
36. **Hemachudha, T.** 1994. Human rabies: clinical aspects, pathogenesis, and potential therapy, p. 121–144. *In* C. E. Rupprecht, B. Dietzschold, and H. Koprowski (ed.), Lyssaviruses. Springer-Verlag, Berlin, Germany.
37. **Hemachudha, T., P. Phanuphak, B. Sriwanthana, S. Manutsathit, K. Phanthumchinda, W. Siriprasomsup, C. Ukachoke, S. Rasameechan, and S. Kaoroptham.** 1988. Immunologic study of human encephalitic and paralytic rabies. Am. J. Med. **84:**673–677.
38. **Hofman, P., H. Bourhy, J. F. Michiels, P. Dellamonica, P. Sureau, C. Boissy, and R. Loubière.** 1992. Encéphalomyélite rabique avec myocardite et pancréatite. Ann. Pathol. **12:**339–346.
39. **Kalmovarin, N., T. Tirawatnpong, R. Rattanasiwamoke, S. Tirawatnpong, T. Panpanich, and T. Hemachudha.** 1993. Diagnosis of rabies by polymerase chain reaction with nested primers. J. Infect. Dis. **167:**207–210.
40. **Kissi, B., N. Tordo, and H. Bourhy.** 1995. Genetic polymorphism in the rabies virus nucleoprotein gene. Virology **209:**526–537.
41. **Krebs, J. W., T. W. Strine, J. S. Smith, D. L. Noah, C. E. Rupprecht, and J. E. Childs.** 1996. Rabies surveillance in the United States during 1995. J. Am. Vet. Med. Assoc. **209:**2031–2044.
42. **LeGuerrier, P., P. A. Pilon, D. Deshaies, and R. Allard.** 1996. Pre-exposure rabies prophylaxis for the international traveller: a decision analysis. Vaccine **14:**167–176.
43. **Macfarlan, D., and E. Dahle.** 1993. Isolating RNA from whole blood—the dawn of RNA-based diagnosis? Nature **362:**186–188.
44. **Mathuranayagan, D., and P. Vishnupriya Rao.** 1984. Antemortem diagnosis of human rabies by corneal impression smears using immunofluorescent technique. Indian J. Med. Res. **79:**463–467.
45. **Meslin, F.-X., D. B. Fishbein, and H. C. Matter.** 1994. Rationale and prospects for rabies elimination in developing countries, p. 1–26. *In* C. E. Rupprecht, B. Dietzschold, and H. Koprowski (ed.), Lyssaviruses. Springer-Verlag, Berlin, Germany.
46. **Müller, W. W.** 1996. Review of reported rabies case data in Europe to the WHO Collaborating Centre Tübingen from 1977 to 1996. Rabies Bull. Eur. **20:**11–36.
47. **Perrin, P., P. E. Rollin, and P. Sureau.** 1986. A rapid rabies enzyme immunodiagnosis (RREID): a useful and simple technique for the routine diagnosis of rabies. J. Biol. Stand. **14:**217–222.
48. **Perrin, P., P. Versmisse, J. F. Delagneau, G. Lucas, P. E. Rollin, and P. Sureau.** 1986. The influence of the type of immunosorbent on rabies antibody EIA: advantages of purified glycoprotein over whole virus. J. Biol. Stand. **14:**95–102.
49. **Read, S. J., K. J. M. Jeffery, and C. R. M. Bangham.** 1997. Aseptic meningitis and encephalitis: the role of PCR in the diagnostic laboratory. J. Clin. Microbiol. **35:**691–696.
50. **Remington, P. L., T. Shope, and J. Andrews.** 1985. A recommended approach to the evaluation of human rabies exposure in an acute-care hospital. JAMA **254:**67–69.
51. **Rupprecht, C., J. Smith, M. Fekadu, and J. Childs.** 1995. The ascension of wildlife rabies: a cause for public health concern or intervention? Emerg. Infect. Dis. **1:**107–114.
52. **Sacramento, D., H. Bourhy, and N. Tordo.** 1991. PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. Mol. Cell. Probes **6:**229–240.
53. **Sang, E., R. Wesley Farr, M. Fisher, and S. D. Hanna.** 1996. Antemortem diagnosis of human rabies. J. Fam. Pract. **43:**83–87.
54. **Schneider, L. G.** 1969. The corneal test: a new method for the intra-vitam diagnosis of rabies. Zentralbl. Veterinaermed. **16:**24–31.
55. **Schuller, E., M. Helary, B. Allinquant, C. Gibert, F. Vachon, and P. Atanasiu.** 1979. IgM and IgG antibody responses in rabies encephalitis. Ann. Microbiol. **130A:**365–372.
56. **Smith, J. S.** 1996. New aspects of rabies with emphasis on epidemiology,

diagnosis, and prevention of the disease in the United States. Clin. Microbiol. Rev. **9:**166–176.

57. **Smith, J. S., P. A. Yager, and G. M. Baer.** 1973. A rapid tissue culture test for determining rabies neutralizing antibody, p. 354–357. *In* M. M. Kaplan and H. Koprowski (ed.), Laboratory techniques in rabies. World Health Organization, Geneva, Switzerland.
58. **Tordo, N., H. Bourhy, and D. Sacramento.** 1995. PCR technology for lyssavirus diagnosis, p. 125–145. *In* J. P. Clewley (ed.), The polymerase chain reaction (PCR) for human viral diagnosis. CRC Press, Inc., Boca Raton, Fla.
59. **Tordo, N., O. Poch, A. Ermine, and G. Keith.** 1986. Primary structure of leader RNA and nucleoprotein genes of the rabies genome: segmented homology with VSV. Nucleic Acids Res. **14:**2671–2683.
60. **Walsh, P. S., D. A. Metzger, and R. Higuchi.** 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques **10:**506–513.
61. **Warrel, M. J., and D. A. Warrel.** 1995. Rhabdovirus infections of humans, p. 343–383. *In* J. S. Porterfield (ed.), Exotic viral infections. Chapman & Hall Medical, London, United Kingdom.
62. **Warrell, M. J., S. Looareesuwan, S. Manatsathit, N. J. White, P. Phuapradit, A. Vejjajiva, C. H. Hoke, D. S. Burke, and D. A. Warrell.** 1988. Rapid diagnosis of rabies and post-vaccinal encephalitides. Clin. Exp. Immunol. **71:** 229–234.
63. **Wilde, H., S. Chutivongse, and W. Tepsumethanon.** 1991. Rabies in Thailand: 1990. Rev. Infect. Dis. **13:**644–652.
64. **World Health Organization.** 1992. WHO Expert Committee on Rabies, eighth report. Technical report series. World Health Organization, Geneva, Switzerland.