Epidemiol. Infect. (2005), **133**, 927–934. © 2005 Cambridge University Press
doi:10.1017/S095026880500405X Printed in the United Kingdom

Detection of multiple strains of rabies virus RNA using primers designed to target Mexican vampire bat variants

E. LOZA-RUBIO[1]*, E. ROJAS-ANAYA[1], V. M. BANDA-RUÍZ[1], S. A. NADIN-DAVIS[2] AND B. CORTEZ-GARCÍA[3]

[1] *Centro Nacional de Investigaciones en Microbiología Animal, INIFAP, Carretera México Toluca Km. 15.5, Colonia Palo Alto, CP 05110, México*
[2] *Rabies Center of Expertise, Ottawa Laboratory* (*Fallowfield*), *Canadian Food Inspection Agency, Ottawa, Canada*
[3] *Centro Nacional de Servicios de Diagnóstico en Salud Animal, CENASA-SENASICA, Km. 37.5, Carretera México-Pachuca, Tecamac, Mexico*

(*Accepted 2 February 2005*)

SUMMARY

A reverse transcription–polymerase chain reaction (RT–PCR), that uses primers specifically designed to amplify a portion of the N gene of vampire bat strains of rabies that circulate in Mexico, but also recognizing most of the rabies variants circulating in endemic areas, was established. This standardized PCR assay was able to detect viral RNA in tenfold serial dilutions up to a 10^7 dilution using stock virus at an original titre of $10^{7.5}$ LD_{50}. The assay was highly specific for rabies virus. Forty different rabies isolates recovered from different species and geographical regions in the country were diagnosed as positive and negative by the fluorescent antibody test (FAT). These same samples were re-examined by both PCR and the mouse inoculation test (MIT). Compared with MIT the PCR exhibited an epidemiological sensitivity of 86% and a specificity of 91% while its positive predictive value was 96%.

INTRODUCTION

The first description of rabies dates from the 23rd century B.C. in Mesopotamia [1]. Over the centuries, this encephalitis has caused millions of human and animal deaths. Nowadays rabies is still recognized as one of the 10 main infectious diseases responsible for mortality worldwide [2]. Although the dog is still the main rabies reservoir in many Latin American countries [2], in Mexico, where extensive urban rabies control programmes have been highly effective, the main reservoir of rabies virus is the hematophagous bat (*Desmodus rotundus*) or vampire bat [3, 4]. *D. rotundus* is present in tropical and subtropical areas from Northern Mexico to Northern Argentina and Chile, and transmits the disease mainly to cattle, thereby resulting in severe economic losses. According to the National Campaign for Controlling Bovine Rabies (NCNBR), there were 1386 laboratory-confirmed cases of bovine rabies in Mexico during the years 1998–2002. However the NCNBR estimates that there are at least 10 additional cases for each one that is reported [5]. According to the Pan-American Health Organisation (PAHO) there were 271 bovine deaths in 2000 [6] in good agreement with the NCNBR data.

Using a panel of eight monoclonal antibodies, two different viral antigenic variants, AgV3 and AgV11, have been identified in Mexican vampire bats and in

* Author for correspondence: Dr E. Loza-Rubio, Centro Nacional de Investigaciones en Microbiología Animal, INIFAP, Carretera México Toluca Km. 15.5, Colonia Palo Alto, CP 05110, México. (Email: loza.elizabeth@inifap.gob.mx)

cattle and other livestock species infected by vampires [7, 8]. Partial sequence comparison of these two viral types has found that, over a 320-bp region of the nucleoprotein gene, AgV3 isolates were more genetically diverse than those of AgV11 [7]. Consideration of the sequences of both viral variants is important when designing primers for PCR detection of all vampire bat isolates.

Unfortunately the establishment of a definitive clinical diagnosis of rabies is difficult and requires the presence of cardinal signs in the encephalitic form. This requirement may not be helpful in areas without endemic canine rabies, where most cases have atypical presentations, associated with exposure to rabid bats or other wild animals. Furthermore, failure of diagnosis in more developed countries is probably related to the lack of a clear bite history, compounded by a lack of medical familiarity with typical clinical features of the disease [9]. The gold standard for rabies diagnosis is the fluorescent antibody test (FAT); however, it has been documented that the FAT may lack sensitivity when testing decomposed brain tissues, an issue which can be very common since rabies outbreaks often occur in tropical and subtropical areas [10]. The World Health Organization (WHO) recommends confirmation of FAT-negative cases by the use of the time-consuming mouse inoculation test (MIT), which requires a large number of mice per sample [9, 11]. However, tests based on polymerase chain reaction (PCR) methodology could provide an alternative to MIT; this strategy would have the advantage of avoiding utilization of large numbers of mice, and could confirm the presence of the rabies virus in a very timely manner. In this study, a PCR method that uses primers targeting conserved regions of the rabies virus N gene and specifically designed to detect vampire bat strains circulating in Mexico was established and investigated for its utility to replace the MIT. The utility of this method in the detection of other rabies strains known to circulate in the country was also evaluated.

MATERIALS AND METHODS

Rabies virus isolates used for method development

Eight rabies virus isolates were intracerebrally inoculated in 21-day-old albino mice (Bioterio Mexico, Mexico City) to produce positive controls for the PCR standardization. Two of the isolates were obtained from vampire bat rabies-related cases in the states of Chiapas and Morelos and antigenically characterized as AgV3 and AgV11 respectively. Two isolates from dog rabies cases were from the states of Puebla and Mexico and antigenically characterized as AgV1. Other samples were from an insectivorous bat infected with AgV9 virus [12] from Mexico City, two skunks from San Luis Potosí and South Baja California, carrying AgV8 and AgV10 types respectively, and a bobcat from Chihuahua infected with AgV7 virus. Additionally, brains from albino mice inoculated with the Pasteur virus (PV) laboratory strain (Productora Nacional de Biológicos Veterinarios (PRONABIVE), Mexico, DF) were also used as PCR-positive controls. Brains from non-inoculated mice were used as negative controls.

Extraction of RNA

Total RNA was extracted directly from brain tissue using Trizol® reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Briefly, 0·1 g brain tissue was homogenized with 1 ml Trizol® and then 200 μl chloroform (Sigma Chemical Co., St Louis, MO, USA) was added. After centrifugation of the sample at 10 000 g for 15 min, the top aqueous layer was recovered and RNA was precipitated by adding 0·5 ml isopropanol. The sample was spun at 10 000 g for 10 min, the liquid removed and the pellet washed with 1 ml of 75% ethanol. The dried RNA pellet was dissolved in 100 μl sterile RNase-free water [13]. RNA was quantified with a spectrophotometer (GeneQuant II Pharmacia, Piscataway, NJ, USA) and stored at -70 °C.

Sequence analysis of N gene

The complete nucleoprotein (N) genes of selected Mexican isolates were amplified by reverse transcription–polymerase chain reaction (RT–PCR) as described previously using a slightly shortened version of primer RabN1 and primer RabN5 [13]. The complete nucleotide sequence of the N gene ORF was determined using an automated Li-Cor 4200L sequencer (Li-Cor Biosciences, Lincoln, NE, USA) with a Thermosequenase cycle sequencing kit (Amersham Biosciences, Baie d'Urfe, PQ, Canada) and IR700- or IR800-labelled primers directed to internal amplicon sequence. Sequences were aligned using the ClustalX, version 1.8, software package [14]. Sequences generated during the course of these studies

https://doi.org/10.1017/S095026880500405X Published online by Cambridge University Press

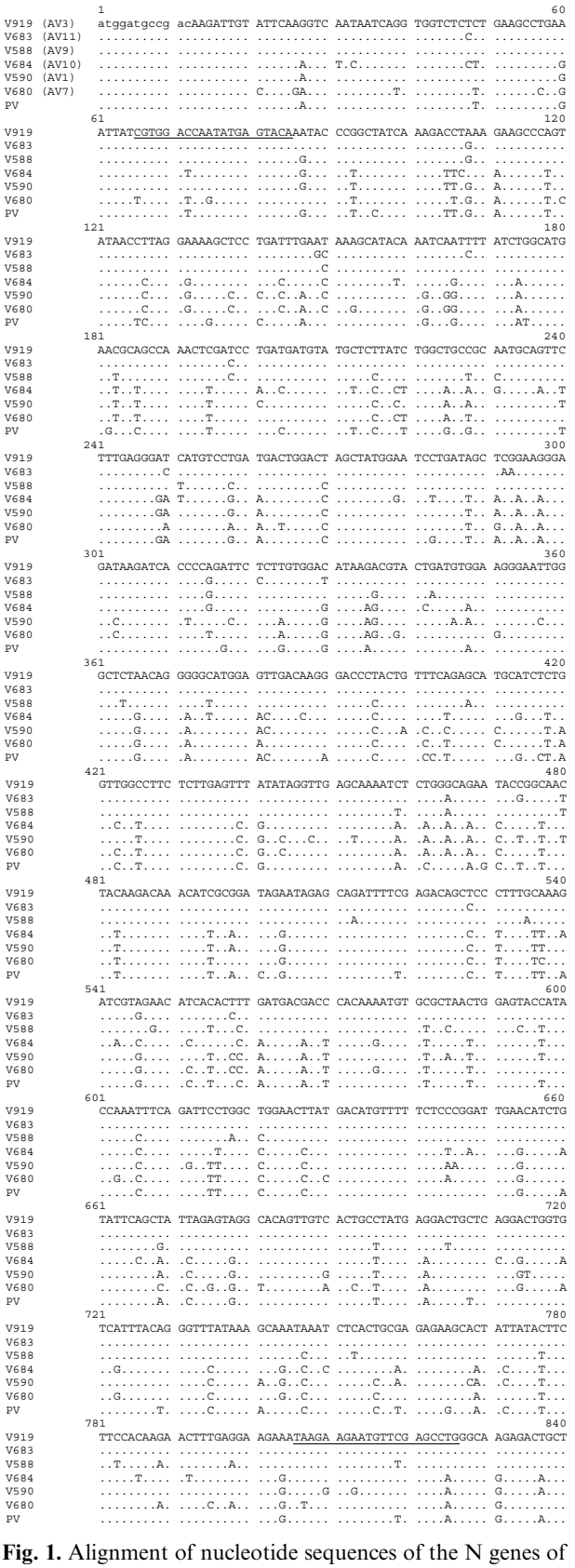

Fig. 1. Alignment of nucleotide sequences of the N genes of six Mexican rabies virus isolates and the PV reference strain. Only the first 840 bases of the N gene coding region, that have been deposited in GenBank and assigned accession numbers AY854587, AY854595 and AY8774335.

Oligodeoxynucleotides for diagnostic RT–PCR

The primers targeting the rabies virus N gene of the vampire bat strain had the following sequences:

Sense (SuEli+):
5′-CGTRGAYCAATATGAGTACA-3′ (66–85)
Antisense (SuEli−):
5′-CAGGCTCRAACATTCTTCTTA-3′ (806–826).

The numbers in parentheses indicate the position of the sequence in the N gene open reading frame with base 1 corresponding to the first base of the initiation codon (see Fig. 1). These primers are slightly modified versions of the universal N gene primers RabNfor and RabNrev that were designed to recognize several divergent strains of rabies viruses that currently circulate in Canada and which target well-conserved sequences of the gene [13].

Diagnostic RT–PCR

Synthesis of complementary DNA (cDNA) was performed with 2 μg RNA, and 50 pmol sense primer. After annealing of primer to its target the mix was brought to a total volume of 20 μl containing 1× first strand buffer, 1 mM dNTPs, 10 mM DTT, RNAsin (1 U) and 200 U M-MLV reverse transcriptase (Invitrogen). This reaction was incubated for 1·5 h at 37 °C and then terminated by heating to 90 °C for 5 min.

For PCR of each cDNA sample the reaction contained: 10 μl 10× PCR buffer as supplied with the

includes the complete amplicon sequence, are shown. The isolates included here are as follows: PV, PV strain; V588, insectivorous bat isolate (AgV9) from Mexico City; V590, dog isolate (AgV1) from Puebla State; V680, AgV7 isolate from a bobcat in Chihuahua State; V683, a bovine sample from Chipas State infected with AgV11 virus; V684, a skunk isolate (AgV10) from South Baja California; V919, an equine isolate of AgV3 strain from Chiapas. Underlined bases show the position of the diagnostic primers (SuEli+/−) specifically designed to amplify a 761-bp fragment of vampire bat strains of rabies virus. The first 12 bases of the alignment are shown in lower case since these bases correspond to the forward amplification primer used to produce the complete N gene and, thus, the definitive sequence at these positions could not be determined for the Mexican viruses.

https://doi.org/10.1017/S095026880500405X Published online by Cambridge University Press

Taq DNA polymerase, 3 μl 50 mM MgCl, 25 pmol each of the two primers SuEli+ and SuEli−, 1·25 μl 10 mM dNTPs, 1 U *Taq* DNA polymerase (Invitrogen) and 20 μl cDNA in a final volume of 100 μl. After determining the optimal annealing temperature, PCRs were cycled in an iCycler (Bio-Rad, Hercules, CA, USA) using the following cycling profile: preheated tubes to 80 °C for 3 min to allow for hot start addition of *Taq* DNA polymerse followed by 30 cycles of 45 s at 95 °C, 30 s at 50 °C, 30 s at 70 °C and 1 cycle of 72 °C for 10 min. After cycling, PCR aliquots were electrophoresed through a 1·5% agarose gel, stained with ethidium bromide (5 μg/ml) (Gibco-BRL, Grand Island, NY, USA), and evaluated under UV light.

A positive control was performed using RNA extracted from a mouse infected with PV strain while the negative control employed RNA extracted from a mouse inoculated with PBS. These two controls were included in every assay.

Analytical sensitivity and specificity of the PCR

The sensitivity of this PCR was evaluated by using serial tenfold dilutions (10^1–10^8) of a 20% suspension of a mouse brain infected with a vampire bat isolate with an original stock titre of $10^{7·5}$ LD_{50} in mice. From each dilution, RNA was extracted for cDNA synthesis and PCR. To evaluate the ability of the PCR to detect all rabies virus variants circulating in Mexico, brains infected with AgV1 (dog), AgV10 (skunk), AgV7 (bobcat), AgV3 and AgV11 (haematophagous bat) and AgV9 (insectivorous bat), were also tested. Specificity for rabies was determined using the following viruses: parainfluenza 3 bovine and bovine viral diarrhoea virus obtained from cell culture of Mexican isolates, both with a titre of $10^{5·7}$ tissue culture infecting dose ($TCID_{50}$) and porcine rubulavirus also grown in cell culture to a titre of 10^6 $TCID_{50}$. RNA extraction and RT–PCR were performed as described for rabies virus.

Field samples

A total of 40 brain samples from six different species and 13 geographical regions of Mexico were diagnosed using FAT at the Reference Centre of the National Animal Health (CENASA-SENASICA); these same samples were also evaluated by the standardized RT–PCR and by MIT; it was noted that some samples were in various levels of decomposition, with no recognizable anatomical structure in the small fragments of nervous tissue submitted.

Diagnostic sensitivity and specificity

Diagnostic sensitivity and specificity were determined using data obtained from the field samples. This was calculated using a 2×2 contingency table using the @Risk software (Palisade Corporation, Newfield, NY, USA) [15]. For this purpose, MIT was taken as the reference test and PCR as the technique under evaluation.

RESULTS

A total of seven Mexican vampire bat strain isolates representative of both antigenic types (AgV3 and AgV11) were sequenced over the complete viral N gene and alignments of the data were performed using Clustal to identify conserved regions suitable for design of PCR primers. Subsequently five additional isolates representative of strains circulating in other reservoirs [insectivorous bat (AgV9), dog (AgV1), skunk (AgV10) and a bobcat isolate (AgV7)] were also characterized over the complete N gene coding region. Consistent with a prior report [7], when nucleotide distance values over the complete N gene were computed between paired samples using the DNADIST program of PHYLIP 3.61 the AgV3 isolates varied by a value of 0·0186 while AgV11 isolates varied by much less (0·0052). Genetic distance of the insectivorous bat isolate between all vampire bat strains varied from 0·0604–0·0668. Strains of terrestrial hosts formed a very distinct grouping with intragroup nucleotide distance values of $\leqslant$0·1 while values between terrestrial and vampire bat strains ranged from 0·169–0·192.

In Figure 1 selected representative sequences are illustrated for the portion of the gene targeted by the diagnostic RT–PCR together with corresponding sequence for the PV reference strain. The sequences targeted by the primers SuEli+ and SuEli− are underlined and correspond quite closely with a set of primers previously found suitable for amplification of many rabies strains of Canadian origin. As evident from the alignment these sequences are amongst the more conserved stretches of the gene that are suitable for targeting by PCR primers; other short but highly conserved segments of the gene often included four or more sequential bases of similar type making these regions suboptimal for use as PCR priming sites. To optimize the design of these new primers, some degenerate nucleotides were incorporated so as to permit recognition of all viruses likely to be circulating in Mexico.

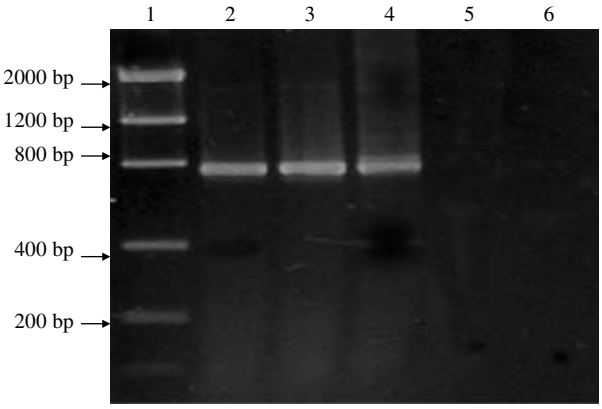

Fig. 2. RT–PCR products obtained using primers designed for vampire bat strains of rabies virus. Products were electrophoresed through a 1·5% agarose gel and stained with ethidium bromide. Lane 1. molecular weights (low DNA mass ladder; Gibco-BRL); lanes 2–4 show RT–PCR products obtained from a vampire bat isolate, the PV strain and a dog strain respectively; lanes 5 and 6, negative controls using non-infected mouse brain and water respectively.

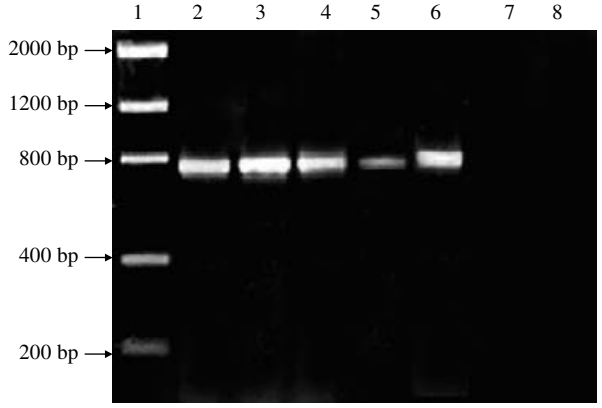

Fig. 3. RT–PCR performed on several additional rabies virus antigenic variants circulating in Mexican host reservoirs. PCR products were analysed as for Figure 2. Lane 1, molecular weight marker (low DNA mass ladder; Gibco-BRL); lane 2, haematophagous bat; lane 3, insectivorous bat; lane 4, skunk; lane 5, bobcat; lane 6, positive control (PV strain); lanes 7 and 8, negative controls using non-infected mouse brain and water respectively.

Amplification with these primers reproducibly generated a single band of the expected size of 761 bp for the PV strain as well as for all of the Mexican strains tested while uninfected mouse brain and water controls did not generate any band (Figs 2 and 3). When tenfold dilutions of rabies virus were tested, this PCR was able to detect up to 10^7-fold dilutions of virus prepared from a stock of titre $10^{7·5}$ LD_{50} (Fig. 4). In Figure 5, it can be observed that this pair of primers specifically amplifies rabies virus, since when

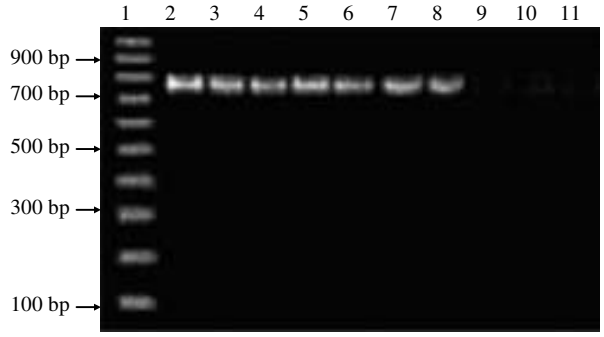

Fig. 4. Sensitivity of RT–PCR for rabies virus as determined from tenfold dilutions of viral stock. Shown is an ethidium bromide stained 1·5% agarose gel after amplicon electrophoresis. Lane 1, molecular weight marker (100 bp); lanes 2–9, RT–PCR performed with dilutions of the rabies virus: 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 respectively; lane 10, negative control (negative mouse brain); lane 11, water.

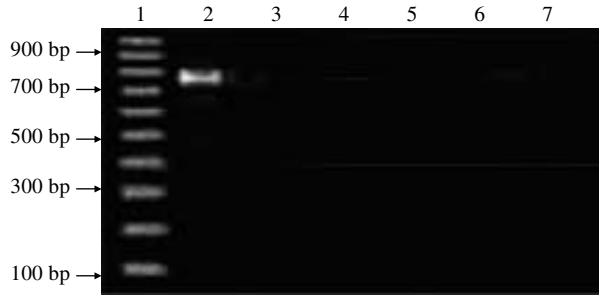

Fig. 5. Specificity of the RT–PCR for rabies. Agarose gel (1·5%) stained with ethidium bromide. Lane 1, molecular weight marker (100 bp); lane 2, positive control (rabies virus); lane 3, bovine parainfluenza virus type 3; lane 4, bovine viral diarrhoea virus; lane 5, porcine rubulavirus; lane 6, negative mouse brain; lane 7, water control.

different RNA viruses, such as: parainfluenza 3 bovine, bovine viral diarrhoea virus and porcine rubulavirus were tested, only the rabies sample generated a detectable PCR product.

The Table shows the results obtained by FAT, MIT and PCR for 40 field specimens that originated from six different species and 13 different states of the Mexican Republic. Of these 40 samples concordance between the results for all three methods was observed for 30 specimens. For the remaining 10 specimens four different scenarios were apparent. In one case (sample no. 6) PCR was positive but the two other tests were negative. Two samples (nos. 11 and 18) were positive by both the MIT and PCR tests but the FAT was negative. Four samples (nos. 17, 25, 28 and 35) were negative only by the PCR test while three samples (nos. 19, 21 and 36) were positive only by FAT. When the PCR test was directly compared to

https://doi.org/10.1017/S095026880500405X Published online by Cambridge University Press

Table. *Summary of rabies status of 40 field specimens according to the three tests under analysis*

Specimen no.	Species	Geographic origin	FAT	MIT	RT–PCR
1	Equine	Chiapas	P	P	P
2	Dog	State of Mexico	P	P	P
3	Dog	State of Mexico	P	P	P
4	Bovine	Michoacan	P	P	P
5	Ovine	Yucatan	P	P	P
6	Ovine	Hidalgo	N	N	P
7	Bovine	Hidalgo	P	P	P
8	Bovine	Veracruz	P	P	P
9	Bovine	Veracruz	P	P	P
10	Bovine	Chiapas	P	P	P
11	Bovine	Chiapas	N	P	P
12	Bovine	Chiapas	P	P	P
13	Bovine	San Luis Potosí	P	P	P
14	Bovine	San Luis Potosí	P	P	P
15	Dog	State of Mexico	N	N	N
16	Human	State of Mexico	P	P	P
17	Bovine	Tlaxcala	P	P	N
18	Bovine	Puebla	N	P	P
19	Bovine	Puebla	P	N	N
20	Cat	State of Mexico	N	N	N
21	Bovine	Chiapas	P	N	N
22	Bovine	State of Mexico	P	P	P
23	Bovine	State of Mexico	P	P	P
24	Bovine	Chihuahua	P	P	P
25	Bovine	Puebla	P	P	N
26	Bovine	Michoacan	P	P	P
27	Bovine	State of Mexico	P	P	P
28	Bovine	Veracruz	P	P	N
29	Bovine	Oaxaca	N	N	N
30	Bovine	Veracruz	P	P	P
31	Bovine	Veracruz	N	N	N
32	Bovine	State of Mexico	N	N	N
33	Bovine	Hidalgo	N	N	N
34	Bovine	Hidalgo	P	P	P
35	Bovine	Michoacan	P	P	N
36	Bovine	Veracruz	P	N	N
37	Bovine	Hidalgo	N	N	N
38	Bovine	Chiapas	P	P	P
39	Bovine	Chiapas	P	P	P
40	Canine	Mexico City	P	P	P

FAT, Fluorescent antibody test; MIT, mouse inoculation test. RT–PCR, reverse transcription polymerase chain reaction; P, Positive; N, negative.

the MIT, it exhibited a sensitivity of 86% and specificity of 91%, with a predictive positive value of 96%.

DISCUSSION

The vampire bat is a major wildlife reservoir of rabies in geographical regions where this host is present, mainly in tropical and subtropical areas from the American continent. Recently, in Mexico it has become the main transmitter of rabies, not only for cattle but also for human beings. This fact prompted the design of a new pair of PCR primers that could be used to standardize a molecular diagnostic tool for rabies that would be effective for the viral strains carried by this vector host. Indeed these primers were successful in detecting the viral genome of Mexican vampire bat rabies strains (AgV3 and AgV11); furthermore, an additional review of N gene sequences of rabies isolates of the vampire bat strain from Brazil and Trinidad, as reported previously [16], confirmed the conservation of the primer target sequences in viruses originating from geographically well-separated areas (data not shown); this suggests that this primer pair may be universally applicable to the detection of the vampire bat strain throughout its American range. In addition it has been demonstrated that this PCR can detect other rabies virus strains, represented by antigenic variants 1, 7, 9 and 10 that circulate in various host reservoirs in Mexico. A single base mismatch near the 3′-end of the reverse primer for the bobcat isolate (see base 807 for isolate V680 in Fig. 1), that was discovered subsequent to primer design, did not surprisingly preclude detection of this strain.

Although FAT has been used as the reference test for many years, its success requires samples in good condition; in tropical regions this demands continuously refrigerated storage of samples prior to testing, a requirement that is frequently not met. A decomposed sample can result in false-negative results, and hence the need for negative results to be confirmed by MIT [10, 17]. However, depending on the level of putrefaction of the tissue, even the MIT technique could produce false-negatives [17]. Consequently, in some countries, post-exposure rabies treatment is started in many patients, particularly those with multiple severe wounds, even when the brains from the offending dogs are negative by FAT [10].

For over a decade, PCR has been utilized as a tool for studies of rabies molecular epidemiology and its potential for diagnostic application as a

https://doi.org/10.1017/S095026880500405X Published online by Cambridge University Press

complementary test to FAT has been indicated [18–21]. However, PCR has proved especially useful for confirming diagnosis in heavily decomposed samples [10, 21, 22]. In accord with these previous findings, the utility of PCR was also shown in this study. Of the 40 field samples diagnosed by FAT, PCR and MIT some discrepancy in results between tests was noted for 10 samples. Sample no. 6, taken from a sheep in Hidalgo State, was received at the laboratory in an advanced state of decomposition (liquefied). This sample was PCR-positive while FAT and MIT were both negative, this finding, however, might be attributed to the high level of decomposition of the sample. Whitby et al. [22] reported the same situation in an Ethiopian wolf brain previously designated rabies negative by both FAT and MIT but positive using RT–PCR. With respect to decomposed samples, another situation in which the specimen is classed as rabies negative by FAT but then yields positive results when re-tested by both PCR and MIT is also typical [10, 21] as exemplified by two samples (nos. 11, 18) of this study. Case nos. 19, 21 and 36, where FAT was positive but PCR and MIT were both negative may be attributed to a false-positive FAT result perhaps due to the quality or dilution of conjugate in the original FAT, these latter points being critical for a good quality diagnosis. Lately Mexican laboratories have tried to standardize methods through the use of only one kind of anti-rabies conjugate. Case nos. 17, 25, 28 and 35 were FAT-and MIT-positive but PCR-negative; this could be a limitation in the sensitivity of the current single-round PCR employed in this study. Kamolvarin et al. [10], reported another single-round PCR method that was capable of detecting only 36 out of 96 samples, but when a nested PCR was used the sensitivity increased dramatically, detecting 96 out of 96 cases diagnosed as positive for rabies by MIT.

The overall findings of this analysis of 40 field samples by FAT, PCR and MIT indicated that, in comparison to the MIT reference test, PCR gave a test sensitivity of 86% and a specificity of 91%. The predictive positive value obtained using these data was 96%, meaning that this PCR could detect at least nine true positive cases out of 10.

Although RT–PCR has not yet been recommended by the WHO for rabies diagnosis, it has proven to be very useful in this and other studies [10, 21, 23, 24]. Given the time-consuming nature of the MIT, even when one animal can be euthanized for performance of FAT after the fifth day post-inoculation [9], PCR could be a useful alternative confirmatory test for FAT-negative results, thereby reducing utilization of mice and confirming the presence of the rabies virus in a short time. In particular, PCR may be especially useful for analysis of brain samples that arrive at the diagnostic laboratory in a severely decomposed state, an issue of some importance in tropical and subtropical countries where preservation of samples during shipping can be problematic; indeed this PCR has detected the rabies virus genome in inoculated mouse brains that were kept at room temperature (25-27 °C) for periods up to 552 h (Rojas et al., unpublished observations). However, some improvements in sensitivity to the current test profile would appear to be needed before this method could reliably replace the MIT.

ACKNOWLEDGEMENTS

This work was supported by funding from the Consejo Nacional de Ciencia y Tecnología (CONACYT) grant number G34635-B (Mexico). We thank the Rabies Unit of the Centers for Disease Control (Atlanta, GA) for supplying the panel of monoclonal antibodies for antigenic typing, and Luis Nieto for his technical assistance.

REFERENCES

1. **Steele JH, Fernandez PJ.** History of rabies and its global aspects. In: Baer GM, ed. The natural history of rabies. Florida: CRC Press, 1991: 1–24.
2. **Meltzer MI, Rupprecht CE.** A review of the economics of the prevention and control of rabies. Part II: Rabies in dogs, livestock and wildlife. Pharmacoeconomics 1998; **14**: 365–383.
3. **Arellano-Sota C.** Vampire bat-transmitted rabies in cattle. Rev Infect Dis 1988; **10** (Suppl): 707–709.
4. **Martinez-Burnes J, Lopez A, Medellin J, Haines D, Loza-Rubio E, Martinez M.** An outbreak of vampire bat-transmitted rabies in cattle in north eastern Mexico. Can Vet J 1997; **38**: 175–177.
5. **Jiménez RA.** The actual situation of bovine paralytic rabies. In: Posadas E, Cano P, eds. Encuentro Nacional de Rabia. Puebla, México: Federación de Médicos Veterinarios, 2003: 18–19 (Sept.): 24–27.
6. **Boletín Vigilancia Epidemiológica de la Rabia en las Américas 2000.** Vol. XXXII. Washington, DC: Organización Panamericana de la Salud, Organización Mundial de la Salud.
7. **De Mattos CC, De Mattos CA, Loza-Rubio E, Aguilar SA, Orciari LA, Smith JS.** Molecular characterization of rabies virus isolates from Mexico: implications for transmission dynamics and human risk. Am J Trop Med Hyg 1999; **61**: 587–597.

8. **Velasco VA, Gómez SM, Hernández RG, et al.** Antigenic diversity and distribution of rabies virus in Mexico. J Clin Microbiol 2002; **40**: 951–958.
9. **Trimarchi CV, Smith JS.** Diagnostic evaluation. In: Jackson AC, Wunner WH, eds. Rabies. San Diego, CA, USA: Academic Press, 2002: 307–349.
10. **Kamolvarin N, Tirawatnpong T, Rattanasiwamoke R, Tirawatnpong S, Panpanich T, Hemachudha T.** Diagnosis of rabies by polymerase chain reaction with nested primers. J Infect Dis 1993; **167**: 207–210.
11. **Hemachuda T. Rabies**. In: Vinken PJ, Bryn GW, Klawans HL, eds. Handbook of clinical neurology. Viral disease. New York: Elsevier Science Publishing 1989: 383–404.
12. **Loza Rubio E, De Mattos CC, Aguilar Setien A, De Mattos CA.** Molecular characterization of rabies virus isolated from a non-haematophagous bat in Mexico City. Vet Méx 2000; **31**: 147–152.
13. **Nadin-Davis S.** Polymerase chain reaction protocols for rabies virus discrimination. J Virol Meth 1998; **75**: 1–8.
14. **Higgins DG, Thompson JD, Gibson TJ.** Using CLUSTAL for multiple sequence alignments. Methods Enzymol 1996; **266**: 383–402.
15. **Thursfield M.** Epidemiología veterinaria. Ed. Acribia. Zaragoza, España; 1990: 339.
16. **Nadin-Davis SA, Huang W, Armstrong J, et al.** Antigenic and genetic divergence of rabies viruses from bat species indigenous to Canada. Virus Res 2001; **74**: 139–156.
17. **Albas A, Ferrari CI, da Silva LH, Bernardi F, Ito FH.** Influence of canine brain decomposition on laboratory diagnosis of rabies. Rev Soc Bras Med Trop 1999; **32**: 19–22.
18. **Sacramento D, Bourhy H, Tordo N.** PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. Mol Cell Prob 1991; **5**: 229–240.
19. **Heaton PR, Johnstone P, McElhinney LM, Cowley R, O'Sullivan E, Whitby JE.** Heminested PCR assay for detection of six genotypes of rabies and rabies-related viruses. J Clin Microbiol 1997; **35**: 2762–2766.
20. **Loza-Rubio E, Aguilar SA, Bahloul C, Brochier B, Pastoret P, Tordo N.** Discrimination between epidemiological cycles of rabies in Mexico. Arch Med Res 1999; **30**: 144–149.
21. **Yacobson DD, Rotenberg D, Dveres N, Davidson I, Stram Y.** Rabies virus detection by RT-PCR in decomposed naturally infected brains. Vet Microbiol 2002; **87**: 111–118.
22. **Whitby JE, Johnstone P, Sillero-Zubiri C.** Rabies virus in decomposed brain of an Ethiopian wolf detected by nested reverse transcription-polymerase chain reaction. J Wildl Dis 1997; **33**: 912–915.
23. **Smith J, McElhinney LM, Heaton PR, Black EM, Lowings P.** Assessment of template quality by incorporation of an internal control into a RT-PCR for the detection of rabies and rabies-related viruses. J Virol Meth 2000; **84**: 107–115.
24. **Tordo N, Bourhy H, Sacramento D.** PCR technology for Lyssavirus diagnosis. In: Clewley JP, ed. The polymerase chain reaction (PCR) for human viral diagnosis. Florida: CRC Press, 1995: 125–145.