Screening of Active Lyssavirus Infection in Wild Bat Populations by Viral RNA Detection on Oropharyngeal Swabs

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Brain analysis cannot be used for the investigation of active lyssavirus infection in healthy bats because most bat species are protected by conservation directives. Consequently, serology remains the only tool for performing virological studies on natural bat populations; however, the presence of antibodies merely reflects past exposure to the virus and is not a valid marker of active infection. This work describes a new nested reverse transcription (RT)-PCR technique specifically designed for the detection of the European bat virus 1 on oropharyngeal swabs obtained from bats but also able to amplify RNA from the remaining rabies-related lyssaviruses in brain samples. The technique was successfully used for surveillance of a serotine bat (*Eptesicus serotinus*) colony involved in a case of human exposure, in which 15 out of 71 oropharyngeal swabs were positive. Lyssavirus infection was detected on 13 oropharyngeal swabs but in only 5 brains out of the 34 animals from which simultaneous brain and oropharyngeal samples had been taken. The lyssavirus involved could be rapidly identified by automatic sequencing of the RT-PCR products obtained from 14 brains and three bat oropharyngeal swabs. In conclusion, RT-PCR using oropharyngeal swabs will permit screening of wild bat populations for active lyssavirus infection, for research or epidemiological purposes, in line not only with conservation policies but also in a more efficient manner than classical detection techniques used on the brain.

Rabies is caused by different rhabdoviruses included within the genus *Lyssavirus*. Land mammals are infected worldwide by the classical rabies virus (RABV) or serotype 1, as well as bats in America (11). This virus causes nearly all the human cases of rabies in the world. A few African land mammals have been found to be infected by a different lyssavirus, the Mokola virus (MOKV) (serotype 3) (25). The hosts for the remaining lyssaviruses are non-American bats: Lagos bat virus (LBV) (serotype 2) (25) and Duvenhage virus (DUVV) (serotype 4) (29) in Africa, the Australian bat virus (ABV) (proposed as genotype 7) in Australia (12), the European bat virus type 1 (EBV1), and European bat virus type 2 (EBV2) (16) in Europe. Both European bat viruses were formerly classified together as DUVV; however, they have recently been divided into two different species (3, 10, 16, 19). Phylogenetic reconstructions show closer relationships between EBV1 and Duvenhage virus than between EBV1 and EBV2. In addition, EBV2 shows a closer relationship with serotype 1 than with EBV1 (4). Two different subgenotypes have recently been described for each (1). The reservoir of EBV1 is the serotine bat (*Eptesicus serotinus* [Vespertilionidae]), while EBV2 is found so far in the species of the genus *Myotis* (Vespertilionidae), *Myotis dasycneme* and *Myotis daubentonii* (1). Other European bat species have rarely been found infected by these viruses (6). More than 500 infected bats have been found in Europe since the first case was found in 1957 (20), and more than 95% of the bats correctly identified were serotines, presumably infected by EBV1 (20). However, the first human case was caused by EBV2 in 1985 (17, 19).

The remaining two known cases were caused by EBV1, in Ukraine in 1977 and Russia in 1985 (19, 24). All three human cases were fatal. Hundreds of Europeans were exposed to rabid bats after 1985 (20), but all received postexposure prophylaxis and none were infected.

A rapid diagnosis of lyssavirus infection should be made on the animal brain after any human exposure. Direct antigen detection by immunofluorescence (IF) is the most widespread screening method (18). Results are usually confirmed by the mouse inoculation test or by viral isolation on murine neuroblastoma cells (18). More recently, RNA detection by reverse transcription (RT)-PCR has been proposed as a rapid and sensitive alternative (13, 14, 15, 21, 23, 26, 31). However, as RT-PCR is not faster than IF, and rabid animal brains usually contain high amounts of virus, very few laboratories have adopted this technique. On the other hand, direct sequencing of RT-PCR products is the most common technique for virus identification and in molecular epidemiology studies (1, 5, 27).

Most European bat species are protected (8) and the brain cannot be used for the screening of active lyssavirus infection in natural bat populations. For this reason, most studies are based exclusively on serology (22, 28); however, total antibody presence merely reflects past exposure to the virus and does not demonstrate active infection. Moreover, conclusive identification of the lyssavirus involved, based on serological techniques, is not possible due to cross-reactivity.

In the present work, a new PCR method is described for detection of RNA from all known rabies lyssaviruses, with further virus identification by genomic sequencing. The presence of viral RNA on bat oropharyngeal swabs as a marker of active lyssavirus infection is evaluated in a bat colony involved in a case of human exposure.

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LISEBL1F									
5 AAGATGTGTGCCAACTGGAG3									
RABV	A				T		T		
LBV	A				A		T		
MOKV			C		T				
DUHV	A				G		T		
EBV1a									
EBV1b									
EBV2a			C						
EBV2b			C						

5 TACTGCTTATGAGGATTGTTTC3									
RABV	A				C				
LBV	A				C				
MOKV	G	A	C	C					
DUHV	C		A		A				
EBV1a									
EBV1b									
EBV2a	G		A		A				
EBV2b	G				A				

TACAAACTCGGTCCCGTTCT5									
ATGTTTGAGCCAGGGCAAGA3									
		C					G		
			A		T	T			
					G				
					C	A		G	
					G			G	
		C			G				

TTCCTTGAAGCTCCTTCTCTAG5									
AAGAACTTCGAGGGAAGAGATC3									
					T				A
					T	A			A
		A	G		T	A	GG		A
					T		G		
					T				
					T	A			

FIG. 1. Primer sequences and mismatches with the different rabies-related lyssaviruses: RABV (genotype 1), LBV (genotype 2), MOKV (genotype 3), DUVV (DVHV) (genotype 4), EBV1a (subtype a, genotype 5), EBV1b (subtype b, genotype 5), EBV2a (subtype a, genotype 6), EBV2b (subtype b, genotype 6). Sequences of primers LISEBL1F, LISEBL1R, LISEBL2F, and LISEBL2R are shown.

MATERIALS AND METHODS

Samples. Group 1 consisted of 12 RNA extracts from all seven rabies-related lyssaviruses distributed as follows: One RABV (CVS strain), two LBV, three MOKV, one DUVV, two EBV1, two EBV2, and one ABV. All were kindly donated by J. Smith from the Centers for Disease Control and Prevention (Atlanta, Ga.). Group 2 included 47 brains from different mammals (25 bats, 15 dogs, six cats, and one horse) from the records center of the Centro Nacional de Microbiología (Majadahonda, Madrid, Spain). Nineteen brains had previously had positive results for rabies antigen detection by IF, and positive confirmation, either using the mouse inoculation test or by virus isolation in murine neuroblastoma cells (30). Fourteen brains (from nine dogs, four cats, and one horse) came from the North African Spanish cities of Ceuta and Melilla, while the other five were from bats from the southern Iberian Peninsula. The remaining 28 brains had previously had IF-negative results. Finally, group 3 consisted of 71 oropharyngeal swabs and 39 brains from 69 different serotine bats (*E. serotinus*) captured or found dead between June 1999 and August 2000 and belonging to the same colony in a public building in Seville (Andalusia, Spain). Six bats were captured twice during follow-up. This colony was studied after a human being was bitten by a bat which tested positive for rabies antigen by IF and viral isolation from the brain. The patient received adequate postexposure immunoprophylaxis and remains symptom free at present. Another 37 samples (one brain and 36 oropharyngeal swabs) from 36 bats captured or found dead in other areas of Seville were also included for comparison.

Collection of oropharyngeal samples. Bats were mist-netted at the exits used by the animals for leaving the building for night feeding. After capture, each animal was aged, sexed, measured, weighed, and ring identified. Oropharyngeal samples were taken with dry cotton swabs stored in tubes containing 1.5 ml of lysis buffer (see below) for transport and daytime storage. On arrival at the laboratory, swabs were applied tightly to the tube walls and the liquids were divided into two different aliquots which were frozen to -80°C until analysis. Each swab was stored with one of the aliquots. Bats were usually released after sampling.

Immunofluorescence. This was performed following standard procedures (9). Ammon's horn and cerebellum were examined for land mammals, while longitudinal sections of the entire encephalon were used for bats. At least two

impressions from different areas were observed before giving a negative result. A commercial RABV-derived fluorescent conjugate (Sanofi-Pasteur, Marnes la Coquette, France) was used for immunologic staining. After 1999, a pan-lyssavirus-specific monoclonal antibody, kindly donated by J. Cox from the WHO Collaborating Center for Rabies Surveillance and Research (Tübingen, Germany), was used in addition for bat specimens.

RNA extraction. Brain samples were homogenized with sterilized glass grinders and resuspended in minimal essential medium. RNA was extracted from the samples as described previously (7). Briefly, 50 μl of each suspension was treated with 200 μl of a guanidinium thiocyanate extraction buffer, followed by isopropanol and 70% ethanol precipitations. An RNA plasmid, supplied as positive control in the Access RT-PCR kit (Promega, Madison, Wis.), was included in the extraction buffer as part of an internal control system (see below) at a concentration of 20 molecules/ μl . Pellets resulting from the final centrifugation were resuspended in 10 μl of distilled water and used immediately. For oropharyngeal swabs, 500 μl of sample was directly treated with 500 μl of isopropanol, continuing the procedure as before.

Primer design and preparation. Sequences of the nucleoprotein gene of each rabies-related lyssavirus were obtained from genomic databases and aligned by using the Macaw program (National Center for Biotechnology Information, Bethesda, Md.). External and nested primer sequences were chosen from regions conserved among all rabies-related lyssaviruses; however, nucleotides matching with EBV1 were selected in variable positions (Fig. 1). For the internal control system, the 1UPS and 1DS primers supplied in the Access RT-PCR kit (Promega) as a part of the positive control system were used as nested primers. External primers were chosen from the plasmid sequence supplied in the kit insert (CONINT1F, 5' CTGGCCTGTTGAACAAGTCT 3'; CONINT1R, 5' GATCTGATCCTTCAACTCAGC 3'). Primer synthesis was undertaken by a commercial customer service (Pharmacia Biotech, Freiburg, Germany).

Reverse transcription, amplification, and product detection. Single-step reverse transcription and primary amplification were performed using the Access RT-PCR kit (Promega). Five microliters of extracted sample was added to an RT-PCR mixture containing 10 μl of $5\times$ reaction buffer; 3 mM magnesium sulfate; dATP, dCTP, dGTP, and dTTP, each at a concentration of 500 μM ; LISEBV1F, LISEBV1R, CONINT1F, and CONINT1R primers, each at a concentration of

FIG. 2. RT-PCR results for bat samples. The upper band (323 bp) is the internal control band. The lower band (117 bp) is the lyssavirus-specific band. Lanes 1, 2, 4 to 9, and 11 are lyssavirus negative, lane 10 is lyssavirus positive, lane 3 has presence of enzyme inhibitors, and lanes 12 and 13 are negative and positive controls (with no internal control).

0.2 μ M; 5 U of avian myeloblastosis virus reverse transcriptase; 5 U of *Thermus flavus* DNA polymerase; and RNase-free distilled water to a final volume of 50 μ l. All reagents except primers were supplied in the kit. Amplification was performed in an Autocycler plus (Linus, Cultek, Madrid, Spain) thermal cycler, programmed for a first retrotranscription step of 45 min at 48°C, followed by two min at 94°C for reverse transcriptase inhibition and cDNA denaturation, and 30 repetitive cycles of 1 min of denaturation at 93°C, 1 min of annealing at 60°C, and 1 min of elongation at 72°C. Elongation was extended for 5 additional min in the last cycle. For nested PCR, 1 μ l of the primary amplification products was added to a new PCR mixture containing 5 μ l of magnesium-free 10 $\times$ reaction buffer (Roche Diagnostics GmbH); 3 mM magnesium chloride; dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech), each at a concentration of 500 μ M; LISEBV2F and LISEBV2R primers, each at a concentration of 0.5 μ M; 1 UPS and 1DS primers, each at a concentration of 0.2 μ M; 1.25 U of *Ampli-Taq Thermus aquaticus* DNA polymerase (Roche Diagnostics GmbH); and distilled water to a final volume of 50 μ l. Thermal cycles were performed as before but skipping the retrotranscription step and using 94°C for denaturation and 50°C for annealing. The PCR products were sized by gel electrophoresis in 2% agarose containing 0.5 g of ethidium bromide per ml of TBE (Tris-borate-EDTA) buffer and seen under UV light. Standard precautions were taken to avoid carryover contamination. Pipetting was performed with aerosol-resistant tips, and different biosafety cabinets were used for master mix preparation, sample and extract handling, and nested reaction. Product detection was undertaken in a different area. Samples showing both the 323-bp internal control band and the 117-bp lyssavirus-specific band were considered positive; those showing only the internal control band were considered negative; those showing no band were tested again and considered to contain enzyme inhibitors, if no band was observed on repetition (Fig. 2). All samples showing positive results were tested again. In the case of oropharyngeal exudates, these repetitions were made from a different aliquot. Only samples with repetitive results were finally considered positive. Concentrations of magnesium, deoxyribonucleotides, and primers were optimized for both reactions, as well as denaturation and annealing temperatures.

Sequencing. First-amplification 262-bp bands were sequenced for lyssavirus species (genotype) identification. First-amplification products were mixed with an equal volume of ammonium acetate and precipitated, first with isopropanol and then with 70% ethanol. Final pellets were resuspended in 10 μ l of distilled water. The sequencing reaction was performed with the ABI PRISM big dye sequencing kit (Applied Biosystems, Foster City, Calif.), following the manufacturer's indications. Both forward and reverse strings were sequenced using LISEBV1F and LISEBV1R as sequencing primers, respectively. Sequencing reactions were performed in a PTC200 (MJ Research, Watertown, Mass.) thermal cycler and consisted of a first-denaturation cycle of 3 min followed by 25 cycles of 10 s of denaturation at 96°C, 10 s of annealing at 50°C, and 4 min of elongation at 60°C. Products were purified by subsequent 80 and 70% ethanol precipitations. Final products were run on an ABI PRISM 377 DNA sequencer (Applied Biosystems). Forward and reverse strings were fitted using the Seqman program of the DNASTAR package (DNASTAR INC, Madison, Wis.). Some

FIG. 3. Results of the PCR test on all rabies-related lyssaviruses. Lanes 1 and 2, LBV; lanes 3 to 5, MOKV; lane 6, DUVV; lanes 7 and 11, EBV1; lanes 8 and 9, EBV2; lane 10, ABV; lane 12, RABV (CVS strain); lane 13, negative control; lane 14, molecular size marker (123 bp; Life Technologies, Gaithersburg, Md.). No internal control was included in this assay.

land mammal brains did not show visible first-amplification bands, and none of the bat oropharyngeal swabs showed these, despite their being positive after nested reaction. Reverse transcription-first amplification reaction was repeated as before on land mammal samples, but using new primers with RABV instead of EBV1 specific nucleotides on variable positions. This reaction was also repeated on bat oropharyngeal swabs, but using other primers (SEQ1F, 5' AAG ATTGTRGAACACCACAC; SEQ1R 5' GCATTGGATGAATAAGGAGA) external to LISEBV1F and LISEBV1R. The nested reaction was then performed as before but using LISEBL1F and LISEBL1R instead of LISEBV2F and LISEBV2R. Sequencing was performed as above, when visible 262 bp bands were obtained.

The readable 220-bp fragments obtained from the automatic sequencer were aligned as above, together with representative strains of all rabies-related lyssaviruses obtained from genomic databases. One sequence from each of French RABV (genotype 1), Moroccan RABV (genotype 1), LBV (genotype 2), MOKV (genotype 3), DUVV (genotype 4), EBV1a (genotype 5), EBV1b (genotype 5), EBV2a (genotype 6), and EBV2b (genotype 6) was included for comparison.

RESULTS

RNA extracts from the different lyssaviruses (group 1). PCR products of the expected size were obtained for all seven rabies-related lyssaviruses (Fig. 3). First-amplification bands were apparent for all samples except for the LBV in lane 1, the DUVV in lane 6, and the ABV in lane 10.

Brain samples from animal rabies diagnosis (group 2). RT-PCR results were totally coincident with previous results. All IF-negative samples were RT-PCR negative and all IF-positive brains were RT-PCR positive.

Bat samples from a bat colony involved in human exposure to EBV1 (group 3) (Table 1). After the human exposure case in June 1999, 27 additional animals were captured between June and August, and all tested negative. However, one bat found moribund in September tested positive in both the brain and on the oropharyngeal swab, as did the brain from another moribund bat found in another part of the city some days later. This last animal was the only one that showed positive results for both antigen detection and RT-PCR using the brain, despite being negative on the oropharyngeal swab. As bats moved to hibernation shelters, no more captures could be made in 1999. Oropharyngeal swabs from 4 of 12 additional bats captured on 21 May 2000 tested positive by RT-PCR. Two of these RT-PCR-positive animals died during capture. Both brains

TABLE 1. Distribution over time of RT-PCR results obtained after follow-up of a serotine bat colony from a public building after a case of human exposure to a rabid bat from this colony^c

Specimen	Result	No. of samples with result at time point									Total
		June 1999	July 1999	Aug 1999	Sept 1999	Dec 1999	May 2000	June 2000	July 2000	Aug 2000	
Brain	Pos.	1 ^a	0	0	1 ^b (1)	0	2 ^c	1	0	0	5 (1)
	Neg.	4 ^b	2 ^b	0	1	1 ^b	0	19	7	0	34
	NA	0	4	17	0	0	10 (13)	0	(22)	5	36 (13)
Oropharyngeal exudates	Pos.	0	0	0	1	0	4	10	0	0	15
	Neg.	1	6	17	1 (1)	1	8 (13)	10	7 (22)	5	56 (36)
	NA	4	0	0	0	0	0	0	0	0	4
Total captures		5	6	17	2 (1)	1	12 (13)	20 ^d	7 ^d (22)	5	75 (36)

^a Bat causing human exposure.^b Bat found dead on the floor of the building.^c Death during capture and sampling.^d Sacrificed after sampling.^e Results in parentheses refer to bats captured in locations other than the public building. NA, not available; Pos., positive; Neg., negative; Aug, August; Sept, September; Dec, December.

were RT-PCR positive, and one of them was also IF positive. Thirteen oropharyngeal swabs from other bats captured the same week in another part of the city showed no virus. As the risk for the human population was considered high, health and conservation authorities agreed to remove the bat colony from the public building. Twenty bats were captured and slaughtered after being sampled in June 2000. Ten of these tested positive for RT-PCR on oropharyngeal swabs, but only one tested positive in the brain. This brain was highly positive using IF. The only bat known to be positive but still alive after the May 2000 campaign was captured again in June. It remained positive on the oropharyngeal swab, although it tested negative in the brain. The last 12 bats captured in July and August 2000 tested negative, as well as 22 additional bats captured at another location very close to the public building. PCR products from six samples (three brains and three oropharyngeal swabs) taken at different times were sequenced for lyssavirus identification (see below). All were classified as EBV1 (genotype 5).

To summarize, only 5 (15%) of the 33 brains from bats with simultaneous oropharyngeal sample were RT-PCR positive. In contrast, virus was detected in 13 (39%) oropharyngeal exudates (Table 2).

Lyssavirus identification (genotyping). All lyssavirus strains from land mammals were classified as RABV (genotype 1) (Table 3). In contrast, all bat-derived strains were classified as EBV1 (genotype 5). Homologies between the same genotype ranged from 93.6 to 100% for RABV and from 89.5 to 99.5% for EBV1. These values ranged from 68.6 to 77.7% for

RABV1, and from 61.8 to 80.5% for EBV1, compared with the other lyssaviruses. Thus, every strain could be easily assigned to one lyssavirus species according to nucleotide homology.

DISCUSSION

The RT-PCR method described here is able to detect all rabies-related lyssaviruses. Only five of all previous PCR-based methods for rabies diagnosis have a similarly wide range of specificity (2, 13, 14, 26, 31), and only two of these provide virus identification by product sequencing (13, 14). As the only bat lyssavirus known so far in Spain is EBV1, the primers described here were optimized for the detection of this particular virus, and sensitivity to the other lyssaviruses may be suboptimal. In fact, no band was obtained after first amplification for some RABV-infected brains, despite their highly positive IF images. Variable positions should be degenerated, or mixtures of individual virus-specific primers should be used, to achieve a better pan-lyssavirus amplification method. Primers were chosen from conserved positions to ensure the detection of all individual EBV1 variants.

An internal control system was included to avoid false-negative results in each individual tube, due to handling errors, or the presence of enzyme inhibitors. In our sampling design, the RNA plasmid was included in the extraction buffer, and this was used as a transport medium for the oropharyngeal swabs. Therefore, the internal control system used here made it possible to monitor the whole process from as early as sample collection. Only one of the previous methods includes an internal control system (26). However, in this study rRNA was used as a target instead of the controlled low number of plasmid molecules used here. As the amount of rRNA is expected to be high in clinical samples, low-grade RNA losses or enzyme inactivation could be missed and false negative results could be shown.

Both RT-PCR and antigen detection using IF showed the same rate of efficiency for detection of lyssaviruses in well-preserved animal brains. However, as the most-widespread commercial antisera are derived from RABV, reactivity with other rabies-related lyssaviruses is usually poorer. In fact, one

TABLE 2. Results obtained with bats for which simultaneous brain and oropharyngeal samples were obtained^a

Result for brain	No. with result for oropharyngeal exudate		Total no.
	Pos	Neg	
Pos	4	1	5
Neg	9	19	28
Total	13	20	33

^a Abbreviations: Pos, positive; Neg, negative.

TABLE 3. Homology between samples sequenced in this work and sequences of different rabies-related lyssaviruses obtained from genomic databases^a

Virus ^b	% Homology with isolate from:															
	Dog								Horse							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
RABV (France)	93.6	94.1	94.1	95	95	95	94.5	93.6	94.5	77.7	77.3	77.7	77.3	77.7	69.1	77.3
RABV (Morocco)	100	98.2	98.2	99.1	99.1	99.1	99.5	100	98.6	75.5	74.5	75.5	75	75.5	66.8	75
LBV	75	75.5	75	75	75	75	75.5	75	74.5	75	74.1	75.5	75	75	66.4	75.5
MOKV	68.6	70	70	69.5	69.5	69.5	69.1	68.6	69.5	70.5	70	70	70	70.5	61.8	70.5
DUVV	75.5	75	76.8	75.9	75.9	75.9	75.9	75.5	75.9	78.6	77.7	79.1	78.6	78.6	69.5	78.6
EBV1a	75	76.4	76.4	76.4	76.4	76.4	77.3	75	76.4	98.2	97.3	97.7	98.6	98.2	89.5	97.7
EBV1b	76.4	77.7	77.7	77.7	77.7	77.7	74.5	76.4	77.7	99.5	99.1	99.1	99.1	99.5	90.9	99.1
EBV2a	73.6	74.1	74.5	75	75	75	75.5	73.6	75	80.5	80.9	80.9	80.5	80.5	70.5	80.5
EBV2b	74	75	75.5	75.9	75.9	75.9	75.5	74.5	75.9	80	80.5	80.5	80	80	70	80

^a All strains were obtained from the brain except for those from bats 6, 7 and 8, which were amplified from oropharyngeal swabs.

^b For the following viruses, EMBL accession numbers are given in parentheses: RABV (France) (U22474), RABV (Morocco) (U22852), LBV (U22842), MOKV (U22843), DUVV (U22848) EBV1a (U22844), EBV1b (U22845), EBV2a (U22847), EBV2b (U22846).

of the lyssavirus-positive bat brains was missed after a first examination with one of these reagents. This sample showed few but clear fluorescence images when tested with a noncommercial monoclonal antibody, and lyssavirus RNA was clearly amplified by nested RT-PCR. Nevertheless, no band was observed after primary amplification, which suggests a low viral load. Other works show that RT-PCR is more efficient than antigen detection for degraded samples (13).

Most of the previous information about the lyssavirus infection in wild bat populations was obtained from serology (22, 28) and direct virus detection in the brain (28). However, brain analysis cannot be used for healthy individuals because all bats throughout the European Union are protected (8), as in many other countries. As collection of oropharyngeal exudates is harmless for bats, RNA detection for this specimen seems a valid alternative for the detection of active lyssavirus infection in wild populations. In fact, all bats with neurological infection showed virus in oropharyngeal exudates as expected, in concordance with classic patterns of rabies pathogenesis, in which brain colonization from peripheral nerves precedes centrifuge dissemination of the virus to the salivary glands (11). The only bat showing infection in the brain but not oropharyngeal excretion was the one with an apparently low amount of virus in the brain (see above). It may have been captured before centrifuge dissemination from the brain to the salivary glands. However, most of the bats with virus on oropharyngeal swab and available brain sample showed no virus in the brain. Consequently, the virus was unable to reach the salivary glands by axonal spread from the brain, and the infection in these animals did not follow the classic pattern of rabies pathogenesis. Some previous works have shown a low active infection rate, despite high RABV (28) or high EBV1 (22) antibody prevalence in healthy bat populations. Some ring-identified individuals were even captured alive years after they had tested positive for lyssavirus antibodies (22). All these data suggest that the clinical expression of the EBV1 and RABV infection in bats is usually a mild, nonfatal extraneurological disease. This could explain the greater efficiency of the oropharyngeal swab compared to the brain for detection of active EBV1 infections in bats, as shown in this study. Only animals with neurological disease can be detected by the using the brain. However, the

use of the oropharyngeal swab also allows detection of healthy carriers of EBV1, which is of major epidemiological interest.

To sum up, the RT-PCR described here is an effective complement to IF for primary diagnosis of rabies in animals, also providing rapid identification of the lyssavirus by automatic sequencing of the products. The use of this technique on bat oropharyngeal swabs, in combination with antibody detection, will permit highly efficient mass screening of wild bat populations for lyssavirus infection, as well as conforming to current conservation policies. This new approach will permit not only new basic research studies but also surveillance of bat colonies for epidemiological purposes.

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