LABORATORY INVESTIGATION OF HUMAN DEATHS FROM VAMPIRE BAT RABIES IN PERU


Abstract. In the spring of 1996, multiple cases of an acute febrile illness resulting in several deaths in remote locations in Peru were reported to the Centers for Disease Control and Prevention (CDC). The clinical syndromes for these cases included dysphagia and encephalitis. Because bat bites were a common occurrence in the affected areas, the initial clinical diagnosis was rabies. However, rabies was discounted primarily because of reported patient recovery. Samples of brain tissue from two of the fatal cases were received at CDC for laboratory confirmation of the rabies diagnosis. An extensive array of tests on the formalin-fixed tissues confirmed the presence of both rabies viral antigen and nucleic acid. The virus was shown to be most closely related to a vampire bat rabies isolate. These results indicate the importance of maintaining rabies in the differential diagnosis of acute febrile encephalitis, particularly in areas where exposure to vampire bats may occur.

The earliest record of vampire bat attacks in Peru is by the French explorer Carlos Maria de la Condamine. In 1743, while traveling in Peru, Condamine recorded his observation of vampire bat attacks on cattle. His description of that event is included in the presentation of his travels to the French Academy of Science on November 7, 1745. Since then, several reports of both human and bovine rabies outbreaks have been recorded. In 1992, Lopez and others reported an outbreak of human rabies in the Peruvian jungle that resulted in 29 fatalities. Most of the victims had a history of bat bite. Epidemiologic and laboratory investigation of this outbreak indicated that the rabies virus involved was of vampire bat origin. The report of this outbreak provided the most extensive description of vampire bat rabies in humans, as well as some historical context for the outbreak. Laboratory examination of samples from one of the fatal cases resulted in virus isolation and monoclonal antibody (MAB) typing. The laboratory findings confirmed the virus to be of vampire bat origin. In April 1996, outbreaks of an acute febrile illness resulting in at least nine deaths in two remote villages in the Amazon region of Peru were reported to the Centers for Disease Control and Prevention (CDC). Because the clinical syndromes for these cases included dysphagia and encephalitis, and because bat bites were a common occurrence in the affected areas, the initial clinical diagnosis of these fatalities was rabies. However, rabies was discounted primarily because of reported patient recovery. Samples from two of the fatal cases in these outbreaks were received at CDC for laboratory studies.

Until recently, laboratory investigation of rabies required non-fixed material (either fresh or frozen), which was particularly difficult to acquire from remote locations. Although techniques for confirming the presence of rabies antigen in formalin-fixed (FF) tissues were described many years ago, these procedures had never been optimized for diagnostic purposes. By the time the Peruvian outbreak samples were received, rapid and reliable laboratory procedures for the detection of both rabies antigen and nucleic acid in FF tissues had been developed. When the FF tissue analysis was completed, a sample of frozen brain tissue from one of the two fatal cases became available and allowed direct confirmation of the laboratory results obtained with the FF tissue. All of these tests indicated the presence of rabies virus in these cases.

SUBJECTS, MATERIALS, AND METHODS

Patient tissues. Formalin-fixed brain tissues from two of the fatal cases of an acute febrile encephalitis were received at CDC from Peru for pathologic evaluation. A sample of frozen brain tissue was available from one of these two cases. Serum specimens from 52 survivors and contacts were also received.

Histopathology. Routine hematoxylin and eosin-stained sections were examined for histopathology. Some of the tissues were deparaffinized and prepared for examination by electron microscopy (EM).

Direct fluorescent-antibody (DFA) detection of rabies antigen in FF tissue. Sections of FF brain were processed and examined for rabies antigen by using a polyclonal antirabies antibody conjugated with fluorescein as previously described.

Immunohistochemical (IHC) detection of rabies antigen in FF tissue. Sections of FF brain were processed and examined for rabies antigen by using both anti-rabies monoclonal and polyclonal antibodies. Detection required a secondary reaction with either Strep-A protein or goat anti-mouse antibody conjugated with alkaline phosphatase.

In situ detection of rabies mRNA in FF tissue. Sections of FF brain were processed and examined for rabies antigen by using both anti-rabies monoclonal and polyclonal antibodies. Detection required a secondary reaction with either Strep-A protein or goat anti-mouse antibody conjugated with alkaline phosphatase.

Polymerase chain reaction (PCR) and sequencing.
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FIGURE 1. Light micrographs of brain tissue from the Peru 1996 cases. All sections are from formalin-fixed tissue of the Purkinje cell layer of the cerebellum. A, hematoxylin and eosin-stained section of a Purkinje cell containing inclusions. B, direct fluorescent antibody staining of the Purkinje cells. The rabies antigen present in the inclusions within the cells appears as green fluorescence. C, immunohistochemical assay of a comparable area, using polyclonal anti-rabies antibody to detect rabies antigen. The rabies antigen appears as a red precipitate. D, in situ detection of mRNA of the rabies L gene, using a digoxigenin-labeled RNA probe. The rabies RNA appears as a red precipitate. A, C, and D were photographed using a 100× oil-immersion objective (original magnification × 250), and B was photographed using a 40× oil-immersion objective (original magnification × 100).

Sections of FF brain were used as a source of template RNA for reverse transcription (RT) and PCR amplification of rabies nucleic acid. The procedure was based on that presented by Stanta and Schneider and optimized by Koopmans and others. The sequences of the PCR primers used for amplification, 5′-TCATGATGAATGGAGG-3′ (upstream) and 5′-GAGTCACCTCGAGTATGTT-3′ (downstream), are within the N gene. From previous work, these primers were known to amplify rabies of vampire bat origin. The amplified product was used as template for sequencing and yielded approximately 120 nucleotides sequence corresponding to nucleotide numbers 1296 to 1419 on the Pasteur virus strain of rabies virus. This sequence corresponds in part to a region in the rabies N gene that has been used in sequence typing of rabies virus. The RNA was extracted from frozen brain tissue and used as template for the RT-PCR amplification of rabies nucleic acid as previously described.

Monoclonal antibody characterization of rabies in frozen tissue. A panel of diagnostic MAbs was used to characterize the rabies virus in touch impressions made from frozen brain tissue as previously described.

Dendrogram and sequence similarity. The sequence obtained from the FF tissue sample was compared with other pertinent rabies sequences by using the software program Pileup contained in the GCG sequence analysis package (Genetics Computer Group, Madison, WI).

Serology. Each of the 52 serum samples received in this investigation was tested for rabies neutralizing antibody.

Animal studies. Four to six-week-old female ICR mice were inoculated intracerebrally (IC) with 30 μl of a 10% suspension of the frozen brain. The animal experiment included in this study was completed in accordance with a protocol reviewed and approved by the Institutional Animal Care Use Committee of CDC.

RESULTS

Histopathology. The tissues showed mononuclear perivascular cuffing and microglial hyperplasia consistent with viral infection. Hyaline eosinophilic cytoplasmic inclusions characteristic of rabies were observed in both Purkinje cells of the cerebellum (Figure 1A) and pyramidal cells of the hippocampus. Examination of the cytoplasmic inclusions in the tissues by EM revealed the presence of rhabdovirus particles (Figure 2).

Detection of rabies antigen in FF tissue. Rabies antigen
FIGURE 2. Electron micrographs of rabies virus inclusions in Purkinje cells of the cerebellum. Within the granular matrix are darkly staining filaments. Bullet-shaped particles are seen in longitudinal (arrows) and transverse (arrowhead) sections. The regions indicated by the arrows in C are shown in A and B at higher magnification (bars: A and B = 100 nm, C = 1 μm).

was detected in FF tissues from both cases by DFA analysis. A photomicrograph of the DFA detection of rabies in Purkinje cells of one of the cases is shown in Figure 1B. These cells contained multiple inclusions as visualized using a polyclonal antibody conjugated with fluorescein. Figure 1C shows Purkinje cells from the same case containing multiple inclusions as visualized using IHC to detect antigen. Interestingly, IHC analysis using the rabies anti-nucleocapsid MAb (802-2), which has been so effective in the detection of many strains of rabies in FF tissues, did not detect this vampire bat virus. In contrast, the anti-glycoprotein MAb (2-1-7), which has detected all of the rabies strains tested thus far, reacted with this vampire bat rabies antigen.

Detection of rabies nucleic acid by in situ hybridization. The results of an in situ detection using a probe that hybridizes to rabies L gene mRNA are shown in Figure 1D. The Purkinje cells have multiple inclusions containing rabies mRNA as visualized with this procedure. The probe that detects the M gene mRNA gave similar results.

Detection of rabies nucleic acid tissue by PCR and sequencing. Sections of the paraffin-embedded FF tissue were used to extract RNA template for RT-PCR amplification of a region of the N gene. The PCR products of the expected molecular weight were obtained from both cases. The amplified DNA fragment from one case yielded approximately 120 nucleotides of sequence. In Figure 3, the sequence obtained from this case is compared with the same sequence from other pertinent rabies isolates. Even this limited sequence is adequate to allow typing of this strain of rabies. The nucleotide sequences that are most similar to the sequence from this case are from human cases of rabies that occurred in Madre de Dios, Peru in 1989. The human case that occurred in Mamayaque, Peru in 1990 is slightly different. The other isolates that share the greatest similarity to
FIGURE 3. Comparison of the sequences of 12 rabies samples along 124 nucleotides within the N gene. All of the sequences in this comparison were obtained from fresh material except that from the present case, which was obtained from formalin-fixed (FF) brain tissue. The first two samples are from dog rabies isolates collected in Mexico$^{12}$ and Venezuela$^{26}$ and are comparable with dog rabies samples from Peru. PV is the Pasteur Virus strain (GenBank M13215). The ft bt isolates are from insectivorous, free-tail bats ($Tadarida rotundus$) from Argentina and Chile, which are found seasonally in Peru. The vm bt isolates are from vampire bats ($Desmodus rotundus$) from Argentina and Brazil. The human (Peru, 1989) samples are from the Madre de Dios outbreak of rabies. The next sample (Peru, 1996) is the sequence obtained from the FF tissue in the present study. The next isolate (vm bt) is from a vampire bat from Venezuela. The final isolate is from the human case reported in Lancet in 1992.$^{3}$ The 124 nucleotides shown constitute the total sequence information used for each strain to create the dendrogram shown in Figure 4.

FIGURE 4. A dendrogram comparing the rabies sequences shown in Figure 3. This dendrogram was created using the Pileup program from GCG software. For additional information about the samples included, see the legend to Figure 3.

the sequence found in this case are from vampire bats from Argentina, Brazil, and Venezuela. In this region of the nucleotide sequence of rabies virus, the free-tail bat samples, the canine samples, and the vaccine strain are all quite different. Another way of comparing this sequence data is shown in the dendrogram in Figure 4. This sequence provided the first direct identification of the strain of rabies in these cases.

Extraction of RNA from the frozen brain tissue provided a source of template for RT and PCR amplification of the entire N gene sequence. The sequence of the entire N gene confirmed the near identity of the rabies virus present in the frozen brain tissue with that of other vampire rabies samples from Peru. This sequence also confirmed the sequence obtained from the FF tissue. The sequence has been assigned the GenBank accession number AF045166. Two of the rabies N gene sequences that were useful in the identification of this isolate of rabies have also been submitted to GenBank. The rabies N gene sequences from a vampire bat from Brazil and from a free-tail bat from Chile have been assigned GenBank accession numbers AF07049 and AF07050, respectively.

Characterization of the rabies MAb reactivity of the frozen tissue sample. The MAb reactivity pattern of the rabies virus from the frozen tissue sample is shown in Figure 5. The pattern is identical with that of the 1990 Peruvian vampire bat case.$^{3}$ The pattern of reactivity is distinct from both that of dog rabies present in Peru and insectivorous free-tail bats, whose migratory range extends into Peru.$^{13}$

SeroLOGY. None of the 52 serum samples from survivors and contacts of the two fatal rabies cases tested positive for rabies neutralizing antibody.

Animal studies. The mice that were inoculated IC with the frozen brain tissue from the fatal human rabies case developed typical signs of acute encephalitis within three weeks of inoculation.

DISCUSSION

For many years the clinical phenomenon of acute ascending myelitis had been observed in various locations throughout the Caribbean and South America.$^{16-18}$ In 1931, Hurst and Pawan described an outbreak of rabies in Trinidad “... consisting solely of cases of the paralytic type”.$^{19}$ Although paralytic rabies was well known at that time in both humans and animals, the outbreak in Trinidad was of special interest because only paralytic rabies was involved. In 1932, Hurst and Pawan presented extensive laboratory work that confirmed that the agent responsible for the “acute ascending myelitis outbreak” in Trinidad was rabies virus.$^{16}$ Eventually, the source of this rabies outbreak was shown to be vampire bats.

In 1936, Pawan described extensive experimental investigations of rabies in vampire bats.$^{20}$ He meticulously documented experimental infection and disease progression in a series of animal experiments. Although he lacked contem-
pore laboratory diagnostic procedures, he clearly demonstrated that vampire bats infected with rabies (both naturally and experimentally) could exist for months without showing signs of illness. He also showed that such apparently healthy vampire bats could cause rabies in animals they attacked and that the saliva of such apparently healthy bats could cause rabies in rabbits. More recent characterization of disease resulting from infection by vampire bat rabies has been presented by Fekadu.

In 1936, Pawan also described outbreaks of rabies in Trinidad that had killed thousands of cattle and 89 humans. All of these deaths resulted from the transmission of rabies virus by the common vampire bat, *Desmodus rotundus*. After Pawan’s documentation of the transmission of rabies virus by vampire bats, the habits of these animals received more attention. In 1989, McCarthy documented the temporary increase in vampire bat feeding on humans after the disruption of their preferred food source. In 1996, Caraballo described an increase in vampire bat predation upon humans in Payapal, Venezuela. In this situation, the disruption of the bats’ preferred food source resulted from gold mining activity in the area. No cases of rabies were reported from either of these locations. In Payapal, all bite victims received rabies postexposure treatment. Extensive disruption of vampire bat habitat in Latin America led Schneider and others to propose a method for predicting the impact this activity will have on the transmission of vampire bat rabies to humans.

The results presented here provide detailed laboratory confirmation of rabies infection in two of the fatal human cases from the Peruvian outbreaks in the spring of 1996. The FF samples from each case contained both rabies antigen and nucleic acid. The limited rabies sequence information obtained from the FF tissue RT-PCR analysis was sufficient to characterize the virus present as being from vampire bat. Detection *in situ* allowed pathologic examination of rabies antigen and nucleic acid directly in FF brain tissue. These results demonstrate the diagnostic possibilities now available for rabies virus detection and characterization in FF tissues. Although each of these individual tests has been used previously, this is the first time they have been used in concert to identify and characterize rabies virus in FF human tissues. The availability of frozen brain tissue from one of the two cases provided an ideal opportunity to confirm the results obtained from the FF samples.

The availability of accurate diagnosis of rabies in FF tissue opens some interesting questions for study. Some of the more intriguing cases of human rabies occur in very remote locations. Obtaining fresh or frozen samples adequate for laboratory confirmation of the clinical diagnosis has been challenging. Now that FF tissue can be used for laboratory confirmation of rabies, samples for testing will be easier to obtain. The samples will not be infectious and sample shipment will be simplified. Although the techniques that provide information about rabies in the FF samples require both trained personnel and a well-equipped laboratory, the samples themselves are considerably easier to handle.

Reports of human disease outbreaks and death from regions inhabited by vampire bats will undoubtedly continue. One of the intriguing aspects of the report received in the spring of 1996 was that there were survivors of the outbreak. Although similar reports have been received on other occasions, they have not been followed up by either epidemiologic or laboratory investigations. Several clinical aspects of these outbreaks indicated rabies. Unfortunately, the clinical signs associated with rabies (fever, dysphagia, pharyngitis, and even encephalitis) are associated with many other ill-
nesses. Reports of survivors of these outbreaks confound the diagnosis of rabies. Laboratory confirmation of the rabies diagnosis in those who do not survive is essential. Procedures now available to identify and characterize rabies in FF tissue ensure the accurate diagnosis of rabies from such cases.

Acknowledgments: We thank Douglas Watts and the Peruvian Ministry of Health (Lima Peru) for assistance in obtaining the samples in this case.

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