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Rapid identification of the raccoon rabies virus variant using a real-time reverse-transcriptase polymerase chain reaction

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ABSTRACT

The raccoon-associated variant of rabies virus (RRV) is enzootic throughout the eastern seaboard of the United States with frequent incursions into Canada. Many wildlife management agencies are actively engaged in control programmes targeting elimination of this disease and rapid identification of raccoon rabies cases is crucial to the success of these operations. This report documents the development of a reverse transcriptase real-time PCR (RT-qPCR) that specifically identifies this rabies virus variant (RRV RT-qPCR) and which can be readily multiplexed with a generic rabies virus RT-qPCR for use as a typing tool. Using a large collection of rabies virus samples representative of the variants circulating around the world, but with a focus on those occurring in the Americas, the RRV RT-qPCR was 100% sensitive and 99.31% specific. To further apply these assays for diagnostic purposes, addition of an RT-qPCR targeting the host β-actin mRNA, which serves as an internal amplification control, in a triplex format was shown to yield highly comparable results using a subset of our viral collection. Use of these assays for early and accurate identification of this viral variant will help to optimize the utilization of resources required for control of this disease.

1. Introduction

Since its emergence in Florida in the 1940s and its subsequent translocation into the mid-Atlantic states in the 1970s the raccoon-associated variant of rabies virus (RRV) has spread rapidly throughout the eastern seaboard of the United States (Jenkins et al., 1988; Wandeler et al., 2000). Further expansion of this epizootic westwards across the Appalachian and Adirondack mountain ranges and northwards across the border into Canada is a continuing threat (Monroe et al., 2016). Indeed several incursions of this disease into the eastern Canadian provinces of Ontario, Quebec and New Brunswick have been recorded (Fehlner-Gardiner et al., 2008a; Nadin-Davis et al., 2006a; Rees et al., 2011; Wandeler and Salsberg, 1999). Despite extensive control measures leading to the eradication of these outbreaks (Rosatte et al., 2009) (see https://www2.gnb.ca/content/gnb/en/departments/10/rabies.html), re-introduction of raccoon rabies by cross-border spread remains a threat. The largest raccoon rabies outbreak in Canada to date has been centred round the city of Hamilton, Ontario; this region reported 449 cases between December 2015, when the index case was identified, and December 2018 with additional cases still being reported. Due to the urban nature of the raccoon host, this disease represents a significant potential public health problem. Indeed although transmission of this variant to humans is rare such instances have been reported (Ma et al., 2018) and accordingly substantial resources are expended towards its eradication. The Hamilton outbreak involved public health education, vaccination of companion animals and the use of oral rabies vaccination to eliminate the disease from the raccoon population (Lobo et al., 2018). Early identification of an RRV outbreak can help to mitigate the extent of the problem and thus minimise the costs associated with such a control programme.

While the fluorescent antibody test (FAT) remains the gold standard method for rabies diagnosis (Dean et al., 1996), PCR methods, and in particular those based on real-time technology which reduces the potential for carryover contamination, have gained increased acceptance in recent years as useful diagnostic tools. A broadly-reactive RT-qPCR for rabies virus was originally developed in this laboratory for human *intra vitam* diagnosis (Nadin-Davis et al., 2009). A modified version of this assay performed comparably to the FAT for routine rabies diagnosis (Dupuis et al., 2015) and was even able to resolve FAT-indeterminate results in some cases (Appler et al., 2019). In addition to rabies diagnosis, viral typing remains a cornerstone of control programmes since this information identifies the host reservoir to be targeted. Although raccoons normally constitute the majority of specimens infected with RRV, spillover to other wildlife and to domestic animals has been reported; indeed the first recorded case during the 2000–2002 outbreak in New Brunswick occurred in a skunk (Nadin-Davis et al., 2006a). On

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Table 1
Primers employed in study.

Name	Sequence 5' to 3'	Location in RRV genome*	Location in bovine actin ORF**	Orientation
RABVD1-F	ATGTAACACCYCTACAATG	55-73		Forward
RABVD1-R1	GCMGGRTAYTTRTAYTCATA	146-165		Reverse
RABVD1-R2	GGCMGGRTAYTTRTAYTCAT	147-166		Reverse
RABVD1 probe	56-FAM/CCGAYAAGA/ZEN/TTGTATTYAARGTCAAKAATCAGGT/3IABkFQ/	78-111		Forward
ssRRV2-F	CAGGACTATGAAGCAGCAGAGTT	1169-1191		Forward
ssRRV2-R	TCCGAGTATAGACCGCCTCTG	1275-1295		Reverse
ssRRV2 probe	5Cy3/CAAAGACTGAGGTCGCTCTGGCAGACGACG/3IAbRQSp/	1194-1223		Forward
β-actin For	GGAYGAGGCTCAGAGCAAG		204-222	Forward
β-actin Rev	TCGTCCCAGTTGGTGACGAT		262-281	Reverse
β-actin probe	5Cy5/ATCCTCACCCTGAAGTACCCCATCGAGCA/3IAbRQSp/		229-257	Forward

* NCBI Accession # EU311738.
** NCBI Accession # AY141970.

the other hand, diagnosis of a rabid raccoon does not necessarily confirm the presence of the RRV variant as raccoons can be infected by other RABV variants (Fehlner-Gardiner et al., 2008a).

Antigenic typing, which depends on the availability of one or more highly specific monoclonal antibodies to yield a unique reactivity pattern with a particular rabies virus variant, has traditionally been the primary tool for variant differentiation (Fehlner-Gardiner et al., 2008b). However small changes in the nature of the epitope that reacts with the Mab can confound such testing and in some situations a confirmatory test is helpful. Nucleotide sequencing of the virus followed by phylogenetic analysis is the most accurate means of identifying the variant involved (Nadin-Davis, 2013) but it is time consuming and can further delay reporting of results. As RT-qPCR-based tests for primary rabies diagnosis are now supported by bodies such as the World Organisation for Animal Health and the World Health Organisation (Franka and Wallace, 2018; WHO, 2018), application of tests based on such a platform for variant identification becomes increasingly attractive and feasible. While a standard RT-PCR method that used variant-specific primers to differentiate several types of Canadian rabies viruses according to amplicon size was previously described (Nadin-Davis et al., 1996), the requirement for product analysis by gel electrophoresis increased testing time and the possibility of carryover contamination. A RT-qPCR that detected the RRV variant was described previously but this assay was not evaluated extensively against other rabies viruses (Szanto et al., 2011). Accordingly, this study describes the development of a RT-qPCR for specific identification of the RRV variant and evaluates its performance, in both duplex and triplex formats, together with assays for rabies virus and host β-actin mRNA. In all cases it is shown to be highly specific and inclusive for the RRV variant.

2. Methods

2.1. Rabies virus sample selection

All samples selected for these studies were comprised of brain tissue previously shown to be infected with rabies virus using the direct FAT (Dean et al., 1996). A total of 416 samples, including 126 RRVs, recovered from much of its range in the Northeastern US and Canada, and another 290 samples, representative of a wide range of non-RRV rabies virus variants, were included in this study. Canadian samples were characterised by both antigenic and genetic methods of viral typing; samples from other countries were, for the most part, characterised by sequence analysis only. A full description of the samples employed including their lineage associations is provided in supplemental Tables S1 (for RRV) and S2 (for non-RRV).

2.2. RNA extraction

Total RNA extraction was performed on 0.1-0.2 g brain tissue using TRIzol reagent according to the supplier's instructions (Life Technologies) or in some cases using a hybrid method in which the aqueous phase from the TRIzol method was purified using a MagMax system with an RNA purification kit AM1830 (ThermoFisher) as previously described (Nadin-Davis et al., 2017b). While the hybrid method has proven useful when amplifying large amplicons from viral RNA for the purposes of full genome characterisation, either extraction method proved to be suitable for generation of the small amplicons produced by RT-qPCR. Final RNA preparations were analysed spectrophotometrically using a Nanovue instrument (GE Healthcare) to determine RNA concentration.

2.3. Assay design

Alignments of the N gene sequences of rabies viruses of all major variants including RRV were generated using MEGA v5 (Tamura et al., 2011) and interrogated manually to identify regions conserved most specifically to RRV and thus suitable for design of 3' nuclease PCR assays. Initial review identified two such regions, designated RRV 1 and 2, neither of which were targeted by a previous assay (Szanto et al., 2011). While preliminary studies were conducted on both targets, the RRV1 assay overlapped with the target sequence used for general rabies virus detection thus rendering it unsuitable for multiplexing; accordingly later studies focused on the RRV2 target only. Since all samples to be assessed by these assays were positive by the FAT, a broadly reactive RT-qPCR (RABVD1) which detects all known variants of rabies virus as described previously (Dupuis et al., 2015; Nadin-Davis et al., 2009) was included as an internal positive control. During the later stages of the study the use of an additional amplification control targeting host β-actin mRNA sequence was also evaluated. Details of all primers and probes employed are described in Table 1.

2.4. RT-qPCR

Extensive evaluation of the RRV RT-qPCR was performed as a duplex assay with the RABVD1 RT-qPCR performed as described previously (Nadin-Davis et al., 2009) except that it was modified so as to include two reverse primers with sequence offset by one position (R1 and R2 – see Table 1) mixed in equimolar amounts to provide the reverse primer stock; this change facilitates improved amplification of a small number of samples which exhibited a mutation at the 3' end of the primer target. All reactions were performed using an Agpath One-step RT-PCR kit (ThermoFisher) and run in an Applied Biosystems 7500 FAST instrument. Two µl of template, generally representing between 0.2–1.0 µg total RNA, was included in a 25 µl reaction containing: 1x buffer, 0.2 µM RABVD1 forward and reverse primers, 0.4 µM RRV2 forward and reverse primers, both RABVD1 and RRV2 probes, labelled with fluorophores FAM and Cy3 respectively, at 0.12 µM and 1 µl of enzyme mix. Thermocycling was 50 °C, 10 min, 95 °C, 10 min, followed

by 40 cycles of 95 °C, 15 s and 50 °C, 1 min. This protocol halved the concentration of the RABVD1 amplification primers from that used previously (Nadin-Davis et al., 2009) to ensure that this assay did not interfere with the RRV assay. During conclusion of the study the value of including an additional assay targeting host β-actin mRNA sequence was also examined. This assay has been found to be a reliable internal control for brain tissue extracts in previous evaluations of the RABVD1 RT-qPCR. Evaluation of these triplex assays was performed with the inclusion of the β-actin primers at 0.2 μM and the Cy5-labelled probe at 0.12 μM and the concentration of the RABVD1 primers was increased to 0.3 μM with all other conditions unchanged.

For all assays dilutions of an Ontario RRV sample (2003ON3406) was used as a positive control and water blanks were included as negative controls. While the assay was not considered quantitative in nature the threshold settings for each run were manually set such that the most concentrated standard yielded a consistent Ct (+/- one cycle) so that the Ct values for samples run at different times were reasonably comparable. Ct values < 36 are considered positive by these assays while values between 36 and 40 are considered suspicious only.

The RRV RT-qPCR specificity confidence interval (CI) was calculated by the Wald Method using GraphPad available at https://www.graphpad.com/quickcalcs/confInterval2/.

3. Results

Since during routine laboratory testing for rabies the initial diagnosis is performed by FAT followed by variant typing, the initial evaluation of the RRV-specific assay was performed using a collection of rabies positive samples. Accordingly the RABVD1 RT-qPCR was included as the internal control with the RRV2 assay. Typical standard curves obtained for both assays when performed in this duplex format are shown (Fig. 1) with the metrics for this run summarised in Table 2. Both assays yielded linear standard curves over a dilution series of seven logs with efficiencies around 90%; however absolute viral sensitivity could not be established given the unknown titre of the sample used as a standard.

Table 2
Metrics of RT-qPCRs obtained in typical runs.

Format	Target	% Efficiency	R2	Slope
Duplex	RABVD1	89.5	0.999	−3.602
	RRV	93.3	0.999	−3.494
Triplex	RABVD1	92.2	0.999	−3.525
	RRV	90.8	0.999	−3.564
	β-actin	82.1	0.999	−3.842

To evaluate the specificity and inclusivity of the RRV RT-qPCR a collection of 416 samples representing a wide range of rabies virus variants from around the world were submitted for testing with the duplex assay. A summary of this collection is provided in Table 3 and includes references describing the genetic characterisation of many of these samples, while the Ct values obtained for all samples are presented in Tables S1 (all RRV samples) and S2 (all non-RRV samples). Notably all 126 samples previously typed as members of the RRV variant by either antigenic analysis and/or nucleotide sequencing were positive with both the RABVD1 and RRV RT-qPCRs. The Ct values for the RRV assay were typically higher than those for the RABVD1 assay, usually by 3–4 cycles and indeed many samples were very strongly positive with Ct values for both targets < 15 cycles. Of the 290 samples representing other RABV variants, 288 were positive for the RABVD1 target but negative for the RRV target. The two exceptions, which were positive for both targets, were: a big brown bat sample infected with a BB5 RABV variant (98RABN03232, RABVD1 Ct31.40, RRV Ct 35.81) and a Mexican skunk infected with the Mexican skunk MP2 variant (V854, RABVD1 Ct 20.05, RRV Ct 30.656). It was notable that for the latter sample the RRV Ct value was significantly higher (by 10 cycles) than the RABVD1 Ct. Repeat testing of these two samples using the same RNA extracts gave consistent results. Based on these observations, overall the RRV RT-qPCR yielded a sensitivity of 100% and a specificity of 99.31% (95% CI 97.35–99.98%).

During the course of this study it was suggested that PCR-based approaches might be used to support enhanced surveillance during a

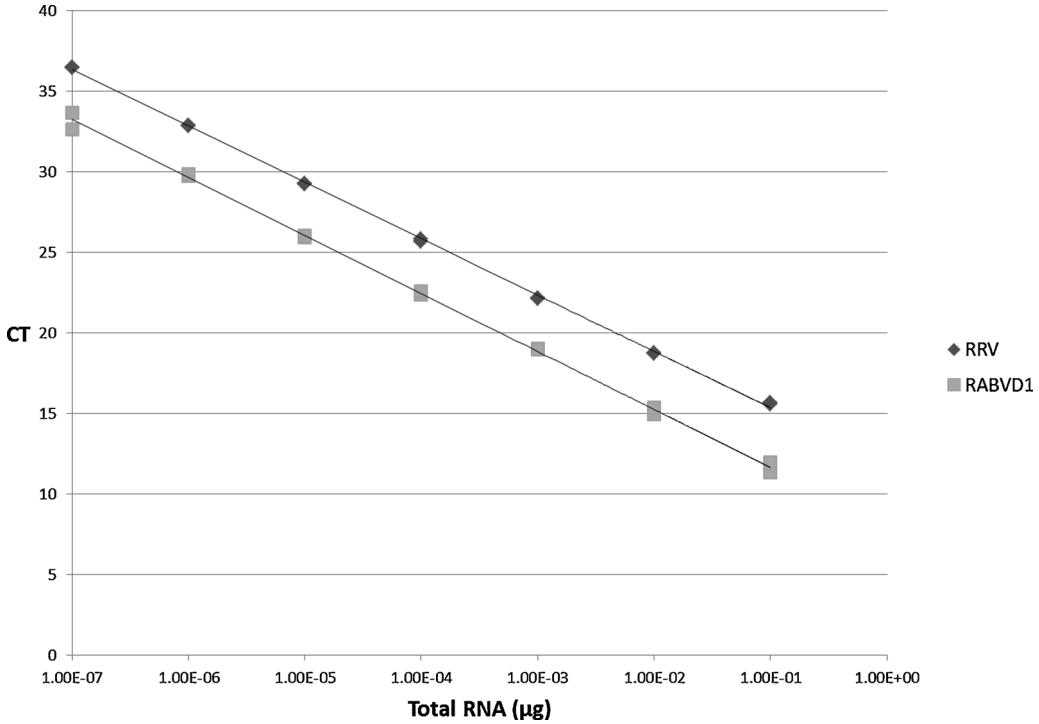

Fig. 1. Typical standard curves for RABVD1 and RRV RT-qPCRs run in duplex format using seven ten-fold dilutions of a RRV sample run in duplicate. The amount of total RNA tested in each assay is represented along the X axis by a logarithmic scale ranging from 0.1 pg to 1 μg RNA.

Table 3
Summary of rabies-positive samples used for assay evaluation.

Rabies variant	No. of Samples	Brief description of viral variant represented by samples	References
RRV	126	Includes 34 Canadian samples from NB (5), ON (15) and QC (14) and 92 US samples recovered as follows: one sample each from FL, MA, NH, NJ and VA and 2 from PA. Border states with Canada are represented by ME (11), NY (47) and VT (27). All apart from the Florida sample represent the Mid-Atlantic variant.	(Nadin-Davis et al., 2017b, 2018; Nadin-Davis et al., 2006a; Trewby et al., 2017)
Other lineages used to assess specificity of the RRV RT-qPCR:			
Africa:CD1	6	East and South Africa canid	(Nadin-Davis et al., 2002)
Africa:CD2	2	West African canid	(Nadin-Davis et al., 2002)
Africa:HP1	6	Southern African mongoose	(Nadin-Davis et al., 2002)
America:CD1	8	Mexican dog / Texas coyote (2) and Cuban mongoose (6)	(Nadin-Davis et al., 2002, 2006b)
America:CD2	4	Brazilian canid	(Bernardi et al., 2005; Nadin-Davis et al., 2002)
America:CD3	1	Peruvian dog	(Nadin-Davis et al., 2002)
America:HP/MP1	21	17 North central skunk (referred to in Canada as Western skunk) and Puerto Rico mongoose (4)	(Davis et al., 2013; Nadin-Davis et al., 2002; Nadin-Davis et al., 2008)
America:IB	93	Representatives of several distinct variants associated with insectivorous bats of the Americas thus: five sub-groups, BB1-5, associated with the big brown bat (34); Lasiurus variant (10); multiple variants associated with bats of the genus *Myotis* (37); silver-haired bat variant (9); Latin American bat variants (3)	(Nadin-Davis et al., 2017a, 2002; Nadin-Davis et al., 2010, 2001)
America:MP2	2	South central skunk	(Davis et al., 2013)
America:MP3	3	California skunk	(Davis et al., 2013)
America:VB	6	Latin American vampire bat	(Bernardi et al., 2005; Nadin-Davis et al., 2002; Nadin-Davis and Loza-Rubio, 2006)
Arctic	112	Mostly samples from northern Canada and a few from Alaska. Four subgroups are represented thus: A1, which has circulated for many years in Ontario (60), A2 (6) and A3 (43) from northern regions of N. America and A4 (1) from Alaska	(Nadin-Davis et al., 2002, 1993; Nadin-Davis et al., 2007)
Arctic-like	4	From Nepal, Korea and Iran	(Nadin-Davis et al., 2007)
Asia:CD1	3	Sri Lankan mongoose	(Nadin-Davis et al., 2002)
Asia:CD2	4	Canid variants from the Philippines and Thailand	unpublished
Europe-Middle East	8	Fox variant that has circulated widely in Europe and middle-eastern countries	(Nadin-Davis et al., 2002, 2003)
Mexico MP1	1	Mexican BC skunk	(Nadin-Davis and Loza-Rubio, 2006)
Mexico MP2	1	Mexican SLP skunk	(Nadin-Davis and Loza-Rubio, 2006)
Vaccine1	5	Represented by the live attenuated ERA strain used for wildlife ORV in Ontario for several years with a small number of cases reported in non-target species	(Fehlner-Gardiner et al., 2008b)
Total	290		

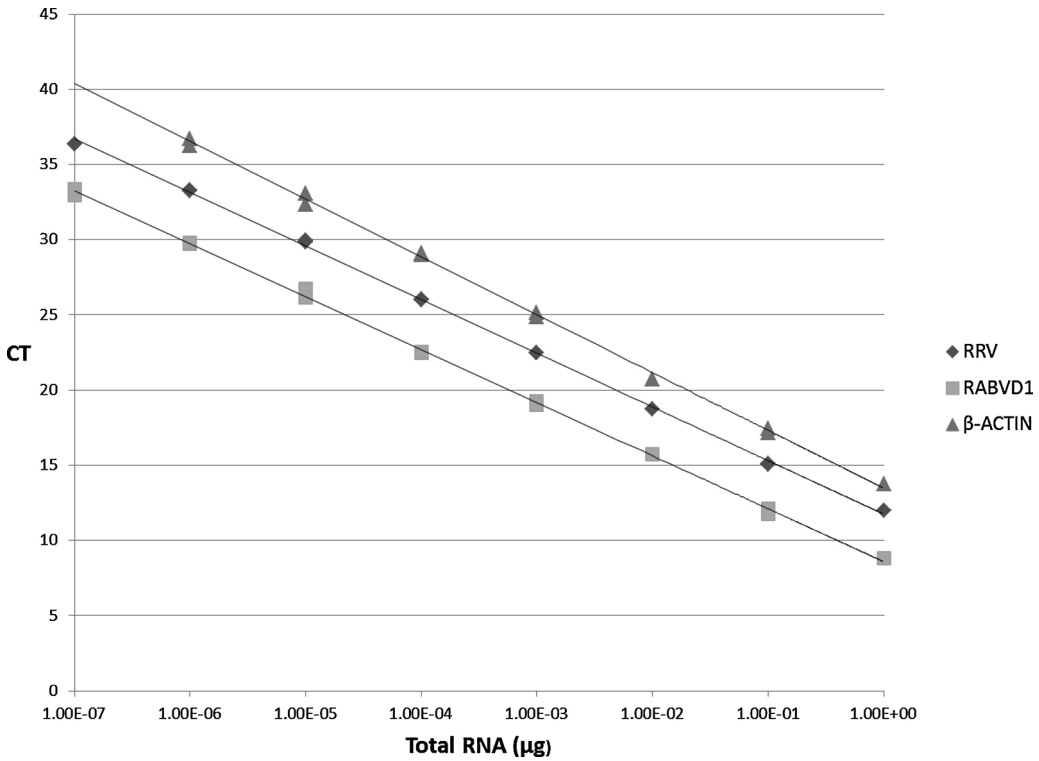

Fig. 2. Typical standard curves for RABVD1, RRV and β-actin mRNA RT-qPCRs run in triplex format. Eight ten-fold dilutions of a RRV sample were run in duplicate. The amount of total RNA tested in each assay is represented along the X axis by a logarithmic scale ranging from 0.1 pg to 1 µg RNA.

wildlife rabies control operation. In such a situation, where the RABVD1 RT-qPCR would be employed as a diagnostic assay to identify rabies negative samples, an alternate internal control would be required to confirm integrity of the input sample. Accordingly assay performance was also evaluated with inclusion of reagents to detect the host β-actin target in a triplex format. As shown in Fig. 2 the RABVD1 and RRV assays performed very similarly in this triplex format compared to the duplex format while the β-actin assay also performed well over a seven log 10 fold serial dilution series; metrics for the illustrated runs are shown in Table 2. A total of 105 samples from the original collection were retested with this triplex format and the resulting Ct values are also included in Tables S1 and S2. All 52 RRV samples were positive for all three targets as expected and yielded Ct values for the RABV and RRV targets that were generally in good agreement with those generated by the duplex assay. A total of 53 non-RRV samples, including the two samples for which aberrant results had been obtained with the duplex RT-qPCR previously, were retested with the triplex assay. Apart from the two aberrant samples all yielded positive results with the RABVD1 and β-actin assays and negative results for the RRV RT-qPCR. The bat sample (98RABN03232) yielded Ct values for the RABVD1 and RRV assays very similar to those obtained with the duplex assay; indeed, the Ct for the RRV target was close to the cut-off value in both assays while the sample was negative with the β-actin. The skunk sample (V854) was positive for all three targets but the RRV Ct was very close to the cut-off of 36 cycles and higher than the RABVD1 Ct by over 15 cycles. Tissue from both of these samples was very limited thereby precluding re-extraction of RNA for additional testing.

4. Discussion

The purpose of this study was to develop a sensitive and highly specific RT-qPCR for RRV variant detection. While a previous assay capable of detecting RRV had been described (Szanto et al., 2011), the design of that assay was based on a much more limited group of RRV samples and never underwent a comprehensive evaluation of its specificity and inclusivity unlike the assay presented here. The method presented here was developed based on a more comprehensive review of RABV sequence data which has become available since the design of the previous assay.

Two distinct testing scenarios were considered for application of these RT-qPCRs: the central laboratory responsible for routine rabies diagnosis and regional laboratories dedicated to enhanced wildlife surveillance specifically related to RRV outbreaks. In recognition of the slightly different workflows that would likely be followed in these two scenarios the RRV RT-qPCR has been evaluated using two different formats. To realise this goal this study has drawn extensively on the prior development of a broadly-reactive and sensitive RT-qPCR capable of detecting a wide range of rabies virus variants (Dupuis et al., 2015; Nadin-Davis et al., 2009), particularly those variants that circulate in the Americas. This same viral collection was of value for establishing the high specificity of the RRV RT-qPCR while a large collection of recently characterised RRV samples, drawn primarily from the north-eastern US states and Canada (Nadin-Davis et al., 2017b, 2018; Trewby et al., 2017) but also including samples from Virginia and Florida, was employed to demonstrate inclusivity.

From the perspective of a centralised diagnostic test facility establishing the specificity of the RRV test using a wide collection of viral variants was necessary given not only the chance of spillover infection of raccoons with other RABV variants from sympatric wildlife reservoirs but also the small but conceivable possibility that exotic rabies virus variants could be introduced into the Americas by transportation of infected animals from other parts of the world. Such long distance transfers have been documented in both western Europe (Ribadeau-Dumas et al., 2016) and more recently in the USA (Hercules et al., 2018). The data presented here yielded a specificity value of 99.3% for the RRV RT-qPCR due to the signal generated for two out of 290 non-RRV samples. The possibility that either of these two exceptional samples had been contaminated with the RRV variant appears slight since the V854 skunk sample originated from Mexico where RRV does not circulate and the 98RABN03232 bat sample from Ontario was processed prior to the known introduction of RRV into the province in 1999.

The Mexican skunk sample represents a skunk-associated variant belonging to the RABV American indigenous lineage and is thus phylogenetically relatively close to the RRV variant (Davis et al., 2013). However, review of the sequence of this sample (GenBank Accession number JQ513553) over the RRV target region indicated that it differed by a total of eight base substitutions compared to that for the RRV primers and probe. While this appears to have been enough to generate some signal for the RRV assay the large difference in Ct values (10–15 cycles) for the RABV and RRV targets would be suspicious given that for true RRV samples the difference in Ct values for these two assays was usually between 2 to 5 cycles. Unfortunately no other isolates of the Mexican MP2 skunk variant were available for testing so it is unknown if this is a true indication of the reactivity of this variant. However, inclusion of guidelines for test interpretation in which Ct differences between the RABV and RRV targets $\geq$10 cycles are to be flagged as suspect could potentially identify such false negatives. Notably the two SCSK samples which represent a distinct skunk variant of the American indigenous lineage did not cross-react with the RRV RT-qPCR. The other sample (98RABN03232), the only one of 14 infected with the BB5 variant associated with Canadian big brown bats that gave a false positive result, consistently yielded high Ct values for both RABV and RRV targets and was negative against the β-actin locus in the triplex test indicating poor sample integrity; the poor quality of this sample may have contributed to non-specific probe degradation leading to a false positive result. In a diagnostic setting the absence of signal from the β-actin target would render the sample unfit for testing.

It should be pointed out that rabies negative samples were not included in this evaluation as previous studies have established the robust performance of the RABVD1 RT-qPCR (Appler et al., 2019; Dupuis et al., 2015; Nadin-Davis et al., 2009). Any RABVD1-negative RRV-positive sample would clearly be flagged as suspicious and require additional testing.

5. Conclusion

The RRV RT-qPCR reported here scored 100% sensitivity, detecting all samples infected with the RRV variant in both the duplex and triplex formats, and a specificity of 99.31%. We thus conclude that this assay is a highly robust addition to this suite of methods of value for rapid typing of the RRV variant and its deployment should prove most beneficial to programmes aimed at control of raccoon rabies.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113713.

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