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MICROBIOLOGY

An RT-rtPCR assay for detection of rabies virus in bovine specimens

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ABSTRACT: *Bovine rabies is endemic in most Brazilian States, including Rio Grande do Sul (RS), which has faced an unprecedented rabies outbreak between 2011 and 2018. We described a real-time reverse transcription quantitative PCR (RT-rtPCR) for detection of rabies virus (RABV) in bovine samples. The primers were designed targeting a highly conserved region of the nucleoprotein (N) gene of RABV obtained from cattle. The detection limit corresponded to 13 DNA copies and the intra- and inter-run repeatability was adequate (CV<9%) in all dilutions tested. Amplification of other pathogens associated with neurological disease in cattle or cross-contamination was not observed. Brain samples from cattle suspicious of rabies (n=21) were tested in triplicate by the RT-rtPCR and by the gold-standard direct fluorescent antibody test (DFAT), resulting in 100% of sensitivity and specificity of the RT-rtPCR. Testing of additional 41 bovine brain samples submitted to the routine DFAT testing yielded 37 (90.2%) concordant results (30 positive/7 negative) and 4 (9.7%) inconclusive in DFAT and RT-rtPCR positive. These results showed a good concordance between the tests and a higher sensitivity of the RT-rtPCR. This assay represents an alternative for RABV detection, either as a confirmatory test or for large-scale diagnosis in endemic regions.*
Key words: *rabies, diagnostic, cattle, PCR.*

Um ensaio RT-rtPCR para detecção de vírus da raiva em espécimes bovinos

RESUMO: *A raiva bovina é endêmica na maioria dos estados brasileiros, inclusive no Rio Grande do Sul (RS), que enfrentou um surto de raiva sem precedentes entre 2011 e 2018. Descrevemos um PCR quantitativo de transcrição reversa em tempo real (RT-rtPCR) para detecção do vírus da raiva (RABV) em bovinos. Os primers foram desenhados visando uma região altamente conservada do gene da nucleoproteína (N) de RABV obtido de bovinos. O limite de detecção correspondeu a 13 cópias de DNA e a repetibilidade intra e inter-ensaios foi adequada (CV <9%) em todas as diluições testadas. Não foi observada amplificação de outros patógenos associados a doenças neurológicas em bovinos ou contaminação cruzada. Amostras de cérebro de bovinos com suspeita de raiva (n = 21) foram testadas em triplicata no RT-rtPCR e pelo teste de anticorpo fluorescente padrão ouro (DFAT), resultando em 100% de sensibilidade e especificidade do RT-rtPCR. O teste de 41 amostras de cérebro bovino adicionais submetidas ao teste de DFAT de rotina rendeu 37 (90,2%) resultados concordantes (30 positivos / sete negativos) e quatro (9,7%) inconclusivos em DFAT e RT-rtPCR positivo. Esses resultados mostraram boa concordância entre os testes e maior sensibilidade do RT-rtPCR. Este ensaio representa uma alternativa para a detecção do vírus da raiva, seja como teste confirmatório ou para diagnóstico em larga escala em regiões endêmicas.*
Palavras-chave: *raiva, diagnóstico, bovinos, PCR.*

INTRODUCTION

Rabies lyssavirus (RABV) is a member of the genus *Lyssavirus*, family *Rhabdoviridae*, order *Mononegavirales* (ICTV, 2018). The genus *Lyssavirus* is divided into seventeen viral species based on nucleotide and amino acid sequence analyses. RABV is the only lyssavirus species identified in the Americas to date, has worldwide distribution and has been classified in six variants (SCHAEFER et al., 2005). This virus is the agent of rabies, a zoonotic disease characterized by severe neurological disease of course generally fatal in domestic and wild mammals (OIE, 2018). In Brazil, RABV is

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maintained in two main cycles: urban rabies (caused by variant 2), where dogs are the main reservoirs, and the sylvatic cycle, where haematophagous bats, particularly *Desmodus rotundus,* are the reservoirs of RABV variant 3. Since these bats are the main source of infection for cattle in Latin America, RABV variant 3 is the most detected in farm animals from Brazil (SCHAEFER et al., 2005; DE ALMEIDA et al., 2020). Viruses involved in these cycles have been identified by genetic analyses (ITO et al., 2001, 2003; SCHAEFER et al., 2002), yet an additional natural cycle involving non-haematophagous bats probably exists (SCHAEFER et al., 2005).

Rabies is a disease listed by the World Organization for Animal Health (OIE) and kills nearly 59,000 people worldwide every year, mostly children in developing countries (OIE, 2018, 2019, 2020). Likewise, bovine rabies costs estimate of hundreds of millions of dollars in Latin America, mainly due to animal deaths (MAPA, 2009). Additional costs include vaccination of millions of cattle and post-exposure treatments of thousands of people exposed to infected/suspect animals (MAPA, 2009). In Rio Grande do Sul (RS), the southernmost Brazilian State, since 2011 has been reported many outbreaks of herbivorous rabies, that cause thousands of cattle deaths each year (KANITZ et al., 2014; ITOU et al., 2016; CARGNELUTTI et al., 2017; DE ALMEIDA et al., 2020; FERNANDES et al., 2020).

The RABV genome consists of a single-stranded RNA of negative sense, encoding five proteins: nucleoprotein (N), phosphoprotein (P), matrix (M), glycoprotein (G) and polymerase (L) (ICTV, 2011). The N gene is the most conserved sequence among RABV isolates and, thus, has been used as target for diagnostic tests based on nucleic acid detection (DEDKOV et al., 2018; FAYE et al., 2017; WADHWA et al., 2017). Considering its conservation, few mutations/variabilities in the N gene may be sufficient to determine RABV lineages and sub-lineages that allow us to suggest the rabies geographical dispersion and cycle, the origins of outbreaks and the groups/colonies of reservoirs of RABV (CARNIELI JÚNIOR et al., 2008; DE ALMEIDA et al., 2020; FERNANDES et al., 2020).

Many reverse-transcriptase quantitative polymerase chain reaction (RT-rtPCR) assays targeting the RABV N gene have been described and, in most cases, their performance has been evaluated using TaqMan or other RT-rtPCR probes (DEDKOV et al., 2018; FAYE et al., 2017; WADHWA et al., 2017). But there is a lack of information about SYBR based RT-rtPCR to detected RABV variant 3 in brain samples. In addition, many studies tested a limited number of samples from natural cases of herbivorous rabies and did not follow the validation process recommended by OIE (OIE, 2013).

Thus, this study o developed and standardize a SYBR green-based RT-rtPCR assay for detection of RABV in bovine samples (generally caused by variant 3), following the guidelines by OIE. This assay would be useful for detection of bovine rabies in endemic areas: to confirm the diagnosis of suspect/inconclusive DFAT testing and for conducting epidemiological investigations in which a large number of samples need to be analyzed.

MATERIALS AND METHODS

To this end, we first designed the primers and positive control based on the N gene of RABV viruses detected in cattle. Then, the assay linear range and efficiency were determined. For validation, the analytical characteristics were evaluated using the positive control. Diagnostic properties were evaluated by testing bovine brain samples that were positive and negative in the conventional gold standard rabies diagnosis (DFAT). Obtained results in the RT-rtPCR were compared with DFAT results.

The RABV N gene was selected as target based on its high conservation and the sequences available in GenBank. Sequences of N gene of RABV variant 3 from samples from cattle of Brazil, were obtained from the database and served as the basis for primer design. The primers (Table 1) were designed using the Primer Quest Tool (available on-line at https://www.idtdna.com/Primerquest/Home/Index) according to the current guidelines for rtPCR primers (RAYMAEKERS et al., 2009; THORNTON & BASU, 2011). Their detection spectrum was analysed *in silico* with 68 RABV sequences obtained from cattle and with 198 bovine housekeeping genes available in GenBank to ensure lack of amplification of host sequences.

The positive control consisted of a 420-bp synthetic DNA fragment (positions 509-928 of the RABV N gene - GenBank access AB685247) (gBlocks Gene Fragments, Integrated DNA Technologies). gBlocks DNA (1ng/µl) was diluted in a brain matrix cDNA solution produced with total RNA extracted from brain fragments a bovine negative to RABV. Total RNA extraction was performed using Trizol reagent (Invitrogen) and cDNA synthesis using the GoScript Reverse Transcriptase (Promega), according to the manufacturer's instructions. The internal control primers were previously described by Ashley et al. (2011) for detection of bovine GAPDH

(Table 1). DNase/RNase-free water was used as a negative control.

The rtPCR assay was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems), using the default program for all genes: 10 min at 95 °C followed by 40 cycles of 15 sec at 95 °C and one min at 60 °C. Individual rtPCR reactions were performed in 20µl in 96-well plates containing 7.2µl of DNase/RNase-free water, 10µl of Power SYBR Green (Applied Biosystems), 0.4µl of each primer and 2µl of the cDNA. Melting curves were obtained at the end of each reaction and analyzed (15 sec at 95 °C, 60 sec at 60 °C, and increased of 0.3 °C until 95 °C).

The linear range of the assay was determined using the synthetic DNA-negative cDNA prepared at an initial concentration of 1ng/µl in a series (6) of 10-fold (1:10; v:v) dilutions. Each dilution was tested 6 times in a single run. PCR efficiencies were determined by the formula: PCR efficiency (%) = $100 \times (10^{1/slope} - 1)$.

For analytical sensitivity and intra- and inter-run variability, 10-fold dilution series consisting of 15 separate dilutions were prepared using the synthetic DNA-negative cDNA prepared at an initial concentration of 1ng/µl, which was examined 6 times in each of 5 independent runs and tested by RABV RT-rtPCR analysis. The results of these analyses were used to calculate the detection limit (i.e. input concentration giving a positive RT-rtPCR result in 95% of the repeats) (BURNS & VALDIVIA, 2008). The copy number of the synthetic DNA-negative cDNA solution was estimated through the formula: number of copies = (amount of DNA in ng x 6.022 x 10^{23}) / (length of template in bp x 1x10^{9} x 650).

The inter-run, intra-run and total standard deviations (SD) were calculated by the formulas: inter-run SD, standard deviation of the means of all runs; intra-run SD, mean of the standard deviations of all runs; total SD, standard deviation of all replicates. The total coefficient of variation (CV) was calculated by the formula: total CV = total SD / (mean Ct-value of all replicates).

The analytical specificity was assessed testing in triplicates by RABV RT-rtPCR brains of cattle infected by pathogens causing similar neurological signs in RS: RABV, *Bovine alphaherpesvirus 5* and *Babesia* sp.

To investigate cross-contamination, 24 RABV positive control and 72 negative control wells (DNase/RNAse-free water) were arranged in a chequerboard pattern (FANG et al., 2007) and submitted to RT-rtPCR analysis in two different days.

Sensitivity and specificity of the RT-rtPCR were evaluated using 21 bovine brains of known infection status (RABV positive x negative), considering diagnostic sensitivity (DSe) and specificity (DSp) estimates of 98%, with an error of 5% margin allowed in estimate of DSe and DSp and a confidence of 90% (OIE, 2013). Thus, 10 RABV positive and 11 RABV negative brains were tested in parallel by DFAT and by the RABV RT-rtPCR. All samples used in this study was submitted to routine testing for rabies and stored at -20 ºC until processing. The samples were received between 2012 and 2017 from farms of different municipalities of Rio Grande do Sul. RNA extraction from fragments of cortex, hippocampus, thalamus and medulla from the samples was performed by the Trizol method and cDNA was produced using the Promega Kit. cDNA samples were then analyzed in triplicate by RT-rtPCR along with controls. The DFAT was performed using a FITC anti-RABV conjugate provided by Pasteur Institute (São Paulo). The DSe was calculated by the formula: total positive (TP)/(TP + false negative (FN)), and the DSp was calculated by formula: total negative (TN)/(TN + false positive (FP) (OIE, 2013). Additionally, we tested by RT-rtPCR 41 bovine brain specimens submitted to routine rabies diagnosis, being 30 DFAT-positive, 7 DFAT-negative and 4 DFAT-inconclusive. The samples were processed essentially as described above, with the exception that there were no replicates.

RESULTS

Linear regression analysis demonstrated that the RABV RT-rtPCR had a linear range

Table 1 - Primer sequences, target and locations.

Sequences (5' to 3')	Target	Nucleotide position
CACTGCGAGAGAAGCACTATTA (forward)	RABV N	687-708
CAAGCCCAATGAACGGAAATG (reverse)	RABV N	796-816
TGACCCCTTCATTGACCTTC (forward)	Bovine GAPDH	167-186
CGTTCTCTGCCTTGACTGTG (reverse)	Bovine GAPDH	239-258

extending from the 10^{-5} dilution to the 10^{0} dilution of the positive control with an $R^2 > 0.99$. Based on the slope, the efficiency of the assay was calculated to be 95.165% (Figure 1).

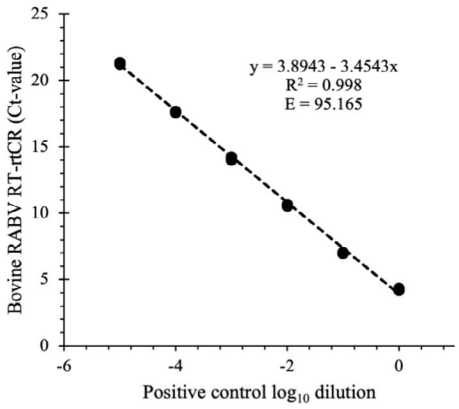
The detection limit (i.e. input concentration giving a positive result in 95% of the replicates), determined by probit analysis, was at a 1.82×10^{-9}dilution, corresponding to 12.99 DNA copies and Ct of 34.08. The mean results and variations for each dilution are summarized in table 2.

Both intra- and inter-run SD were low with maxima at 1.30 Ct and 2.71 Ct, respectively. The coefficient of variation (CV) ranged from 1.46% to 8.46%, indicating low variation among repetitions and runs. The CV increased towards and beyond the limit of detection. The mean Ct for the RABV RT-rtPCR assay was 22.81 ± 0.47 over 25 runs of the 15 dilutions of the positive control.

No false-positive or false-negative results were observed at the analytical specificity assay. The positive control samples on the chequerboard plate yielded mean Ct values of 18.01 ± 0.42. No false positives or cross-contamination were recorded in each of the runs.

The results of the RABV RT-rtPCR and DFAT in samples of rabies-suspect cattle are summarized in table 3. No false-positive or false-negative results were recorded. Estimates of the sensitivity and specificity of the assay are summarized

A



B

		Number of references samples	
		Known positive (10)	Known negative (11)
Bovine RABV RT-rtPCR results	Positive	10 TP	FP 0
	Negative	0 FN	TN 11
		Diagnostic sensitivity TP/(TP + FN) **100%** (72.2 to 100%)*	Diagnostic specificity TN/(TN + FP) **100%** (74.1 to 100%)*

Figure 1 - A. Scatter plot with the regression line (solid line) of a 10-fold (v:v) dilution of a bovine RABV synthetic prepared with a nucleic acid load covering the linear range. Each dilution was tested 6 times in a single run. B. Diagnostic sensitivity and specificity estimates calculated from set of results for samples bovine brain samples of known infection status to rabies virus based on direct immunofluorescence. TP and FP = True Positive and False Positive, respectively. TN and FN = True Negative and False Negative, respectively. *95% exact binomial confidence limits for diagnostic sensitivity and diagnostic specificity calculated values.

Table 2 - Inter- and intra-run variation for 15 two-fold dilutions of RABV synthetic DNA.

Samples	----------Results (Ct)----------				
Log_{10} dilution	Mean	Inter-run SD	Intra-run SD	Total SD	Total CV %
0	4.479	0.457	0.047	0.374	8.350
-1	7.024	0.392	0.038	0.359	5.106
-2	10.527	0.462	0.053	0.424	4.029
-3	14.058	0.408	0.067	0.377	2.683
-4	17.655	0.421	0.047	0.386	2.185
-5	21.289	0.424	0.044	0.388	1.825
-6	25.005	0.521	0.084	0.481	1.923
-7	28.396	0.367	0.218	0.415	1.460
-8	31.902	0.338	0.533	0.627	1.967
-9	34.321	1.416	0.633	1.422	4.143
-10	34.458	1.305	0.521	1.151	3.342
-11	33.707	1.928	1.302	2.344	6.953
-12	34.655	1.168	0.441	1.231	3.552
-13	32.313	2.709	0.691	2.733	8.459
-14	33.434	1.749	0.362	1.279	3.825

The line between the −8 and −9 log_{10} dilution represents the limit of detection. Below of this line, it is considered unspecific amplification.

in figure 1. Additional testing of 41 bovine samples submitted to routine rabies diagnostic yielded 30 samples that were positive and 7 samples negative in both DFAT and RT-rtPCR. Four samples inconclusive by DFAT yielded positive by RT-rtPCR.

DISCUSSION

Despite of a number of nucleic-acid based assays for RABV detection developed to date (DEDKOV et al., 2018; FAYE et al., 2017; WADHWA et al., 2017), none has been validated for RABV detection in cattle. The RT-rtPCR assay described herein is unique amongst published studies in which it has been designed using sequences from several field RABV circulating in cattle. The assay also incorporates a proprietary synthetic positive control to verify proper functioning of the reaction components. The linear range, efficiency and limit of detection were similar to those reported previously for other RT-rtPCRs for RABV (DEDKOV et al., 2018; FAYE et al., 2017; WADHWA et al., 2017). The intra- and inter-run repeatability for the RABV component of the assay was adequate, with CVs of less than 9% in all dilutions, and were similar to those reported previously for RABV detection (FAYE et al., 2017).

The assay was designed to detect the RABV variant 3, usually identified in *Desmodus rotundus* and, consequently, in cattle and, eventually, in horses and other domestic and wild species. Nonetheless, the high conservation of the N gene in lyssaviruses (ICTV, 2011) would likely allow for the assay to detect other RABV variants and viruses of this genus in other animal species as well. The detection of other RABV variants by designed primers was confirmed *in silico* using Primer-BLAST software. The assay

Table 3 - Data table for bovine brain samples of known infection status included in the analysis of sensitivity and specificity of the RABV RT-rtPCR (PCR) and direct fluorescent antibody test (DFAT) assays for detection of RABV in bovine brains.

Test results	DFAT+	DFAT-	Total
PCR+	10	0	10
PCR-	0	11	11
Total	10	11	21

is also RABV specific, since no other neurological pathogens cattle were detected.

The sensitivity and specificity of the RT-rtPCR were estimated to be 100% (sensitivity: 72.2 to 100%; specificity: 74.1 to 100%) using 21 bovine brains of known infection status, considering DSe and DSp estimates of 98%, with an error of 5% margin allowed in estimate of DSe and DSp and a confidence of 90%, according suggested by OIE (OIE, 2013). These results indicated an adequate sensitivity of the RABV RT-rtPCR, equivalent to DFAT, the gold standard for RABV diagnosis. In despite of the limited number of clinical samples used in the validation assay, the RT-rtPCR presented a higher sensitivity that the DFAT. Testing 41 additional samples submitted to routine rabies diagnosis reinforced the usefulness of the RT-rtPCR as a complementary diagnostic assay. Thus, this assay represents a valuable tool for the diagnosis of bovine rabies, caused by variant 3.

The severe nature of rabies and the implications of false negative or inconclusive results justify the need of a complementary assay for confirmation of the diagnosis with higher accuracy. Complementary detection tests – e.g. rabies tissue culture infection test (RTCIT) and mouse inoculation test (MIT) are frequently used upon DFAT inconclusive results and/or in strongly suspected cases followed by DFAT-negative results. However, these tests recommended by OIE may take 2 to 4 (RTCIT) or 5 to 28 days (MIT) to get the results (OIE, 2013). In addition, MIT faces concerns about ethics in animal use. In addition, post-exposure treatment is frequently recommended in cases of human exposure to suspect animals, requiring a prompt and definitive result. Furthermore, this RT-rtPCR can contribute in situations when a large number of samples need to be tested; it can be standardized in laboratories that do not have the structure to perform DFAT and MIT; it can replace DFAT in the possibility of lack of reagents; and it can be standardized for portable equipment and field testing.

CONCLUSION

Hence, the use of a RABV RT-rtPCR standardized and validated for bovine samples may bring more agility to get the final diagnosis of rabies in cattle, especially in inconclusive cases. In addition, the development of rapid and suitable molecular tools for large-scale detection of RABV may be important for routine diagnostic testing and epidemiological surveillance, particularly in RABV endemic areas (MAPA, 2019).

DECLARATION OF CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUITIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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