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TITLE:

Evaluation of a Universal Nested Reverse Transcription Polymerase Chain Reaction for the Detection of Lyssaviruses

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SUMMARY:

A pan-lyssavirus nested reverse transcription polymerase chain reaction has been developed to detect specifically all known lyssaviruses. Validation using rabies brain samples of different animal species showed that this method has a sensitivity and specificity equivalent to the gold standard fluorescent antibody test and could be used for routine rabies diagnosis.

ABSTRACT:

To detect rabies virus and other member species of the genus *Lyssavirus* within the family *Rhabdoviridae*, the pan-lyssavirus nested reverse transcription polymerase chain reaction (nested RT-PCR) was developed to detect the conserved region of the nucleoprotein (N) gene of lyssaviruses. The method applies reverse transcription (RT) using viral RNA as template and oligo $(dT)_{15}$ and random hexamers as primers to synthesize the viral complementary DNA (cDNA). Then, the viral cDNA is used as a template to amplify an 845 bp N gene fragment in first-round PCR using outer primers, followed by second-round nested PCR to amplify the final 371 bp fragment

using inner primers. This method can detect different genetic clades of rabies viruses (RABV). The validation, using 9,624 brain specimens from eight domestic animal species in 10 years of clinical rabies diagnoses and surveillance in China, showed that the method has 100% sensitivity and 99.97% specificity in comparison with the direct fluorescent antibody test (FAT), the gold standard method recommended by the World Health Organization (WHO) and the World Organization for Animal Health (OIE). In addition, the method could also specifically amplify the targeted N gene fragment of 15 other approved and two novel lyssavirus species in the 10th Report of the International Committee on Taxonomy of Viruses (ICTV) as evaluated by a mimic detection of synthesized N gene plasmids of all lyssaviruses. The method provides a convenient alternative to FAT for rabies diagnosis and has been approved as a National Standard (GB/T36789-2018) of China.

INTRODUCTION:

Rabies is a worldwide zoonotic disease caused by viruses within the genus *Lyssavirus*[1]. Lyssaviruses (family *Rhabdoviridae*) are single-negative-stranded RNA viruses with an approximately 12 kb genome that encodes five proteins: N, phosphoprotein (P), matrix protein (M), glycoprotein (G), and the large protein or polymerase (L). Based on nucleotide sequences of the N gene, genetic distance, and antigenic patterns, the lyssaviruses have been divided into 16 species, comprising classical rabies virus (RABV) and the rabies-related viruses (RRV): Lagos bat virus (LBV), Duvenhage virus (DUVV), Mokola virus (MOKV), European bat lyssavirus 1 (EBLV-1), European bat lyssavirus 2 (EBLV-2), Australian bat lyssavirus (ABLV), Aravan virus (ARAV), Ikoma virus (IKOV), Bokeloh bat lyssavirus (BBLV), Gannoruwa bat lyssavirus (GBLV), Irkut virus (IRKV), Khujand virus (KHUV), West Caucasian bat virus (WCBV), Shimoni bat virus (SHIBV), and Lleida bat lyssavirus (LLEBV)[2]. Recently, two additional lyssaviruses have been identified: Kotalahti bat lyssavirus (KBLV) isolated from a Brandt's bat (*Myotis brandtii*) in Finland in 2017[3] and Taiwan bat lyssavirus (TWBLV) isolated from a Japanese pipistrelle (*Pipistrellus abramus*) in Taiwan, China in 2016–2017[4].

All mammals are susceptible to rabies; however, no gross pathognomonic lesions or specific clinical signs permit its identification, and diagnosis can only be made in the laboratory[5]. The most widely used method for rabies diagnosis is the FAT, which is considered as the gold standard by both the WHO and the OIE[5,6]. Nevertheless, the FAT can produce unreliable results on degraded/autolyzed brain tissue samples. Additionally, it cannot be used to assay biological fluid specimens such as cerebrospinal fluid (CSF), saliva, and urine, thereby largely precluding its employment in antemortem diagnosis[7]. Alternative conventional diagnostic tests, such as the rabies tissue culture infection test (RTCIT) and the mouse inoculation test (MIT), require several days[6], a major drawback when a rapid diagnosis is essential.

Various molecular diagnostic tests (e.g., the detection of viral RNA by RT-PCR, the PCR–enzyme-linked immunosorbent assay [PCR-ELISA], in situ hybridization, and real-time PCR) are used as rapid and sensitive techniques for rabies diagnosis[8]. RT-PCR is now recommended by OIE for routine rabies diagnosis, and a heminested (hn) PCR is described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals to detect all lyssaviruses[5]. Here we describe a pan-

lyssavirus nested RT-PCR, which allows the specific and sensitive detection of all 18 lyssavirus species comparable to or exceeding that obtained by the FAT. The principle of the method is an RT of the target RNA (conserved region of the lyssavirus N gene) into cDNA, followed by the amplification of the cDNA by two rounds of PCR. The cDNA undergoes the first-round PCR with outer primers to amplify an 845 bp fragment; then, the second-round PCR uses the first-round PCR product as a template to amplify a 371 bp fragment with inner primers. The two rounds of PCR significantly increase the sensitivity of the assay.

PROTOCOL:

The use of mice in this protocol was approved by the Administrative Committee on Animal Welfare of the Institute of Military Veterinary Medicine, the Academy of Military Medical Sciences, China (Laboratory Animal Care and Use Committee Authorization, permit number JSY-DW-2010-02). All institutional and national guidelines for the care and use of laboratory animals were followed.

1. RNA extraction

1.1. Extract RNA from rabies-suspected brain tissue, skin biopsies, saliva, or CSF or from RABV-infected cell culture, using guanidinium isothiocyanate-phenol-chloroform-based extraction methods or commercially available viral RNA extraction kits. Use the prepared RNA immediately or store it at -80 °C until required.

2. Reverse transcription of the viral RNA

2.1. Remove the RT reagents listed in **Table 1** from the freezer, keep them on ice, and thaw and vortex them before use.

2.2. Prepare 12 µL of RT reaction mix in a 0.2 mL PCR tube with the reagents listed in **Table 1**. Allow for pipetting variations by preparing a volume of master mix at least one reaction size greater than required.

2.3. Add 8 µL sample, positive control RNA or negative control to the RT reaction mix within a PCR workstation in a template room. The RT positive control is RNA extracted from the cell culture infected with fixed RABV strain CVS-11 (challenge virus standard-11) and stored at -80 °C. The negative control contains RNase-free ddH_2O.

2.4. Mix the contents of the RT tubes by vortexing; then, centrifuge briefly.

2.5. Load the reaction tubes into a thermal cycler. Set up the cDNA synthesis program with the following conditions: 42 °C for 90 min, 95 °C for 5 min, and 4 °C on hold. Set the reaction volume to 20 µL. Start the RT run.

3. First-round PCR

3.1. Keep the PCR reagents listed in **Table 2** on ice in a clean room until use; then, thaw and vortex them.

3.2. Prepare the first-round PCR mix in a 0.2 mL PCR tube with the reagents listed in **Table 2**.

3.3. Add a 2 µL sample of cDNA or plasmid into the first-round PCR mix within a PCR workstation in a template room. The PCR positive control is CVS-11 cDNA prepared as mentioned in step 2.3 for the above RT method. The PCR negative control is ddH_2O.

3.4. Transfer the sealed tubes to a PCR thermal cycler and cycle using the parameters listed in **Table 3**.

4. Second-round PCR

4.1. Prepare the second-round PCR mix in a 0.2 mL PCR tube using the reagents listed in **Table 4**.

4.2. Add 2 µL of first-round PCR product into the second-round PCR mix. In addition, include ddH_2O as a negative control of the second-round PCR.

4.3. Perform PCR thermal cycling using the same parameters as given in step 3.4.

5. Analysis of the PCR products by electrophoresis on agarose gels

5.1. Prepare a 1.5% agarose gel by adding 1.5 g of agarose to 100 mL of Tris-acetate-EDTA (TAE) and dissolving it thoroughly by heating it in a microwave oven.

5.2. Add ethidium bromide (EB) (at a final concentration of 0.01%) or another commercial EB substitution. Pour the gel into the mold and leave it to solidify at ambient temperature for at least 30 min.

5.3. Prepare the loading samples by mixing 5 µL of each PCR product with 1 µL of 6x loading buffer.

5.4. Load the samples and suitable DNA marker separately into the wells and run the gel for approximately 30–45 min at 120 V until the dye line is approximately 75%–80% down the gel.

5.5. Turn off the power, disconnect the electrodes from the power source, and then, carefully remove the gel from the gel box.

5.6. Use a UV gel documenting device to visualize and photograph the DNA fragments.

6. Characterization of nested RT-PCR

6.1. Specificity and sensitivity for the detection of 18 lyssaviruses plasmids

6.1.1. Order 18 commercial plasmids containing the full N gene of each lyssavirus (16 ICTV species and two novel species) for the PCR.

6.1.2. Calculate the copy number of the plasmid using Avogadro's number (N_A) and the following formula.

[(g/µL plasmid DNA)/(plasmid length in bp x 660)] x 6.022 x 10^{23} = number of molecules/µL

6.1.3. Prepare stock solutions (2.24 x 10^9 molecules/µL) of all 18 plasmids in ddH_2O.

6.1.4. Perform nine 10-fold serial dilutions of all 18 plasmids in ddH_2O. Dilute 10 µL of each plasmid stock with 90 µL of ddH_2O. Vortex and centrifuge briefly.

6.1.5. Perform PCR amplification as described in sections 3–5.

6.1.6. Analyze the specificity and sensitivity of the nested PCR by detecting a series of lyssavirus plasmids.

6.2. Determination of the detection limit

6.2.1. Adjust the titer of the rabies virus strain CVS-11 cell culture to $10^{5.5}$ $TCID_{50}$/mL (virus titer determined according to the OIE Manual)[5].

6.2.2. Perform five 10-fold serial dilutions of CVS-11 (stock solution is $10^{5.5}$ $TCID_{50}$/mL) as described in step 6.1.4.

6.2.3. Perform the RNA extraction and nested RT-PCR amplification procedures of all viral dilutions as described in sections 1–5.

6.3. Comparison of the nested RT-PCR with the "gold standard" FAT

6.3.1. Test all clinical samples by nested RT-PCR, and then, confirm the results with the FAT[5] and N gene sequencing[9].

6.3.2. Use normalized data for a statistical analysis with SAS 9.1. Use the kappa test and McNemar's chi-squared test for a statistical comparison of the diagnostic tests (SAS command: proc freq; table/agree). Calculate confidence intervals assuming abinomial distribution.

6.4. Evaluation of the efficacy in testing degraded samples

6.4.1. Expose two confirmed clinical brain tissue samples of rabid dogs at 37 °C.

6.4.2. Assay the two samples on each day of exposure at 37 °C by nested RT-PCR, the FAT, and the MIT[5].

REPRESENTATIVE RESULTS:

Results of nested RT-PCR to detect 18 lyssavirus species are shown in **Figure 1**. All PCR positive controls showed the expected 845 bp in the first- and 371 bp in the second-round amplifications with no band in the negative control. All 18 lyssaviruses produced the expected 845 and/or 371 bp bands, indicating that the nested RT-PCR detected all 18 lyssaviruses. Sixteen lyssaviruses plasmids had efficient amplification in two rounds of PCR, but two, namely ARAV and IKOV, had amplification in either the first- or second-round PCR. The sensitivity of the method varied in the detection of different lyssavirus plasmids, with limits ranging from 2.24×10^0 to 2.24×10^5 molecules/µL, as shown in **Table 6**. These differences can be attributed to the mismatches between the primers and templates due to viral sequence diversity. Furthermore, the sensitivity of detecting rabies virus CVS-11 in cell culture was $10^{2.5}$ $TCID_{50}$/mL.

A total of 9,624 brain tissues from clinical specimens were tested by nested RT-PCR in comparison with the FAT and the results are summarized in **Table 7**, which shows that nested RT-PCR had a 100% sensitivity (CI, 97.75% to 100%) and a 99.97% specificity (CI, 99.91% to 99.99%). The accordance between the two methods was 99.07%. Three tests that were positive by nested RT-PCR but negative by the FAT were of highly decayed clinical material. These three specimens were confirmed as RABV positive by N gene sequencing.

Comparison of the test performance in the detection of the two brain specimens incubated at 37 °C (step 6.4.1 of the protocol) indicated that the nested RT-PCR could effectively detect virus in decayed brain tissues for least 17 days postdegradation, which is for a longer period of time when compared with only 7 days by the FAT and not even 1 day by the MIT. This result shows that nested RT-PCR is more sensitive in the detection of degraded samples than the FAT and the MIT.

To further validate the nested RT-PCR, 10 rabies laboratories in China were invited to conduct tests on a set of specimens. Of these, eight laboratories were provided a set of 10 blinded animal brain tissues from our laboratory, including RABV positive and negative specimens. The other two laboratories used their own specimens. All specimens had been archived and confirmed by the FAT previously. All 10 laboratories obtained results by nested RT-PCR in 100% accordance with the FAT, with no false-negatives or false-positives (**Table 8**), indicating that the nested RT-PCR had a high specificity and reproducibility.

FIGURE AND TABLE LEGENDS:

Table 1: Reagents of reverse transcription for cDNA synthesis.

Table 2: Reagents of the first-round PCR.

Table 3: Cycling parameters of the first- and second-round PCR.

Table 4: Reagents of the second-round PCR.

Table 5: Primer sequences of the first- and second-round PCR. Degenerate bases: N (A/T/C/G), K (G/T), R (A/G), Y (C/T), WV (G/A/C).

Table 6: Detection limit of nested RT-PCR of 18 lyssaviruses.

Table 7: Correlation between nested RT-PCR and the FAT in the detection of RABVs in clinical specimens.

Table 8: Validation results of nested RT-PCR by 10 laboratories.

Figure 1: Detection of 18 lyssaviruses by nested RT-PCR. (**A**) The result of the first-round PCR. (**B**) The result of the second-round PCR. M = DL 2000 DNA marker. Lanes 1–18 = RABV, LBV, MOKV, DUVV, EBLV-1, EBLV-2, ABLV, ARAV, IKOV, BBLV, GBLV, IRKV, KHUV, LLEBV, SHIBV, WCBV, KBLV, and TWBLV, respectively. Lane 19 = PCR positive control; lane 20 = negative control for the first-round PCR; lane 21 = negative control for the second-round PCR. A positive PCR result shows a band at 845 bp in the first round and 371 bp in the second-round PCR. The amplicon of ARAV is not visible in the first round, but visible in the second-round PCR (lane 8), while the amplicon of IKOV is visible in the first round, but not visible in the second-round PCR (lane 9).

Figure 2: Comparison with primer sequences shows the differential nucleotides in primer regions of 18 lyssavirus species. N127 and N829 were outer primers, N371F and N371R were inner primers.

DISCUSSION:

Currently, RABV is a major lyssavirus responsible for nearly all human and animal rabies in China, as well as in other countries. In addition, an IRKV variant was first identified from a *Murina leucogaster* bat in the Jilin province in Northeast China in 2012[10], and it has been reported to cause a dog's death in the Liaoning Province in 2017[11]. Most recently, a novel lyssavirus, TWBLV, was also identified from a Japanese pipistrelle bat in Taiwan, China in 2017. These results suggest that the effective detection of other lyssaviruses is also important to prevent the spill-over of the bat-borne lyssaviruses. In this regard, the pan-lyssavirus nested RT-PCR targeting the most conserved N gene region is a very useful tool, and the results have shown that it can effectively detect all 16 ICTV-approved and two novel lyssavirus species identified so far, including genetically divergent RABVs and IRKV in China (data on IRKV strain not shown). However, it is also interesting to note that ARAV was detected only by the second-round PCR primers, while IKOV was detected only by first-round PCR primers (**Figure 1**). To investigate the cause of this

discrepancy, sequence comparison of all 18 lyssaviruses within the four primer regions was conducted, with the results showing that the 3' end nucleotide T of outer primer N829 in the first-round PCR and the second nucleotide T at the 3' end of inner primer N371F in the second-round PCR were not identical to the corresponding positions of ARAV (G) and IKOV (A) (**Figure 2**). Once the T of primer N829 was changed to the G of ARAV and the T of primer N371F changed to the A of IKOV, both viruses were successfully detected in PCR (data not shown). This result demonstrates the critical role of the 3' end or near-end nucleotides of primers in the successful amplification of the target region.

To evaluate the sensitivity and specificity of the method in the detection of rabies virus for clinical diagnosis and surveillance, 9,624 animal brain tissue samples were tested in the last 10 years by the FAT and nested RT-PCR in parallel. Of the 165 samples that tested rabies positive by nested RT-PCR, 162 were detected as positive by the FAT; therefore, the two methods show 99.07% accordance. The RABVs detected in these 165 samples could be classified into different lineages in Asian, Arctic-related, and Cosmopolitan clades[12,13], indicating that nested RT-PCR can cover the various genetic clades of RABVs. Three highly decayed clinical specimens were tested positive by nested RT-PCR but negative by the FAT. This result is consistent with the evaluation of the efficacy in testing two degraded samples as shown in the representative results section, indicating that nested RT-PCR is more sensitive than the FAT in the detection of viruses in highly decayed specimens.

The performance of nested RT-PCR was further confirmed by participation in the International Laboratory Comparison Test (ILCT) organized by the EU Reference Laboratory for Rabies at the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) in 2010, using a set of 12 samples (one each of RABV, EBLV-1, EBLV-2, and ABLV as positive controls, one negative control, and seven blinded samples). In the test, nested RT-PCR successfully identified all the lyssaviruses with a 100% consistency. In 2018, the method was approved as a National Standard of Rabies Diagnoses (GB/T36789-2018) by the National Technical Standardization Committee of Animal Health of China.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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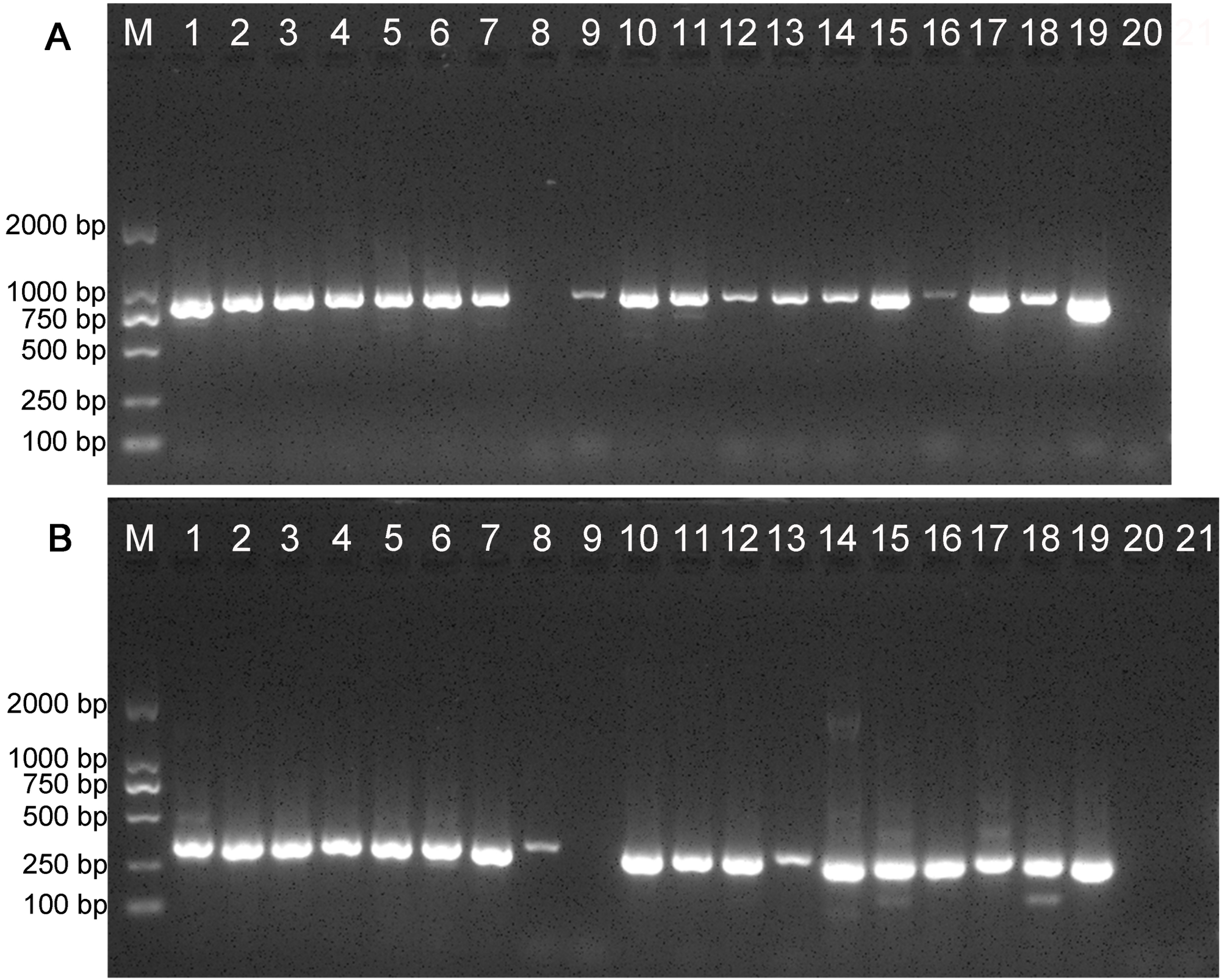

Figure

N127	A	T	G	T	A	A	C	N	C	C	T	C	T	A	C	A	A	T	G	G
RABV GQ918139.1	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
LBV EU293110.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
MOKV KF155005.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
DUVV AY062080.2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
EBLV-1 EF157976.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
EBLV-2 KF155004.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
ABLV GU992312.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
ARAV NC_020808.1	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
IKOV NC_018629.1	·	A	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·
BBLV NC_025251.1	·	G	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
GBLV NC_031988.1	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
IRKV NC_020809.1	·	·	·	·	·	·	·	·	·	·	C	·	·	·	A	·	·	·	·	·
KHUV NC_025385.1	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
LLEBV NC_031955.1	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
SHIBV NC_025365.1	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
WCBV NC_025377.1	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
KBLV MF960865.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
TBLV MF472710.1	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·

N371F	A	C	A	A	T	G	G	A	K	K	C	T	G	A	C	A	R	A	T	T	G
RABV GQ918139.1	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
LBV EU293110.1	·	·	·	·	·	·	·	·	·	·	·	A	·	·	A	·	G	·	·	·	·
MOKV KF155005.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
DUVV AY062080.2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	G	·	·	·	·
EBLV-1 EF157976.1	·	·	·	·	·	·	·	·	·	·	T	·	A	·	·	·	·	·	G	·	·
EBLV-2 KF155004.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·
ABLV GU992312.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·
ARAV NC_020808.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
IKOV NC_018629.1	·	·	·	·	·	·	·	·	·	C	·	·	·	·	A	C	·	·	G	·	A
BBLV NC_025251.1	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·
GBLV NC_031988.1	·	·	·	·	·	·	·	·	·	·	·	C	·	·	T	·	·	·	·	·	·
IRKV NC_020809.1	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·
KHUV NC_025385.1	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	G	·	·	·	·
LLEBV NC_031955.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	C	·	·	G	·	C
SHIBV NC_025365.1	·	·	·	·	·	·	·	·	C	·	·	·	·	·	A	·	·	·	·	·	·
WCBV NC_025377.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	C	·	C	·	·	·
KBLV MF960865.1	·	·	·	·	·	·	·	·	·	·	·	C	A	G	·	·	G	·	·	·	·
TBLV MF472710.1	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	G	·	·	·	A

N829	G	C	C	C	T	G	G	T	T	C	G	A	A	C	A	T	T	C	T
RABV GQ918139.1	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·
LBV EU293110.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
MOKV KF155005.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
DUVV AY062080.2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
EBLV-1 EF157976.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
EBLV-2 KF155004.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
ABLV GU992312.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
ARAV NC_020808.1	·	T	·	·	C	·	·	·	·	·	A	·	·	·	·	·	C	·	G
IKOV NC_018629.1	·	A	·	·	A	·	·	C	·	T	·	·	·	·	·	·	·	·	·
BBLV NC_025251.1	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	C	·	·
GBLV NC_031988.1	·	T	·	·	·	·	·	C	·	·	A	·	·	·	·	·	C	·	·
IRKV NC_020809.1	·	A	·	·	A	·	·	C	·	·	·	·	·	·	·	·	·	·	·
KHUV NC_025385.1	·	A	·	·	·	·	·	C	·	·	A	·	·	·	·	·	C	·	·
LLEBV NC_031955.1	·	·	·	·	A	·	·	·	C	T	A	·	·	·	·	·	·	·	·
SHIBV NC_025365.1	·	A	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·
WCBV NC_025377.1	·	A	T	T	G	·	·	A	C	G	·	·	·	·	·	·	·	·	·
KBLV MF960865.1	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	C	·	·
TBLV MF472710.1	·	T	·	·	A	·	·	·	·	·	A	·	·	·	·	·	·	·	·

N371R	C	C	T	G	Y	Y	W	G	A	G	C	C	C	A	G	T	T	V	C	C	Y	T	C
RABV GQ918139.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·
LBV EU293110.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·
MOKV KF155005.1	·	·	G	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·
DUVV AY062080.2	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
EBLV-1 EF157976.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
EBLV-2 KF155004.1	·	·	·	·	·	·	·	·	T	·	·	·	·	·	A	·	·	·	·	·	·	·	·
ABLV GU992312.1	·	·	A	·	·	·	·	A	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·
ARAV NC_020808.1	·	·	A	·	·	·	·	·	T	·	·	·	·	·	A	·	·	·	·	·	·	·	·
IKOV NC_018629.1	G	·	A	A	·	·	·	·	T	·	·	·	·	·	·	·	T	·	·	·	·	·	·
BBLV NC_025251.1	·	·	G	·	·	·	·	A	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GBLV NC_031988.1	·	·	·	·	·	·	·	A	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·
IRKV NC_020809.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·
KHUV NC_025385.1	·	·	G	·	·	·	·	A	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·
LLEBV NC_031955.1	G	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·
SHIBV NC_025365.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	T	·	·	·	·	·
WCBV NC_025377.1	·	·	A	·	A	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·
KBLV MF960865.1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	G	·
TBLV MF472710.1	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·

Table 1. Reagents of reverse transcription for cDNA synthesis	
Components	**Volume per reaction (μL)**
dNTPs (2.5 mM)	4
Random Primer (50 μM)	1.5
Oligo(dT) $_{15}$ (50 μM)	0.5
M-MLV buffer (5 ×)	4
M-MLV reverse transcriptase (200 IU/μL)	1
RNasin (40 IU/μL)	1
Total volume	12

Table 2. Reagents of the first round PCR	
Components	**Volume per reaction (μL)**
dNTPs (10 mM)	1
Ex-Taq (5 U/μL)	0.3
Taq Buffer (10×)	5
N127 (20 μM)	1
N829 (20 μM)	1
dd H_2O	39.7
Total volume	48

Table 3. Cycling parameters of the first and second round PCR		
Temperature	**Time**	**Cycles**
94 ℃	2 min	1
94 ℃	30 s	35
56 ℃	30 s	
72 ℃	40 s	
72 ℃	10 min	1
4 ℃	∞	

Table 4. Reagents of the second round PCR	
Components	**Volume per reaction (µL)**
dNTPs (10 mM)	1
Ex-Taq (5 U/µL)	0.3
Taq Buffer (10×)	5
N371F (20 µM)	1
N371R (20 µM)	1
dd H_2O	39.7
Total volume	48

Table 5. Primer sequences of the first and second round PCR. Degenerate bases: N (A/T/C/G), K (G/T), R(A/G), Y(C/T), WV (G/A/C).

Primer Name	Details	Direction	Sequence (5'-3')	Nucleotide position	Product Size
N127	The first round PCR	Forward	ATGTAACNCCTCTACAATGG	-19～0	845bp
N829	The first round PCR	Reverse	GCCCTGGTTCGAACATTCT	807～825	
N371F	The second round PCR	Forward	ACAATGGAKKCTGACAARATTG	-6～15	371bp
N371R	The second round PCR	Reverse	CCTGYYWGAGCCCAGTTVCCYTC	345～367	

Table 6. Detection limit of nested RT-PCR on 18 lyssaviruses		
Lyssavirus species	**Strain**	**Template plasmid (molecules /μL)**
RABV	GQ918139.1	2.24×10^{1}
LBV	EU293110.1	2.24×10^{2}
MOKV	KF155005.1	2.24×10^{1}
DUVV	EU293119.1	2.24×10^{1}
EBLV-1	EF157976.1	2.24×10^{1}
EBLV-2	KF155004.1	2.24×10^{1}
ABLV	GU992312.1	2.24×10^{0}
IRKV	NC_020809.1	2.24×10^{1}
WCBV	NC_025377.1	2.24×10^{1}
KHUV	NC_025385.1	2.24×10^{3}
ARAV	NC_020808.1	2.24×10^{2}
SHIBV	NC_025365.1	2.24×10^{1}
BBLV	NC_025251.1	2.24×10^{1}
IKOV	NC_018629.1	2.24×10^{5}
LLEBV	NC_031955.1	2.24×10^{3}
GBLV	NC_031988.1	2.24×10^{5}
KBLV	MF960865.1	2.24×10^{1}
TWBLV	MF472710.1	2.24×10^{1}

Table 7. Correlation between nested RT-PCR and FAT in detection of RABVs in clinical specimens

Standard method and result		RT-nPCR		Correlation (%)
		Positive	Negative	
FAT	Positive	162	0	99.07
	Negative	3	9459	

Table 8. Validation results of nested RT-PCR by 10 laboratories

No.	Name of specialized laboratory	Number of verified samples				
		FAT		nested RT-PCR		Total
		Positive	Negative	Positive	Negative	
1	National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (China CDC)	6	6	6	6	12
2	Department of Veterinary Medicine, Zhejiang University (ZJU)	5	5	5	5	10
3	College of Veterinary Medicine,Huazhong Agriculture Unversity （HZAU）	8	2	8	2	10
4	Center for Animal Disease Control and Prevention of Chongqing	5	5	5	5	10
5	College of Animal Science and Technology,Jilin Agriculture University (JAU)	5	5	5	5	10
6	Institute of Special Wild Economic Animal and Plant Science,Chinese Academy of Agriculture Sceinces (CAAS)	8	2	8	2	10
7	Guangxi Veterinary Research Institute (GVRI)	8	2	8	2	10
8	College of Veterinary Medicine, South China Agricultural University (SCAU)	2	0	2	0	2
9	Guangdong Academy of Agriculture Sciences (GAAS)	8	2	8	2	10
10	College of Veterinary Medicine, Jilin University (JLU)	4	6	4	6	10
Total		61	33	61	33	94

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
50 × TAE	Various	Various
6 × loading buffer	TakaRa	9156
Agarose	US Everbright® Inc	A-2015-100g
ddH_2O	Various	Various
DL 2,000 Marker	Takara	3427A
dNTPs (10 mM)	TakaRa	4019
dNTPs (2.5 mM)	TakaRa	4030
Electrophoresis System	Tanon	EPS300
Ex-Taq (5 U/ μ L)	TakaRa	RR001
Gel Imaging System	UVITEC	Fire Reader
Microcentifuge tubes	Various	Various
M-MLV reverse transcriptase (200 IU/μL)	TakaRa	2641A
NanoDrop 1000 Spectrophotometer	Thermoscientific	ND1000
Oligo $(dT)_{15}$	TakaRa	3805
PCR Machine	BIO-RAD	T100
PCR Tubes	Various	Various
Phusion High-Fidelity DNA Polymearase	NEW ENGLAND BioLabs	M0530S
Pipettors	Various	Various
Random Primer	TakaRa	3801
RNase Inhibitor (40 IU/μL)	TakaRa	2313A
RNase-free ddH_2O	TakaRa	9102
Taq Buffer (10×)	TakaRa	9152A
Tips	Various	Various
Vortex mixer	Various	Various

Comments/D escription

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Author(s): Yuyang Wang, Weidi Xu, Huancheng Guo, Wenjie Cong, Biao He, Changchun Fu, Ye Feng

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Editorial comments:
One reviewer declined to review the manuscript. The comment should be included.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response 1: The manuscript has been edited by a native English-speaking virologist and the grammar has now been improved.

2. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please check the iThenticateReport attached to this email and rewrite lines 59-70, 74-77, 79-81,

Response 2: Modified. Lines 59, 75-82 has been rewritten. The lines 60-62 were overlapping with ICTV but unmodified due to they were the definition of lyssavirus and introduction of lyssavirus genomes. This part was added according to the suggestion of reviewer 3.

3. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc.

Response 3: Modified.

4. Step 1.1: Please write this step in the imperative tense.

5. 6.3.1: Please write this step in the imperative tense.

6. 6.3.2: Please write this step in the imperative tense.

7. 6.3.3: Please write this step in the imperative tense.

8. 6.4.1: Please write this step in the imperative tense.

9. 6.4.2: Please write this step in the imperative tense.

Response 4: Modified.

10. Figure 1: Please add a unit for each panel.

Response 5: Modified.

Changes to be made by the author(s) regarding the manuscript:

Reviewer #1:

Rabies is an important NTD. Globally, this encephalitis is caused by more than 16 lyssavirus species. The most important member is rabies virus, primarily in dogs and other mammalian carnivores, but newly characterized species are found primarily among bats. Improved laboratory-based surveillance for lyssavirus detection would be ideal. This sop on nested PCR was able to detect all known species of lyssaviruses. In addition, the sensitivity and specificity were both high in thousands of animal brains examined, from a variety of species, some in less than ideal conditions. Moreover, other major laboratories throughout China were also able to corroborate these findings.

One question that comes to mind while reading this paper is, what kinds of lyssaviruses have been found in China to date?

Response 1: The induction of epidemiology of lyssaviruses in China has been added in discussion, see in lines 246-250.

In the results, were the authors satisfied with the lower limit of detection for CVS?

Response 2: The 9624 animal brain tissue samples were tested in last ten years by nested RT-PCR in parallel with FAT, and no false negative was founded. It suggests the detectionlimit of this method is acceptable.

In the discussion, has this test been accepted as a primary diagnostic method throughout China?

Response 3: The nested RT-PCR has been accepted as National Standard in 2018, see lin 282. The laboratories in China could use the OIE standard and national standard for disease diagnosis according to the national policy.

Will the sop be submitted for review by OIE and WHO.

Response 4: The description of RT-PCR recommended by OIE was added in instruction, see lines 88-90. No specific PCR method was described by WHO Laboratory Techniques in Rabies.

Can the technology be shared with other Asian countries?

Response 5: Yes. As the OIE Reference Laboratory for Rabies, we have supplied the nested RT-PCR reagents and protocol to the technicians of 13 Asian countries during the Regional Hands-on Laboratory Trainings on Rabies Diagnosis organized by OIE

in 2014 and 2017. This method was easy to perform according to technicians' feedbacks. Some Asian laboratories are going to use this method for rabies diagnosis.

Also, the MS should be carefully examined to detect any editorial errors and improve the clarity of writing for the general reader.

Other minor comments follow.

Line 49: what is a rabies-related disease?

Response 6: Modified, see line 59.

Lines 58-60: Please separate the Genus species names.

Response 7: The names of two novel species Kotalahti bat lyssavirus and Taiwan bat lyssavirus in lines 70-73 were defined in reference 3-4.

Lines 67-69: how do the biosafety levels differ between different tests?

Response 8: Deleted. RTCIT and MIT require performance in a BSL 2 or 3 laboratory according to the different national policies.

Line 73: how does this test compare to other nested PCR tests for rabies?

Response 9: The hn PCR described in OIE Manual also could detect all lyssaviruses. However, we did not compare our nested RT-PCR with hn PCR due to the sensitivity and specificity of hn PCR were not published.

Reviewer #2:

Manuscript Summary:

This manuscript developed a pan-lyssavirus nested RT-PCR which shown equivalent sensitivity and specificity, had 98.18% and 100% identity with standard FAT, and verified to be a specific diagnostic tool. Overall, this is a straightforward study and easy to follow.

An accurate and reliable detection method for RABV and RRVs meets the demands of clinical veterinarians, and this study showed that the RT-nPCR method is sensitive and specific enough.

Basically, the manuscript is suitable, and consider to publish in JoVE.

Nevertheless, some questions need to improve, and some minor issues should be addressed in this manuscript.

Minor Concerns:

#1. Fig 1 legend: It is better to delete the sentence "The positive control demonstrated that the PCR occurred as expected" in the illustration of fig. 1 (row 217) , because there was no positive control in this experiment and in fig. 1.

Response 1: Figure 1 was modified by adding PCR positive and negative controls. The legend was rewritten, see lines 231-239.

#2. About fig. 2, could the authors provide a higher quality image? The fig. 2 isn't clear enough in this manuscript.

Response 2: Figure 2 was changed to a higher quality image.

#3. How many animal brain samples were tested both by RT-nPCR and FAT? 9624 (row 185) or 9454(row 244)? And how many negative samples by FAT were tested as positive by RT-nPCR? Two (row 246) or three (table 7)? Also, about the table 7, how to calculate the correlative percentage ?

Response 3: Sorry for the clerical error. In fact, 9624 animal brain tissue samples were tested both by FAT and RT-nPCR and 3 FAT negative samples tested positive by RT-nPCR, see lines 191, 267-270 and table 7. And then the sensitivity and specificity of nested RT-PCR also have been recalculated and modified, see lines 47-49 and 191-193. The statistical analysis method has been added as 6.3.2, see lines 173-175.

#4. Explain or discuss the differences of detection limit among the template plasmids (molecules/μl) in 18 lyssavirus species? From $2.24x10^0$ to $2.24x10^5$ (table 6).

Response 4: The difference of detection limit presumably according to the mismatches in the primer binding region due to viral diversity. See lines 188-189.

Reviewer #3:

Manuscript Summary:

This manuscript describes a universal nested RT-PCR for detection of lyssaviruses. It provides a useful and alternative method for rabies diagnosis, particularly for clinical samples that have degraded.

Major Concerns:

1. Please describe the RT-PCR chapter in the OIE manual and also make clear that this is not the only pan-lyssavirus RT-PCR available.

Response 1: Added, see lines 88-90.

2. This manuscript requires re-writing to an acceptable academic/scientific writing standard and to improve the manuscripts clarity and 'flow' so the assay is adequately introduced and discussed and the technical procedure is clearly explained.

Response 2: The manuscript has been edited by a native English-speaking virologist and the grammar has now been improved. We hope therevised manuscript meets the requirements.

3. The scientific accuracy in some parts of the manuscript also requires amending. For example, Lyssavirus species are delineated based on demarcation criteria such as genetic distance and antigenic patterns, rather than solely 'based on the nucleoprotein gene' as stated in the manuscript.

Response 3: Modified. See line 63.

4. The introduction requires additional information:

a. inclusion of the rationale for performing second round PCR

Response 4: Done, see lines 95-98.

b. Prior to mentioning the N gene, a brief mention that lyssavirus genomes contain five structural proteins would be beneficial.

Response 5: Done, see lines 60-62.

5. The use of positive and negative controls 'during important key steps' is mentioned and included for the reverse transcription stage (step 1), however other key steps that require additional controls (such as during first and second round PCR set-ups) need to be included.

Response 6: Modified. RT positive and negative controls have been added in lines 114-115, PCR positive and negative controls have been added in lines 126-127 and 133-134.

6. The protocol is too detailed in non-critical steps, such as removing reagents from a freezer, but scant in important steps, i.e. mixing agarose powder with TAE buffer and microwaving before the addition of ethidium bromide/Super GelRed is not included.

Response 7: Modified protocol, and added the important steps, see lines 138-142.

7. The results for the assays reproducibility between Chinese laboratories are difficult to interpret, and the results column in table 8 requires explanation and further interpretation.

Minor Concerns:

1) Line 36 - RT-PCR and N gene requires writing out in full

Response 8: Done, see lines 40-41.

2) Line 38 - ICTV requires writing out in full

Response 9: Done, see line 53.

3) Line 41 - The OIE manual now accepts and includes PCR methods for rabies diagnosis

Response 10: Added the description of PCR method of OIE manual, see lines 88-90.

4) Line 41 - OIE and WHO require writing out in full

Response 11: Done, see lines 78-79.

5) Line 49 - Remove 'various'

Response 12: Modified, see line 59.

6) Line 50 - RNA requires writing out in full

Response 13: Done, see line 86.

7) Line 50-51 - What about antigenicity?

Response 14: Modified, see line 63.

8) Line 65 - Change "does not" to "can produce unreliable"

Response 15: Done, see line 79.

9) Line 65 - Change 'decomposed' to degraded (and change throughout rest of manuscript)

Response 16: Done, see line 80.

10) Line 69 - Change 'certain' to 'higher'

Response 17: Deleted.

11) Line 71 - ELISA requires writing in full

Response 18: Done, see line 87.

12) Line 71 - In situ hybridization

Response 19: Done, see line 87.

13) Line 73 - "All lyssavirus species" - is this specific to known lyssaviruses or does this include novel lyssaviruses?

Response 20: RT-nPCR was specific to 18 lyssaviruses. Modified, see line 92.

14) Line 75 - State rationale of 2nd round PCR and include product lengths

Response 21: Added, see lines 95-98.

15) Line 78 - What is meant by 'decayed' test?

Response 22: Modified to “Evaluation of the efficacy in testing degraded samples”, see line 176.

16) Line 85 - Total or viral RNA can be isolated

Response 23: Total RNA isextracted by using TRizol methods or the commercially kits. Modified, see lines 102-105.

17) Line 87 - Remove 'total'

Response 24: Modified, see line 104.

18) Line 91 - change 'mix' to 'mastermix'

Response 25: Note was deleted.

19) Lines 99, 111 and 117- Change 'recipe' to 'reagents'

Response 26: Modified, see lines 110, 124 and 132.

20) Line 112 - Need to include addition of controls into master mix and also the addition of cDNA controls

Response 27: Done, see response 6.

21) Line 118 - Include additional second round negative control

Response 28: Done, see response 6.

22) Line 121 - Include mixing agarose powder with TAE buffer and microwaving before the addition of ethidium bromide. SYBR safe can also be used

Response 29: Done, see response 7. The commercial EB substitution was added due to GelRedand SYBR safe are unacceptable commercial language, see lines 140-141.

23) Line 128 - Give an example of a suitable DNA marker

Response 30: Added in figure 1.

24) Lines 134-147 - This section requires more clarity. For example, what fragment is relevant (line 136); and more clarity is needed for like 143 - what are the 9 ten-fold dilutions made from?

Response 31: The commercial plasmids containing the full N gene of each lyssaviruses, see line 153. The step of ten-fold serial dilutions of plasmids was been added, see lines 158-159.

25) Line 158 - were these samples previously FAT +ve, or were they collected for the study regardless of the test result and then tested by FAT?

Response 32: The 9624 samples collected by clinical rabies diagnoses and surveillance were tested by FAT and nested RT-PCR in parallel. The descriptions were modified, see lines 171-172 and 267-269.

26) Line 165 - What is meant by 'everyday'

Response 33: Modified, see line 178.

27) Line 176 - Include that all controls should pass. If a negative control is positive, or a positive control negative, the run should be disregarded.

Response 34: Modified, see lines 181-183.

28) Line 193 - does this mean the labs only detected RABV positive samples?

Response 35: The specialized laboratories tested the RABV positive and negative samples provided by our laboratory or stored in their own laboratories. This part was modified, see lines 204-210 and table 8.

29) Lines 199, 201 and 205 - Change 'condition' to 'reagents'

Response 36: Done, see lines 213, 215 and 219.