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TITLE:

Pan-Lyssavirus Real Time RT-PCR for Rabies Diagnosis

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KEYWORDS:

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SUMMARY:

This real-time RT-PCR using dsDNA intercalating dye is suitable to diagnose lyssavirus infections. The method begins with RNA extracted from rabies suspected ante-mortem or post-mortem samples, detailing master mix preparation, RNA addition, setup of the real-time machine and correct interpretation of results.

ABSTRACT:

Molecular assays are rapid, sensitive and specific, and have become central to diagnosing rabies. PCR based assays have been utilized for decades to confirm rabies diagnosis but have only recently been accepted by the OIE (World Organisation for Animal Health) as a primary method to detect rabies infection. Real-time RT-PCR assays provide real-time data, and are closed-tube systems, minimizing the risk of contamination during setup. DNA intercalating fluorochrome real-time RT-PCR assays do not require expensive probes, minimizing the cost per sample, and when the primers are designed in conserved regions, assays that are specific across virus genera rather than specific to just one virus species are possible. Here we describe a pan-lyssavirus SYBR real-time RT-PCR assay that detects lyssaviruses across the *Lyssavirus* genus, including the most divergent viruses IKOV, WCBV and LLEBV. In conjunction with dissociation curve analysis, this assay is sensitive and specific, with the advantage of detecting all lyssavirus species. The assay has been adopted in many diagnostic laboratories with quality assured environments, enabling robust, rapid, sensitive diagnosis of animal and human rabies cases.

INTRODUCTION:

Diagnosis of rabies using molecular methodologies was accepted by the OIE in 2018[1], recognizing the advantages of these techniques in confirming rabies cases, particularly in situations when the samples are sub-optimal, or for ante-mortem diagnosis, as there is no requirement for live virus or fresh samples. PCR assays for lyssaviruses require a reverse transcription (RT) before PCR can commence as the genome is RNA. RT-PCR assays that detect the 3' proximal region of the genome are considered the most sensitive, as transcriptional gradients occur during lyssavirus replication. Commonly used RT-PCR assays can be divided broadly into two categories, end-point (or gel-based) and real-time. Both approaches are sensitive and specific; however, the real-time assay has some additional benefits such as obtaining results in 'real-time' and being performed in an entirely closed tube system, thereby reducing the potential for operator contamination. There are two main approaches to detect lyssavirus-specific amplicons obtained using real-time assays. The first utilizes hydrolysis probes (such as TaqMan probes) that contain a fluorophore and a quencher. When the probe binds to the target region during amplification, the exonuclease activity of the polymerase results in dissociation of fluorophore and quencher, enabling the resulting fluorescence to be measured. The second utilizes a DNA intercalating dye (fluorochrome such as SYBR Green) that binds to double stranded DNA during amplification. The bound fluorochromes emit fluorescence that is detected at each cycle, allowing real time detection and quantification of the product. Due to the non-specific nature of binding to any dsDNA, a dissociation curve analysis is undertaken to confirm the specificity of the reaction. Real-time RT-PCRs are rapid due to the small amplicon sizes, typically less than 200 bp in length; however, identifying suitable regions to design primers and probes in conserved regions, can prove challenging, therefore removing the requirement for a probe is a distinct advantage.

A number of real-time RT-PCRs have been designed for specifically detecting individual strains or lineages of RABV[2] and also to detect lyssaviruses across the genus[3-9]. All assays will have a limit of detection dependent on how conserved the primer (and if necessary, the probe) sequences are across the genus. Indeed, emerging or novel virus strains may render the highly specific probe-based assays ineffective. The choice of the real-time detection (dye vs. probe) will depend on the intended application. For a laboratory conducting surveillance on locally sourced brain material and expecting high numbers of negative samples, the use of the cheaper intercalating dye is a sensible choice. The SYBR Green approach would also be optimal when conducting scanning surveillance where the presence of novel or divergent lyssaviruses would remain undetected by more restricted probe-based assays.

All members of the genus *Lyssavirus* cause the disease rabies, which is fatal once symptoms appear. The vast majority of human and animal rabies cases are due to rabies virus (RABV), the dominant reservoir for which is the domestic dog[10]. Bats are important host reservoirs for lyssaviruses and all but two lyssavirus species characterized have been identified directly in bats - Ikoma lyssavirus (IKOV) and Mokola virus (MOKV) - and of these two, IKOV has been speculated to have a bat host reservoir[11]. In addition to the 16 recognized lyssavirus species[12], there are two lyssaviruses that have been recently described: Taiwan bat lyssavirus (TWBLV)[13] and Kotalahti bat lyssavirus (KBLV)[14]. Lyssaviruses can be genetically divided into three phylogroups, with the majority of lyssaviruses, including RABV, belonging to phylogroup I. However, the most divergent lyssaviruses belong to phylogroup III and are

unlikely to be detected by RT-PCRs designed to target RABV or phylogroup I virus sequences.

The assay described here utilizes the real-time primer pair JW12-N165, first described in 2005[3]. The primers were designed to be pan-lyssavirus albeit the original application was as a TaqMan assay with probes to differentiate lyssavirus species. Subsequent confirmation that the primer pair was pan-lyssavirus in specificity was achieved utilizing a 2-step SYBR real-time assay on all lyssavirus species available including WCBV[15]. The primer pair is described here in a one-step RT-PCR real-time assay utilizing an intercalating fluorochrome, validated using representatives from all 16 recognized lyssavirus species. This one-step real-time assay is a rapid, sensitive, lyssavirus-specific assay and demonstrates that the robustness of the primer set to identify even highly divergent lyssavirus species.

PROTOCOL:

Samples from diagnostic material received at APHA after natural infection, or obtained by inoculating mice using protocols assessed by the APHA ethics and statistical committee under UK Home Office regulations under licence 70/7394.

1. Quantification of RNA using a Micro-Volume Spectrophotometer

1.1. Ensure the settings of the spectrophotometer are set to RNA.

1.2. Use 1-2 µL of molecular grade water to initialize the machine and set a baseline.

1.3. Use 1-2 µL of each test RNA sample to assess the RNA quantity.

1.4. Save the readings and document.

1.5. Adjust the RNA to 1 µg/µL, if required.

NOTE: RNA must be kept on ice (or in a cool block) at all times. If RNA is obtained using a column, or bead-based method the RNA is usually less than 1 µg/µL. In this situation use the RNA neat.

2. Preparation of RNA dilution series to determine end point sensitivity

2.1. Make a 10-fold serial dilution of the RNA.

2.1.1. Label tubes with the dilution series (e.g., 10^{-1}, 10^{-2}, etc.) and the RNA details.

2.1.2. Add 45 µL of molecular grade water to each tube.

2.1.3. Add 5 µL of the RNA (previously diluted to 1 µg/µL) and mix well.

2.1.4. Dispose of pipette tip in appropriate disinfectant and replace with a fresh tip.

2.1.5. Take 5 µL from the 10^{-1} and add to 10^{-2} tube and mix well.

2.1.6. Repeat 2.1.3-2.1.5 with the remaining dilutions.

NOTE: RNA must be kept on ice (or in a cool block) at all times.

3. Preparation of real-time RT-PCR reactions

3.1. Using a spreadsheet, plan the plate layout according to the number of test samples and control samples, for both the lyssavirus and ß-actin assays.

NOTE: If 4 samples are to be tested in duplicate with a positive and negative control, this equates to 10 reactions for both assays.

3.2. In a 'clean room' or area separate from the RNA template, wipe down the surfaces with an appropriate disinfectant prior to use or prepare a PCR workstation (if using). To prepare the workstation, wipe the cabinet surface with an appropriate disinfect and place the items required into the workstation and close the doors. Switch on the UV light for 10 minutes

3.3. Remove regents and primers from the freezer and thaw (reagents listed in **Table 1** and primers in **Table 2**).

NOTE: The enzyme mix is stored in glycerol so does not require thawing and must be kept on ice (or in a cool block) at all times. All other reagents can be thawed at room temperature.

3.4. Once thawed, mix the reagents and centrifuge briefly to collect liquid.

NOTE: Do not vortex the enzyme mix, just centrifuge briefly.

3.5. Prepare separate master mixes for lyssavirus and ß-actin. For each reaction, add 7.55 µL of molecular grade water, 10 µL of 2x Universal SYBR green reaction mix, 0.6 µL of forward primer, 0.6 µL of reverse primer, 0.25 µL of iTaq RT enzyme mix.

3.5.1. Use a spreadsheet to calculate correct volumes to avoid errors in manual calculating. Ensure enough master-mix is prepared to compensate for pipetting error. Therefore if 10 reactions are required (see NOTE in 3.1), prepare 12 reactions

3.5.2. Prepare the master-mix on ice (or in a cool block) and remain on ice until placed into the real-time machine.

3.6. Mix the prepared master-mixes, centrifuge briefly and dispense 19 µL into the relevant wells of strip tubes or a 96 well plate compatible with the real-time machine in use.

NOTE: Minimize the production of bubbles in the wells whilst pipetting.

3.7. In a separate room, or in a UV cabinet prepared as described in 3.2, carefully add 1 µL of the RNA previously adjusted to 1 µg/µL (see step 1.5) below the surface of the appropriate master-mix well and mix gently. Discard pipette tip into disinfectant directly after use (under the surface).

3.7.1. Add the controls after the test samples, with the positive control added next and the no template control (NTC – molecular grade water) added last. The amount of RNA used can be altered depending on the sample type, and RNA extraction used. The amount used must be validated to ensure the reaction is optimized.

3.8. Seal plate using strip lids or sealer taking care to ensure all the lids are firmly closed and labelled sufficiently to orientate the samples. Label the edge of the plate/strip tubes.

3.9. Spin down the samples using a centrifuge to collect all the liquid at the bottom of the wells.

3.10. Transfer plate to the real-time PCR machine, open the door and place in the holder ensuring the correct location/orientation of the samples according to the plate layout.

NOTE: If tube strips are used, ensure the holder is in place.

3.11. Open up the real-time PCR machine program and choose the option for SYBR real-time experiment with dissociation curve. Program the real-time PCR machine using the thermal cycling conditions specified in **Table 3**, including the data collection points.

3.12. Select **SYBR** as the fluorescent dye and select **unknown** as the sample type and insert a name into the correct sample name box.

NOTE: Differentiate between the replicates and also between the lyssavirus and ß-actin wells.

3.13. Choose a file location to save the experimental data, ensure the lamp will be switched off at the end of the run, then start the run.

NOTE: As the first step is an RT stage, no data is collected during this time, therefore, if the lamp requires a warm up period this can occur during the RT stage. The real-time machine and software will display the amplification curves in real-time, while the melting curve will be generated at the end of the cycle.

4. Data analysis

4.1. Once the run has been completed perform the data analysis as follows.

4.1.1. First analyze the amplification plot results of the test samples alongside the control samples. Positive samples display exponential ramps, usually followed by plateau and a C_t value. Negative samples display flat amplification plots with no C_t values (**Figure 1A**). The C_t

value is automatically calculated by the software, although this should be checked and manually altered if required.

4.1.2. Second, analyze the dissociation curve results of the test samples alongside the control samples. A positive sample will have a melting temperature (T_m) 77 – 80 °C, and overlap with the positive control **Figure 1B**).

4.1.3. Obtain the overall diagnostic result by ensuring the controls are valid. Use **Table 4** to interpret the results in relation to the internal ß-actin control. If the positive control samples are negative and/or the negative samples are positive, the run should be disregarded.

4.1.4. Record the C_t and T_m values obtained for the control RNA in a 'control card' to enable trend analysis and help identify drifts in the assay sensitivity.

REPRESENTATIVE RESULTS:

Following the protocol describe above, the sensitivity of the pan-lyssavirus RT-PCR was demonstrated on a dilution series of the control standard virus (CVS) (**Figure 1**) and a range of other lyssaviruses (**Figure 2** and **Figure 3**). SYBR Green I dye was utilized as a universal one-step RT-PCR, where cDNA synthesis and PCR amplification are carried out in a single tube. As the amplification of the specific target occurs, more dye is bound, resulting in real-time increased levels of fluorescence. All dye intercalating real-time assays must be interpreted in two phases: amplification and dissociation. The amplification phase is identical to any real-time amplification (**Figure 1A**). There is a linear 'early phase' during the early cycles where DNA amplification cannot be calculated due to insufficient signal in relation to the background. The length of this is directly related to the amount of target in the sample. Subsequently, there is an exponential phase where the doubling of DNA molecules is detected and recorded. Finally, the plateau phase is reached (apart from the highly diluted samples which may not reach this phase before the end of the program). In this phase, the intensity of fluorescence levels out, due to the exhaustion of reagents. The amplification plots observed using a 10-fold serial dilution of CVS, conformed to the expected plots (**Figure 1A,C**) where the lyssavirus assay demonstrated a higher sensitivity than the ß-actin assay. The dissociation curve was calculated after amplification, where the dsDNA was dissociated into ssDNA by an incremental increase in temperature and the fluorescence monitored as a function of temperature (**Figure 1B, D-F**). The threshold temperature at which the specific amplicon dissociates into ssDNA, caused a release of fluorescence, which was measured by the thermocycler software (T_m). This dissociation phase provided data on the amplicon size, enabling the user to interpret the result in comparison to a positive control, resulting in a negligible likelihood of false-positive results. The T_m observed for CVS using the pan-lyssavirus assay (**Figure 1B**) and ß-actin assay (**Figure 1D**) are distinct, and aided the user to confirm the correct assay analysis by noting the T_m obtained (**Figure 1E**). Furthermore, the C_t and T_m values between runs and operators was assessed and shown to be reproducible (**Table 5**). The threshold used to calculate the C_t value was calculated automatically by the software and dependent on many factors, including the reaction mix or instrument used. Over the 12 independent runs the mean C_t was 20.66 (SD 0.63) for the lyssavirus assay and 27.5 (SD 1.13) for the ß-actin assay. In contrast the variation observed in the T_m values was markedly lower, due to the lack of external influences on this measurement. For example, the mean T_m for the CVS lyssavirus

assay was 78.92 (SD 0.16) (**Table 5**), when compared to the mean of all lyssaviruses 78.81 °C (SD 0.531) (**Table 6** and **Figure 1F**). This lack of variation in the T_m across the *Lyssavirus* genus is advantageous as the same control RNA can be used irrespective of the lyssavirus in the sample, however differentiating between the lyssavirus species using the T_m is not possible, particularly because different RABV sub-lineages spanned the range of T_m values observed (**Table 6**). Non-specific amplification plots are rarely observed with this assay; however, specific parameters to define a positive result vs. a non-specific negative result are required. The SD observed across all lyssaviruses (0.531) was applied to the lowest (77.34 – LBVa) and highest (79.67 – IKOV) observed T_m values to set a range 76.8 °C – 80.2 °C for positive specific bands. Therefore, T_m values outside this range were considered non-specific and therefore a negative result. Occasionally multiple peaks are observed for a sample. If the dominant peak is at the correct T_m (for each replicate) then the sample is considered positive. The most common reason a non-specific peak is observed is due to primer-dimers, the assay has been optimized to minimize primer dimers. Primer dimers typically result in an amplicon smaller than that of the target sequence, therefore would have a T_m lower than the specific product.

A 10-fold serial dilution of three lyssavirus positive brain sample RNAs extracted using TRIzol, were run in parallel and plotted (**Figure 2**). The limit of detection for the three lyssaviruses varied, but none exceeded C_t 36. The R^2 coefficient values for the viruses plotted in **Figure 2** ranged from 0.9637 and 0.996. For all viruses analyzed (**Table 6**) the range did not exceed this, furthermore, 7 of the 29 lyssavirus had R^2 >0.99 (data not shown). Taking into account that the preparation of the dilution series is from total RNA extractions, the linearity observed provides evidence that the assay is robust. Finally, detection of all lyssavirus species (particularly the most diverse phylogroup III viruses) was investigated using a panel of RNAs spanning all three phylogroups in the *Lyssavirus* genus. RNA extracted from either original, or experimentally infected mice, brain material, was utilized using the protocols described above. The results confirm that the primers amplify all lyssavirus species, including the divergent phylogroup III lyssaviruses IKOV, WBCV and LLEBV (**Table 6** and **Figure 3**). A diverse panel of non-lyssavirus rhabdoviruses, originally collected and analyzed antigenically[16], and more recently genetically[17] were screened and no-cross reactivity was detected, indicating that the primers are specific for members of the *Lyssavirus* genus only (data not shown). The pan-lyssavirus real-time assay has been included in the EURL (EU Reference Laboratory) inter-laboratory proficiency schemes since 2013, demonstrating 100% concordance with other molecular assays such as the pan-lyssavirus TaqMan assay and the conventional RT-PCR assay in addition to the FAT (Fluorescent Antibody test).

Figures:

Figure 1: 10-fold serial dilution of CVS positive control RNA, run on the pan-lyssavirus RT-PCR assay, visualized as the amplification plot (A), and dissociation curve (B), and run on the ß-actin RT-PCR assay visualized as the amplification plot (C), and dissociation curve (D). NTC – no template control. Comparison of the CVS control RNA run on both the pan-lyssavirus RT-PCR assay (blue) and the ß-actin RT-PCR assay (red) demonstrating the difference in dissociation curves (E) – see **Table 5** for mean values; and finally dissociation curves for LBVa (blue) and IKOV (red) demonstrating the range of T_m values observed across the *Lyssavirus* genus (**F**).

Figure 2: 10-fold serial dilutions for three lyssavirus species: RABV (RV108), DUVV (RV131) and ARAV (RV3379). R^2= 0.996, 0.9962 and 0.9637 respectively. Data points at 10^{-7} (0.0001 ng/µL) reached the limit of detection (where a value was obtained).

Figure 3: Representative 10-fold serial dilution data from across the *Lyssavirus* genus (see individual legends for lyssavirus identity and Table 6 for tabulated results in comparison to other lyssaviruses). Panels **A, C, E, G** amplification plots and **B, D, F, H** dissociation curves for A, C, E, and G respectively.

Table 1: Pan-lyssavirus real-time RT-PCR master mix reagents.

Table 2: Pan-lyssavirus real-time RT-PCR primer details.

Table 3: Pan-lyssavirus real-time RT-PCR cycling conditions.

Table 4: Summary of outcomes and overall results for pan-lyssavirus real-time RT-PCR. Negative is designated to a sample with no C_t value (amplification) and no melt temperature (dissociation), or a melt temperature which is outside of the T_m range for positive lyssaviruses (76.8 °C – 80.2 °C). Positive is designated to a sample with a C_t value (amplification) and a melt temperature (dissociation) which is inside of the T_m range for positive lyssaviruses.

Table 5: Inter-run analysis of CVS positive control across 12 independent runs including multiple operators.

Table 6: Summary of pan-lyssavirus real-time RT-PCR specificity, sensitivity and T_m for representative lyssaviruses across all three phylogroups. The mean T_m across lyssaviruses was 78.81 (SD 0.531).

DISCUSSION:

The pan-lyssavirus real-time RT-PCR assay described is a closed-tube, one-step assay which detects lyssaviruses across all three phylogroups. The assay has been validated for both animal and human rabies diagnosis, including post-mortem brain tissue (optimally brainstem), and ante-mortem samples such as skin biopsy, serially collected saliva, or cerebral spinal fluid (CSF). The primers utilized in this assay were first designed and utilized for a probe-based assay to differentiate between RABV, EBLV-1 and EBLV-2[3], which has been used in many OIE rabies laboratories and performs consistently in the EURL proficiency schemes. Subsequently the 'pan-lyssavirus' nature of these primers has been confirmed using a 2-step real-time assay[15]. The assay described here has utilized the primers to further optimize the RT-PCR in a one-step SYBR assay enabling a closed-tube, rapid system. Furthermore, training in rabies endemic countries using this assay has confirmed suitability to implement in any laboratory with basic PPE and quality systems to reduce cross contamination and trace samples, facilities to store the reagents and a real-time machine with SYBR detection. The assay is extremely robust and has 100% correlation with the FAT, with improved sensitivity for decomposed samples[3]. One of the main advantages for a DNA intercalating dye based assay in comparison to a probe based assay is the relative cost. An

additional benefit is that the assay only utilizes two primers, therefore there is less risk of failed detection due to sequence divergence, which has been a weakness in previously published probe-based assays. Indeed, representatives of all lyssavirus species (apart from TWBLV and KBLV) are detected using this assay, and sequence analysis of TWBLV and KBLV across the primer sites reveals no significant divergence strongly suggesting that they will also be detected using this method. The range of limit of detection observed across the viruses analyzed, can be considered to be due to two main factors. The first is that the RNA was isolated from clinical brain material, therefore the amount of genome copies in each undiluted sample is not directly comparable. The RNA was 'normalized' by adjusting the total RNA to 1 µg/µL; however, the proportion of viral genome RNA within that sample will vary. The second is the diversity of lyssavirus sequences, despite the primer sites being located in conserved regions, there remain positions of variation. Therefore, it is not surprising that the majority of lyssaviruses with lower sensitivity for the assay are phylogroup II and III viruses. The dissociation curve analysis represents an essential parameter, minimizing a false positive result which could otherwise occur due to the formation of primer dimers, or amplification of a non-specific region in the host genome. In reality, this is a rare occurrence and the dissociation curve analysis is equivalent to running an agarose gel to visualize correctly sized conventional RT-PCR amplicons. The range of acceptable T_m values has been provided (77-80 °C), based on the data collected in our laboratory. It is strongly recommended that individual laboratories collate in-house data to ensure the range is transferrable and amend accordingly. Interpretation of the results from both the amplification and dissociation plots, alongside the positive and negative controls and the ß-actin results, enables robust and reproducible diagnostic outcomes.

Outside the scope of this protocol is the RNA extraction method used to obtain high quality RNA. All RNA analyzed in this protocol was prepared using TRIzol; however, there are many suitable guanidium-based extractions RNA extraction protocols available, including column and bead-based extraction kits. Handling of lyssavirus positive (or suspected positive) samples must be within licenced biocontainment facilities approved within country. However, the total RNA extracted is non-infectious, therefore handled within low containment laboratories. Depending on the extraction method used, the requirement to quantify and dilute the RNA would need to be assessed. For phenol-based extractions, including TRIzol, this step is required and prevents inhibition of the assay from contaminating gDNA; however, column and bead-based extractions (particularly those with a DNA depletion stage) do not require dilution prior to testing.

Throughout the protocol, it is essential that care and diligence are used to prevent cross-contamination and accurate addition of sample to the correct wells. A spreadsheet with the reagent calculations and plate layout is available for download as a supplemental file. Good laboratory practice, including clean work surfaces, regular changes of gloves, use of barrier tips and different rooms/UV cabinets to separate each stage will minimize the chance of contamination. To ensure the test is performing with expected parameters, positive and negative controls must be included and all test samples run in duplicate (or triplicate).

The inclusion of controls is an essential feature of any PCR, particularly for diagnostics. Positive control RNA was prepared from CVS (challenge virus standard) infected mouse brains in batches and validated and calibrated to ensure consistency between batches. The

control RNA was quantified and diluted to 1 µg/µL. RNA for which a positive result was obtained in a serial dilution down to at least 10^{-4} (equal to 100 pg/µL) was considered fit for purpose. The positive control RNA was stored at -80 °C in 10^{-1} aliquots. When required, an aliquot was diluted 1:100 to provide a working stock at 1 ng/µL and stored at -80 °C in 5 µL single use aliquots. The diluted positive control RNA was used to represent low level positive samples, and to ensure that any reduction in sensitivity of the assay was detected. A 'control card' was kept for each control to monitor the C_t values and identify trends (**Table 5**). **Table 5** demonstrated good inter-run comparability for the CVS positive control sample C_t and T_m values across multiple days and operators, providing reassurance that the assay is robust and reproducible. Results that deviate from these measurements should be investigated and test samples repeated if necessary. Molecular grade water was included in every run as an NTC to confirm the reagents were free from contamination with lyssavirus RNA and confirm a negative sample. Furthermore, to ensure RNA extraction efficacy, ß-actin was tested alongside the test samples in a separate tube. The lyssavirus positive control RNA was also used for the ß-actin positive control. Other endogenous genes or heterologous internal control systems can be utilized. Use of these controls ensured that all steps were analyzed under the same conditions as the test samples. Occasionally, if the sample was highly degraded or did not contain sufficient host RNA (such as saliva or CSF), the endogenous gene PCR can fail. In this instance, where the lyssavirus real-time RT-PCR result was positive, confirmation on an independent RNA extraction – to rule out contamination during RNA extraction on the original test or by a secondary test (either molecular, such as conventional RT-PCR), or FAT. Use of **Table 4** during analysis of a diagnostic sample ensured the correct interpretation.

Regardless of the molecular assay used to confirm rabies infection, follow up investigations using Sanger sequencing to determine the lyssavirus species and classical techniques in virology such as FAT or virus isolation should also be undertaken to allow for further virus characterisation and support the notification of positive cases to OIE and WHO.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 OIE. in *OIE Terrestrial Manual 2018* 8-14 (2018).

2 Panning, M. et al. Comparative analysis of rabies virus reverse transcription-PCR and virus isolation using samples from a patient infected with rabies virus. *Journal of Clinical Microbiology.* **48** (8), 2960-2962 (2010).

3 Wakeley, P. R. et al. Development of a real-time, TaqMan reverse transcription-PCR assay for detection and differentiation of lyssavirus genotypes 1, 5, and 6. *Journal of Clinical Microbiology.* **43** (6), 2786-2792 (2005).
4 Wang, L. et al. A SYBR-green I quantitative real-time reverse transcription-PCR assay for rabies viruses with different virulence. *Virologica Sinica.* **29** (2), 131-132 (2014).
5 Wadhwa, A. et al. A Pan-Lyssavirus Taqman Real-Time RT-PCR Assay for the Detection of Highly Variable Rabies virus and Other Lyssaviruses. *PLOS Neglected Tropical Diseases.* **11** (1), e0005258 (2017).
6 Nadin-Davis, S. A., Sheen, M., Wandeler, A. I. Development of real-time reverse transcriptase polymerase chain reaction methods for human rabies diagnosis. *Journal of Medical Virology.* **81** (8), 1484-1497 (2009).
7 Hoffmann, B. et al. Improved safety for molecular diagnosis of classical rabies viruses by use of a TaqMan real-time reverse transcription-PCR "double check" strategy. *Journal of Clinical Microbiology.* **48** (11), 3970-3978 (2010).
8 Faye, M. et al. Development and validation of sensitive real-time RT-PCR assay for broad detection of rabies virus. *Journal of Virological Methods.* **243** 120-130 (2017).
9 Dacheux, L. et al. Dual Combined Real-Time Reverse Transcription Polymerase Chain Reaction Assay for the Diagnosis of Lyssavirus Infection. *PLOS Neglected Tropical Diseases.* **10** (7), e0004812 (2016).
10 Fooks, A. R. et al. Rabies. *Nature Reviews Disease Primers.* **3**, 17091 (2017).
11 Marston, D. A. et al. Ikoma lyssavirus, highly divergent novel lyssavirus in an african civet(1). *Emerging Infectious Diseases.* **18** (4), 664-667 (2012).
12 ICTV. *Virus Taxonomy: 2018 Release: Email ratification October 2018 (MSL #33)*, <https://talk.ictvonline.org/taxonomy/> (2018).
13 Hu, S. C. et al. Lyssavirus in Japanese Pipistrelle, Taiwan. *Emerging Infectious Disease.* **24** (4), 782-785 (2018).
14 Nokireki, T., Tammiranta, N., Kokkonen, U. M., Kantala, T., Gadd, T. Tentative novel lyssavirus in a bat in Finland. *Transboundary and Emerging Diseases.* **65** (3), 593-596 (2018).
15 Hayman, D. T. et al. A universal real-time assay for the detection of Lyssaviruses. *Journal of Virological Methods.* **177** (1), 87-93 (2011).
16 Calisher, C. H. et al. Antigenic relationships among rhabdoviruses from vertebrates and hematophagous arthropods. *Intervirology.* **30** (5), 241-257 (1989).
17 Aznar-Lopez, C. et al. Detection of rhabdovirus viral RNA in oropharyngeal swabs and ectoparasites of Spanish bats. *Journal of General Virology.* **94** (1), 69-75 (2013).

Figure 1

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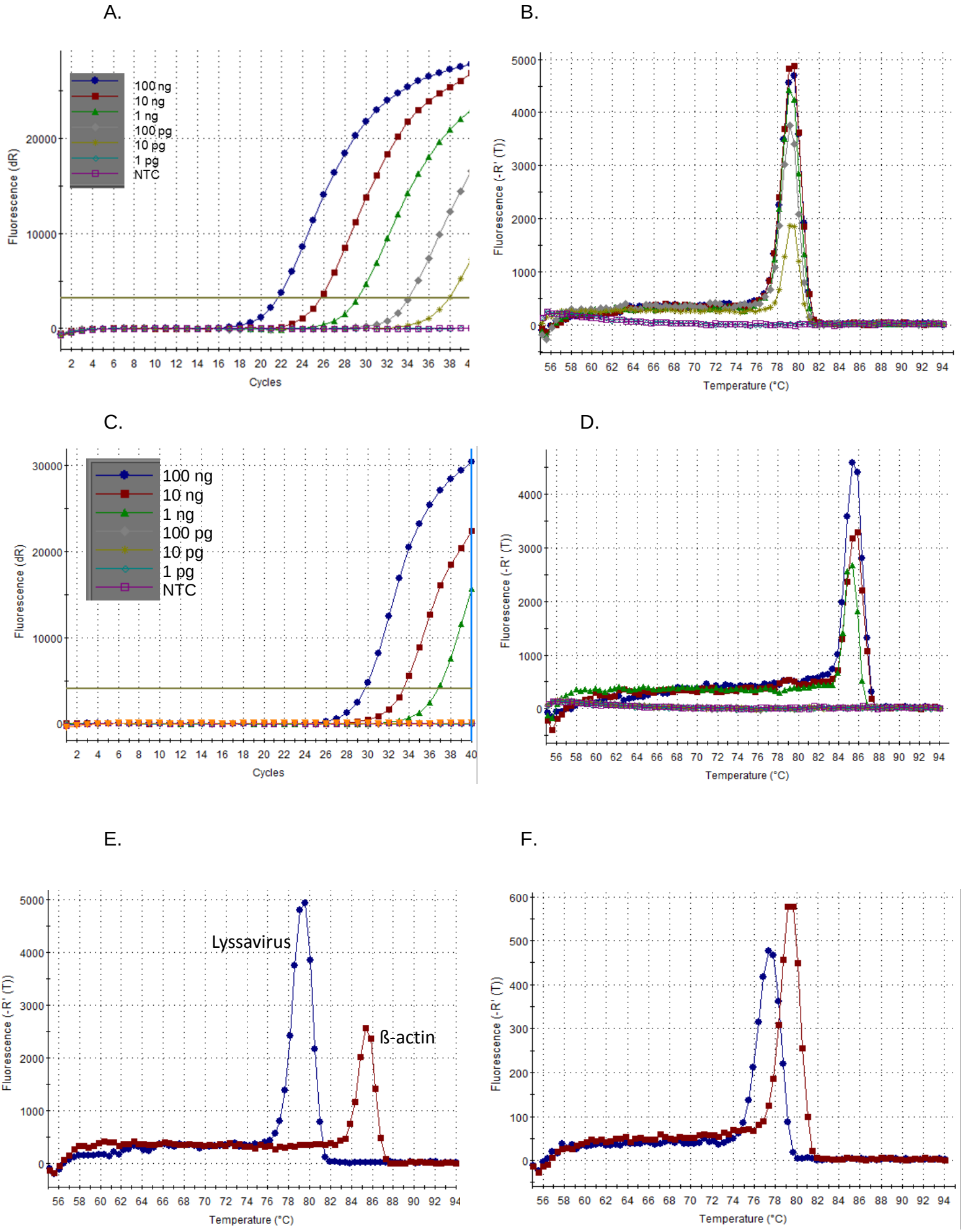

Figure 2

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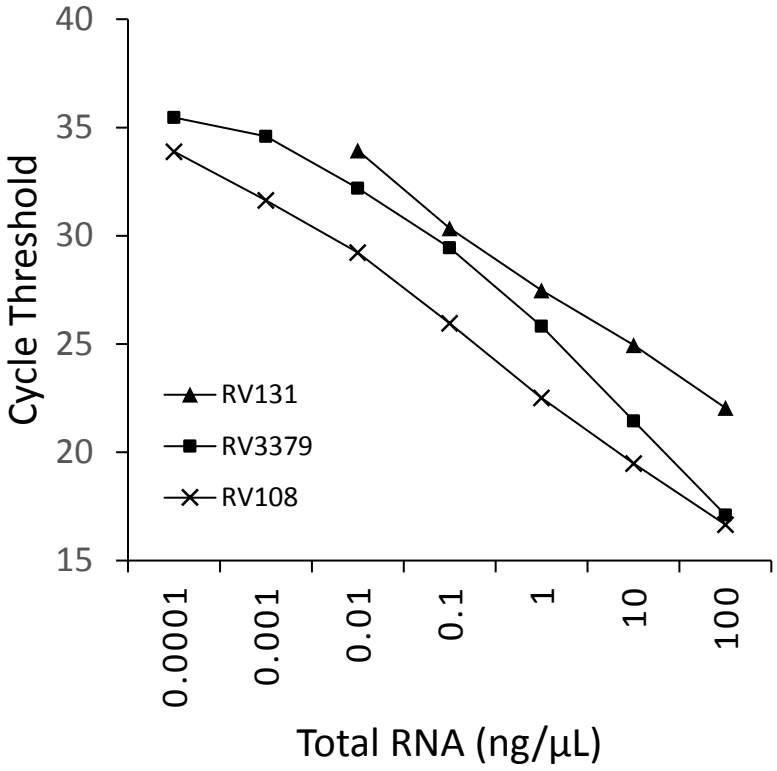

Figure 3

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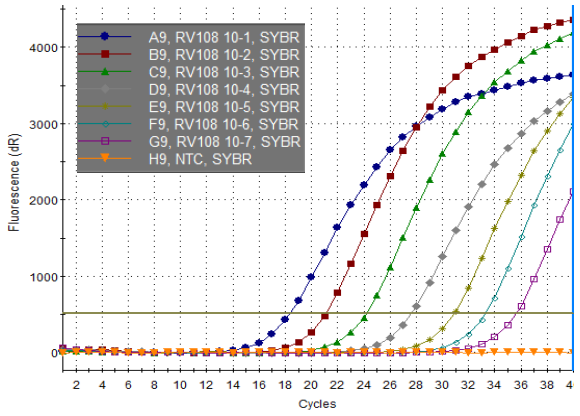
A.



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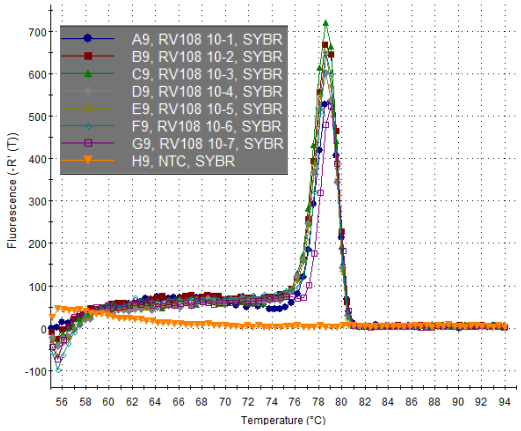

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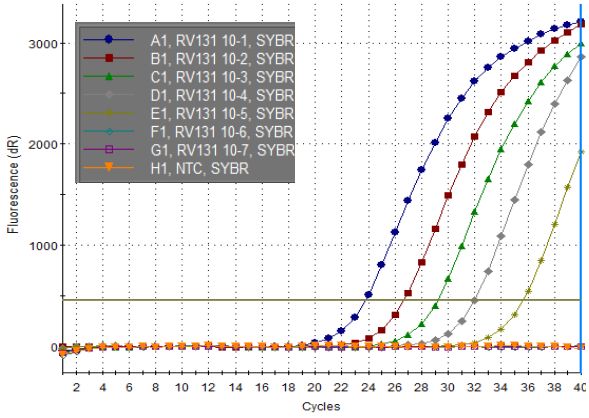

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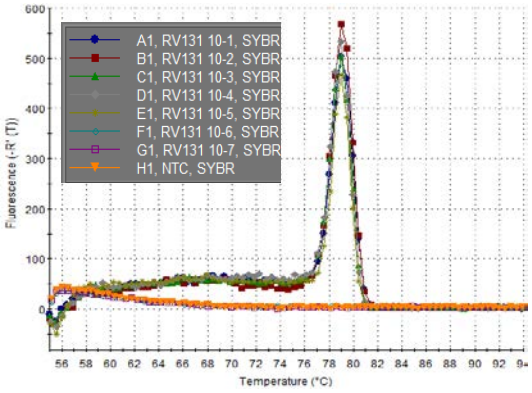

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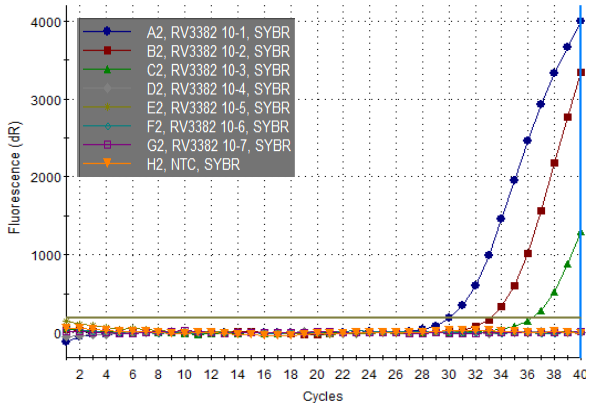

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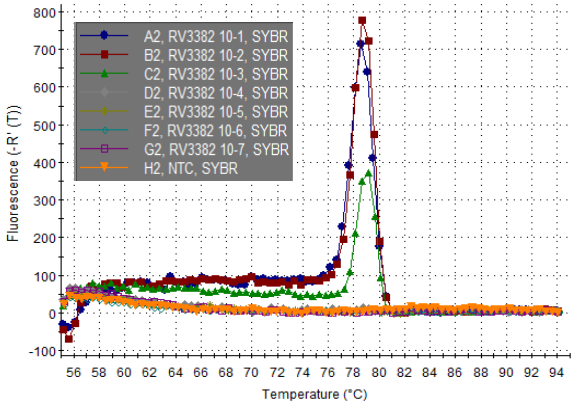

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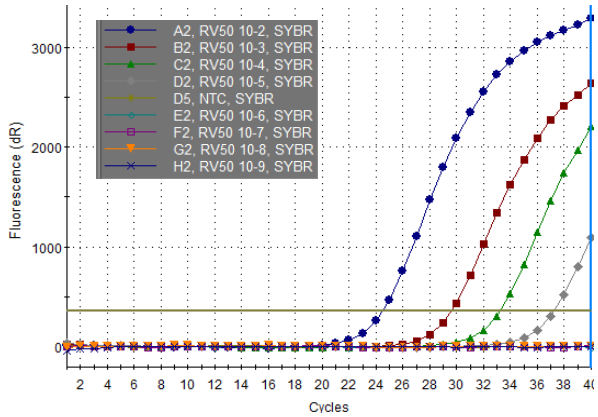

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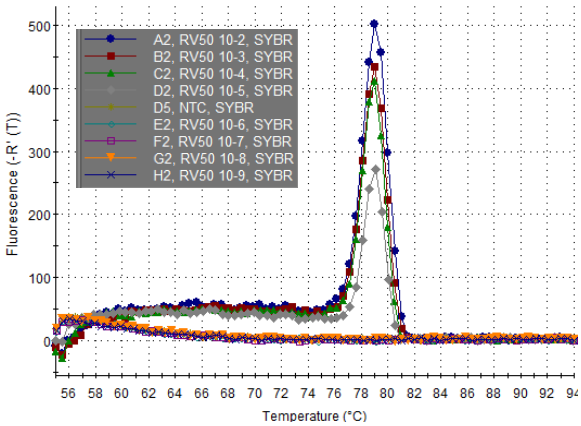

Table 1

Reagent	µL / Reaction
Molecular grade water	7.55
2 × Universal RT PCR reaction mix	10
Primer Forward [20 µM]	0.6
Primer Reverse [20 µM]	0.6
RT enzyme mix	0.25
Total per reaction	**19**

Table 2

Assay	Primer name	Primer role	Sequence 5'-3'
Lyssavirus	JW12	RT-PCR	ATG TAA CAC CYC TAC AAT G
	N165	PCR	GCA GGG TAY TTR TAC TCA TA
ß-actin	ß-actin intronic	PCR	CGA TGA AGA TCA AGA TCA TTG
	ß-actin reverse	RT-PCR	AAG CAT TTG CGG TGG AC

Primer positions are given in relation to Pasteur virus sequence (M13215) and mouse ß-actin g

Position[1]
53-73
165-146
1051-1072
1204-1188

gene sequence (NM_007393)

Table 3

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Stage	Cycles	Temperature	Time	Data Collection
Reverse Transcription	1	50 °C	10 min	
RT inactivation/initial denaturation	1	95 °C	5 min	
Amplification	40	95 °C 60 °C	10 s 30 s	 end point
Dissociation curve analysis	1	95 °C 55 °C 55 - 95 °C	1 min 1 min 10 s	 all points

Table 4

Test Result	Internal ß-actin control	Overall result
Negative	Negative[1]	Invalid. Repeat extraction and assay[2]
Negative	Positive	Negative result reported
Positive	Positive	Positive result reported
Positive	Negative[1]	Repeat extraction and assay[3]

[1]Use of heterologous external control would be beneficial during repeat extraction.

[2] If a second negative result is obtained for the internal control, the sample will be reported as untestable by this assay

[3] If a second negative result is obtained for the internal control, alongside a positive test result a secondary rabies diagnostic test should be undertaken to confirm this result.

y.

Table 5

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	Lyssavirus assay		ß-actin assay	
	Ct	Tm	Ct	Tm
Mean	20.66	78.92	27.5	85.26
SD	0.63	0.16	1.13	0.35
LCL (95%)	19.39	78.59	25.23	84.56
UCL (95%)	21.93	79.25	29.76	85.96

Table 6

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Phylogroup	Species	Virus ID	Lineage	Limit of detection	Tm
I	RABV	RV50	US bat	10-5	79.5
I	RABV	RV51	US Fox	10-7	77.6
I	RABV	RV108	Chile bat	10-7	78.63
I	RABV	RV313	European Fox	10-9	78.53
I	RABV	RV437	European RacDog	10-7	78.09
I	RABV	RV1237	European Deer	10-8	78.76
I	RABV	RV334	Chinese Vaccine	10-8	79.03
I	RABV	RV102	Africa 2	10-7	78.58
I	RABV	RV995	Africa 3a	10-8	79.66
I	RABV	RV410	Africa 3b	10-7	79.03
I	RABV	RV2324	Africa 4	10-7	79.17
I	RABV	RV2417	Sri Lanka Dog	10-9	78.71
I	RABV	CVS-11		10-7	79.17
I	EBLV-1	RV20	Germany	10-6	79.05
I	EBLV-2	RV1787	UK	10-7	78.76
I	BBLV	RV2507	Germany	10-9	78.71
I	ABLV	RV634		10-8	78.25
I	DUVV	RV131		10-5	79.03
I	GBLV	RV3269		10-7	79.15
I	ARAV	RV3379		10-7	79.46
I	KHUV	RV3380		10-7	78.97
I	SHIBV	RV3381		10-7	78.59
I	IRKV	RV3382		10-3	78.59
II	LBVa	RV767		10-5	77.34
II	LBVd	RV3383		10-7	78.59
II	MOKV	RV4		10-3	78.81
III	IKOV	RV2508		10-5	79.67
III	LLEBV	RV3208		10-4	79.15
III	WCBV	RV3384		10-3	79

Name of Material/ Equipment	Company	Catalog Number
Art Barrier pipette tips (various sizes)	Thermofisher	various
Centrifuge	Beckman	Allegra 21R
Centrifuge (mico)	Sigma	
Finnpipettes (to dispense 0.5-1000 µL)	Thermofisher	various
iTaq Universal SYBR Green One-Step RT-PCR kit	Bio-Rad	172-5150
MX3000P or MX3005P real-tme PCR system	Stratagene	N/A
MicroAmp reaction plate base	Any suitable	
Optically clear flat clear strips (8)	ABgene	AB-0866
Perfect fit frame (if using tube strips)	Stratagene	N/A
Primers: for primer details see Table 2.		
Thermo-Fast 96 well plares, non skirted	ABgene	AB-600
Thermo-Fast strips (8) Thermo-tubes	ABgene	AB-0452
Vortex machine / Whirlimixer	Fisons Scientific equipment	SGP-202-010J

Unless stated, alternative equipment can be used

	Comments/Description
	Rotor capable of holding 96 well plates required. Step 3.8.
	Equivalent kits can be used if validated Eqivalent machines can be used if validated Used to hold tube strip and plates securely.
	Specific to machine Ordered at 0.05 µmole scale HPLC purified.

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Kind regards, Denise Marston

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15. Please upload each Table individually to your Editorial Manager account as an .xlsx file. *Complete*
16. Table 1: Please remove commercial language (SYBR®, Kit Bio-Rad iTaq™ Universal SYBR™ Green One-Step RT-PCR kit. Cat No: 172-5150). Please include a space between all numerical values and their corresponding units (20 µM). *Complete*
17. Table 3: Please change the time unit "sec" to "s". Please include a space between all numerical values and their corresponding units (50 °C, 10 s, etc.). *Complete*
18. Please reference Table 5 in the manuscript. *Complete*
19. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Complete
20. References: Please do not abbreviate journal titles. Apologies, as the Endnote file was downloaded from the JoVE website the specific details of the formatting were assumed to be correct. The issue appears to be how Endnote holds the citations in the library that caused this issue. To amend this took time but all journal titles are now in full.
21. Table of Materials: Please remove trademark (™) and registered (®) symbols. Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment. *Complete*

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript describes a diagnostic method for rabies, SYBR Green real time RT-PCR, which can detected all 16 recognized lyssavirus species in one step. This method has been accepted by OIE as a primary method, and adopted in many diagnostic laboratories. For readers to learn and adopt this method in their own laboratories more details should be added in the paper. In this regard following comments have been made.

Major Concerns:
1. Regarding controls: What are the positive and negative controls as well as the

standard/parameters to prepare them? *A section in the discussion has been added to describe how the positive control is prepared and address these concerns (Lines 335-359).*

What are the standard/parameters to prepare internal control ß-actin? Is there a range of CT value specialized to define positive control amplification? The amplification plot of ß-actin should be included in the results.

The lyssavirus positive control is also used as the ß-actin positive control as the RNA is prepared from lyssavirus positive mouse brain. Table 5 has been added to include inter-run analysis of the positive control with mean and SD values to aid the reader. The amplification and dissociation plots are included in Fig 1 (C and D).

2. As mentioned in Abstract, this assay has been adopted in many diagnostic laboratories. It would be helpful if authors can extend some information to show how the assay was validated in detection of clinical samples or in different laboratories. Also the authors claimed that the assay can detect all lyssaviruses, including the most divergent viruses IKOV, WCBV and LLEBV, but in Result section no data show the detection of WCBV. In addition, the capacity of this method to detect newly identified TWBLV and KBLV should be discussed.

Addition details of clinical sample data and other laboratories is provided (Lines 284-294). Until recently, we only had data from plasmids containing WCBV sequence, therefore the data was omitted as all other samples were clinically derived brain tissue. However, we now have WCBV grown in mice, therefore brain material could be utilised and compared. This data has now been added to table 5. Comment regarding capability of detecting TWBLV and KBLV has been added (Lines 295-301).

3. In Materials section authors should provide brief description of sample types of rabies virus and other 15 lyssaviruses used to establish and validate the method.

Table 6 provides information regarding the different lyssavirus samples. An extra column has been added with the virus ID (according to our laboratory) which will aid the reader to identify the specific virus strain.

4. The amplification plot of detecting all 16 lyssaviruses (similar to Fig 1A) should be provided in Results. This plot can also be used to accompany Table 5.
The amplification plots are all from multiple dilution series runs. Therefore a selection of 4 (chosen to represent the whole dataset) have been used to create an extra figure (figure 3), including the dissociation curves. In addition, to link figures together, Figure 2 has been updated to present data from the same viruses.

5. Table 5: The sensitivity (lowest positive dilution) varied largely in distinct lyssaviruses from 103-109. What is the initial titer of each sample? Can these be converted to TCID50 or genome copy?

The RNA was prepared from brain material and normalised to 1 µg/µL. Therefore they cannot be converted into TCID50 or genome copy. Sentences have been added to discuss this (Lines 302-305).

Minor Concerns:

6. In Fig 1A: Is the indicator above NTC 10 pg (not ng)? *Yes the legend has the indicator above the NTC as 10 pg this has been made clearer.*
7. Line 71. The name of two lyssavirus species identified in animals rather than bats should be given. *Complete*
8. "Samples should be tested in at least duplicate (ideally triplicate)." Is repeated (lines 93 and 107). The one at line 93 is redundant, should be deleted. *Due to editorial changes these sections have been moved to the discussion, and only one remains.*
9. Figure 1A. Change "ug" to "µg". *Complete*

Reviewer #2:
Manuscript Summary:
In their manuscript entitled "Pan-lyssavirus SYBR Green real time RT-PCR for rabies diagnosis", the authors described the protocol of a real time RT-PCR for the detection of lyssavirus. The manuscript is well written and understandable. However, I think some practical details can be added. In addition, I have questions about the version of the protocol compared to the version already published recently the OIE Manual terrestrial 2018, which can be confusing. Details of these comments are indicated below, with other remarks/comments.

Major Concerns:
The protocol is detailed but probably more practical and basic information could be added to complete this protocol. For example, the version found in the OIE Manual terrestrial 2018 cited by the authors (and which must also have been written by these same authors) is more detailed, even if it seems to have been almost copy/paste for this JoVE manuscript (without some of these details). As examples, the authors did not indicate that the enzyme mix should kept on ice, and that the remaining reagents can be thawed at room temperature for point 2 in line 130, or that in line 125 and line 130 that to keep all reagents on ice during the preparation of the reaction master mix. Authors should also indicate the number of supplementary samples, such as in the Manual "Allow for pipetting variation by preparing at least two extra reaction mixes".

These points have been added as 'NOTES' in the protocol as per request of editors.

I notice some discrepancies between the protocol described recently in the OIE Manual terrestrial 2018 and the protocol presented by the authors, which is confusing for me and probably for the other readers. For example, the volume of RNA used is 1 µl here (line 133), but 2 µl of test RNA (where possible at concentration of 0.5-1 µg/µl for extracted samples) in the Manual. Another example is the volume of the primer N165 (-146): 0.6 µl per sample in the protocol of the manuscript and 1 µl in the OIE Manual terrestrial.

The discrepancies noticed are due to the changes that the kit manufacturer made which altered the final volume of the master mix. We validated the amount of RNA from 2 µL to 1 µL when this happened, but the change wasn't reflected in the OIE manual protocol. Either volume can be used but 1 µL is preferred. A note has been added at this step. The primer amount should have been 0.6 in the OIE manual so this protocol is correct.

- Line 32 and line 84: The authors referred to the WCBV, but I was not able to see the results with this species in the Table 5. Was this important species (in terms of genetic divergence) forgotten, or just not tested?

Until recently, we only had data from plasmids containing WCBV sequence, therefore the data was omitted as all other samples were clinically derived brain tissue. However, we now have WCBV amplified in mice, therefore brain material could be utilised and compared. This data has now been added to table 6.

- Line 119: Can the authors comments about the limitation of use of the cellular internal endogenous gene beta-actin and CSF or even some saliva samples (indicated line 102)? Indeed, by experience, we not that this internal control is quite often negative with acellular CSF (with low number of cells), and with some saliva samples. This issue can be solved with the use of an heterologous internal control, directly introduced in the sample for example.

Table 4 has a note added to it to suggest an external control to be used in such samples if the ß-actin is negative, also a section has been added in the text (lines 349-359).

- Lines 126-127: What are the evidences that the master mixes can be prepared and stored frozen up to one year?

Apologies this was data obtained for our probe based assay which used individual reagents in the master mix (dNTPs, $MgCl_2$ etc). The kit used here has all the reagents on one reaction mix (apart from the enzyme) so it is not necessary to make large master mixes and freeze in batches. Reference to his has been removed.

- Lines 149-151 and lines 181-184: Can the authors add more precision/details about this critical step? For example, it is known that the Tm value will be different from the Tm of the positive control used, depending of the base composition of the amplicon. What are the range of Tm values acceptable for the authors to consider a sample as positive, compared to the control? In particular line 159: what do the authors mean with "equivalent to the positive control"? Or what does a "melt curve which is different to the positive control" (line 183)? Also, the authors present in Figure 1 a single and clear peak, but we know that sometime we can have a principal peak and other secondary peaks. What are the interpretation of the authors when we faced to multiple peaks, especially if one of them match with the one of the positive control?

These points have been addressed in the 'results section' including adding ranges of acceptable Tm results to aid the reader (lines 214-232). In the discussion it is emphasised that the range needs to be calibrated according to each laboratory setting (lines 312-315).

- Line 163-164: For my part, this looks more like the limit of detection rather than the sensitivity, even if the first one is a part of the analytical sensitivity (OIE, Principles and methods of validation of diagnostic assays for infectious diseases). So I suggest the authors to modify, adapt or justified the term used here. Here the auhors mainly look to the efficiency of the real-time RT-PCR. Also they claim that the sensitivity for all the three lyssavirus is comparable. How it is justified, measured?

The results section has been modified in view of the reviewer's comments

- Line 166: Where are the results with WCBV? *Until recently, we only had data from plasmids containing WCBV sequence, therefore the data was omitted as all other samples were clinically derived brain tissue. However, we now have WCBV grown in mice, therefore brain material could be utilised and compared. This data has now been added to table 6.*

- Line 168: It will be nice to have the list of the different rhabdoviruses tested (in supplementary material for example). *References to two papers investigating the same virus panel have been cited (lines 244-245).*
- Table 4: For the two conditions with repeated experiments, what are the decision after them, if the results are still the same? *Notes have been added to the table and this is discussed in the results section (Lines 353-359).*
- Table 5: This table is difficult to interpret. Indeed, the dilution factor will depend of the initial concentration of the tested sample. But we do not have any information about them (primary samples, virus culture?). We do not have even any reference of the strain used (GenBank number if available or reference of the strain). I suggest to the authors to modify/complete this table to give to the readers the necessary information to interpret these results.

Table 5 (which is now table 6) has been modified to add the reference for each virus and the results section updated to clarify the samples are from brain material, which have been standardised to 1 µg/µL total RNA (Lines 302-304). The T_m for each virus has also been added.

Minor Concerns:
- Line 26: Develop "OIE" *Complete*
- Line 94: I do not have access to the Excel spreadsheet. *Uploaded in this revision*
- Line 164: This is not "specificity" but rather "sensitivity". Please correct. *Complete*
- Line 167: No capital letter for "Rhabdoviruses" *Complete*
- Line 169: Develop "EURL" *Complete*
- Line 186: There is both specificity and sensitivity. *Updated*
- Line 190: Can the authors provide some references to associate with "ante-mortem samples"? *Examples have been given*
- Figure 1A: Precise the legend (RNA?, what is NTC, what does +ve mean)? *Complete*
- Figure 1B: There is no legend. *Added*
- Figure 2: Why the authors do not indicate the parameters of the amplification curve for each samples (slope, R2, etc.)? *The figure has been altered to show data used in Figure 3, and R2 calculated. It should be noted the data is not used as controls to standardise results, rather to indicate the robustness of the assay across the genus. The data is discussed (lines 233-239).*

- Table 2: Indicate the position of the primers on a reference sequence (both for lyssavirus and beta-actin). These are provided in a previous publication, which has been cited. *Complete*

reagents calculation sheet

Lyssavirus SYBR Green real-time RT-PCR

iTaqUniversal SYBR Green One-Step Kit (Cat # 172-5150 or 172-5151)

Number of tests	1	(include 2 spare reactions)

Lyssavirus SYBR Master Mix

Reagent	Reagent Lot/Batch number	Vol. per tube (µl)	Vol. required (µl)
Nuclease-free water		*7.55*	**7.55**
iTaq Universal SYBR Green reaction mix		*10.00*	**10.00**
JW12 (20pmol/ul)		*0.60*	**0.60**
N165-146 (20pmol/ul)		*0.60*	**0.60**
iScript reverse transcriptase		*0.25*	**0.25**
Total Volume		*19.00*	**19.00**

ß-actin Master Mix

Reagent	Reagent Lot/Batch number	Vol. per tube (µl)	Vol. required (µl)
Nuclease-free water		*7.55*	**7.55**
iTaq Universal SYBR Green reaction mix		*10.00*	**10.00**
Beta-actin Intronic (20pmol/ul)		*0.60*	**0.60**
Beta-actin Reverse (20pmol/ul)		*0.60*	**0.60**
iScript reverse transcriptase		*0.25*	**0.25**
Total Volume		*19.00*	**19.00**

Aliquot **19µl** master mix per well

Add **1µl** of RNA template @ 1µg/µl

Final reaction volume is **20µl**

pipettes details:

Controls:

	Lyssavirus species	**Batch number**
Positive Control		
NTC (molecular grade water)		

Plate/Strip Layout

	Test	*Beta-actin*	*Test*	*Beta-actin*	*Test*	*Beta-actin*	*Test*	*Beta-actin*	*Test*	*Beta-actin*	*Test*	*Beta-actin*
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

NOTE: Test samples should be set up in duplicate (or triplicate)

PCR Machine used:

Set-up by:
Date:

Comments/Notes: