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MICROBIOLOGY

Feasibility of replacing the viral isolation technique in mice with RT-qPCR for the diagnosis of rabies

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ABSTRACT: Rabies is a viral encephalitis that affects mammals, including humans. Rapid and effective laboratory diagnosis of the rabies virus is critical for public health. This study evaluated the operational, technical, and financial viability of the RT-qPCR in replacement of inoculation in mice for diagnosing rabies in the laboratory routine. A total of 316 samples of mammalian brains were analyzed, 121 positives and 195 negatives, previously diagnosed by direct immunofluorescence (DIF) and mouse inoculation test (MIT). The samples were submitted to the duplex TaqMan RT-qPCR technique. The accuracy, sensitivity, and specificity of RT-qPCR were analyzed. We analyzed the costs for performing the RT-qPCR technique and compared it with the cost of MIT. The results showed 99.37% accuracy, 99.17% sensitivity, and 99.49% specificity by RT-qPCR when related to DIF and MIT results, which proved to be a robust and repeatable technique. The minimum time for a positive diagnosis was reduced in RT-qPCR (1 day) if compared to MIT (9.64 days), with a 17.7% reduction in the cost of the molecular technique. The present study demonstrated that the molecular biology technique is an efficient tool to diagnose rabies in the laboratory routine, being able to replace MIT.
Key words: laboratory diagnosis, molecular techniques, one health, viruses, zoonosis.

Viabilidade da substituição da técnica de isolamento viral em camundongos pela RT-qPCR para o diagnóstico da raiva

RESUMO: A raiva é uma encefalite viral que acomete os mamíferos, incluindo humanos. O diagnóstico laboratorial rápido e eficaz do vírus da raiva é importante para a saúde pública. Este estudo teve como objetivo avaliar a viabilidade operacional, técnica e financeira da RT-qPCR em substituição à inoculação em camundongos para o diagnóstico da raiva na rotina laboratorial. Foram analisadas 316 amostras de cérebros de mamíferos, 121 positivas e 195 negativas, previamente diagnosticadas por imunofluorescência direta (IFD) e isolamento viral em camundongos, por inoculação intracerebral em camundongos. As amostras foram submetidas à técnica duplex TaqMan RT-qPCR. A precisão, sensibilidade e especificidade da RT-qPCR foram analisadas. Os custos para realizar a técnica de RT-qPCR foram analisados e comparados com o custo do isolamento viral em camundongos. Os resultados demostraram 99,37% de acurácia, 99,17% de sensibilidade e 99,49% de especificidade pela RT-qPCR quando relacionada aos resultados de IFD e isolamento viral em camundongos, que se mostrou uma técnica robusta e com repetibilidade. O tempo mínimo para diagnóstico positivo foi reduzido na RT-qPCR (1 dia) quando comparado ao isolamento viral em camundongos (9,64 dias), e com redução de 17,7% no custo pela técnica molecular. O presente estudo demonstrou que a técnica de biologia molecular é uma ferramenta eficiente para o diagnóstico da raiva na rotina laboratorial, podendo substituir o isolamento viral em camundongos.
Palavras-chave: diagnóstico laboratorial, técnicas moleculares, saúde única, vírus, zoonose.

INTRODUCTION

Rabies is a zoonotic viral disease that causes progressive and fatal inflammation of the central nervous system. This encephalitis represents a concerning public health problem worldwide, resulting in the death of approximately 59,000 people annually. Asia and Africa are the continents with the highest mortality, mainly affecting children between 4 and 15 years (WHO, 2022). In Brazil, 45 cases of human rabies were recorded from 2010 to 2022, and 24 were transmitted by bats, nine by dogs, five by felines, four by non-human primates, and two by foxes (BRASIL, 2022a). Rabies causes massive harm to both livestock and public health. In 2021, 642 cases of rabies in ruminants were registered in Brazil (BRASIL, 2022b).

The diagnosis based on the clinical ground alone is difficult and often unreliable. Therefore, a

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clinical case of rabies should be verified by laboratory diagnosis (WHO, 2022). In this context, laboratory diagnosis occupies a central position in the fight against rabies and is essential for choosing strategies for the prophylaxis of human and animal rabies (BRASIL, 2021).

The Centers for Disease Control and Prevention considers Direct Immunofluorescence (DIF) the "gold standard" diagnostic method for rabies (CDC, 2011). In Brazil, most laboratories included in rabies epidemiological surveillance systems carry out the diagnosis using DIF and Mouse Inoculation Test (MIT) techniques. The DIF technique is fast and effective. However, it does not have 100% sensitivity and specificity.

In a study with 6,514 samples from different host species in Brazil, DIF showed a sensitivity of 93.58% and a specificity of 95.9% (NASRAUI & KAWAI, 2021). Thus, it is interesting that the diagnosis of rabies, especially in cases of aggression to humans, is performed by more than one diagnostic technique, the first diagnosis made by DIF and another supplementary test, which could be viral isolation or molecular. MIT has good sensitivity (SINGH et al., 2017); although, its use to guide post-exposure prophylactic treatment is questionable due to the long time to deliver the results. In addition, good practices in the use of animals recommend the reduction, replacement, and refinement of the use of laboratory animals (RUSSEL & BURCH, 1959).

In-vitro techniques are preferable to in-vivo ones if the diagnostic quality is maintained. The isolation of the rabies virus is possible in cell culture. This technique has been proven efficient in terms of both diagnostic sensitivity and speed of the result when compared to MIT. The diagnosis result by cell culture can take up to 96 hours (4 days), while the MIT should extend for at least 40 days (RUPPRECHT et al., 2018).

Diagnosis by molecular techniques is even faster than viral isolation in cells and has high sensitivity and specificity. RT-PCR has been used as a diagnostic method for rabies for decades but has only recently been recommended by WOAH (WOAH, 2023a). In addition, with the implementation of a molecular biology laboratory, it is possible to expand, in a relatively simple way, the differential diagnosis for other neurological diseases, such as bovine and equine herpesvirus and equine encephalomyelitis. Thus, this study evaluated the operational, technical, and financial viability of the RT-qPCR technique for diagnosing rabies, replacing MIT, in the laboratory routine of the state of Espírito Santo, Brazil.

MATERIALS AND METHODS

Samples

Within the standardization phase of RT-qPCR, the standard virus strain, CVS (challenge virus standard), was used. RNA extracted from infected mouse brain suspension was diluted 1:1000 for standardization tests.

To test the sensitivity and specificity of the technique in field samples, 316 mammalian brain samples (121 positive and 195 negative for rabies) from the State of Espírito Santo, Brazil, were used. Espírito Santo is located in the Southeast region. It borders the Atlantic Ocean to the east, Bahia state to the north, Minas Gerais state to the west and northwest, and Rio de Janeiro State to the south. Its area is 46,086.907 km² and it has 78 municipalities. There are six climatologically homogeneous regions: North, Northeast, Northwest, Metropolitan, Serrana, and South. The samples came from 50 different municipalities, of which 40 detected the presence of the rabies virus. Among the samples positive for rabies, 29.75% were from the Northwest region, 24.79% from the South region, 21.49% from the Metropolitan region, 13.22% from the Mountain region, 9.92% from the Northeast region and 0.83% from the North region.

Samples from animal rabies surveillance and control programs were stored at -20 °C at the Institute of Agricultural and Forestry Defense from Espírito Santo (IDAF). All samples were previously submitted to rabies diagnosis by direct immunofluorescence (DIF) and mouse inoculation test (MIT). Samples exhibiting positive results in at least one of the techniques (DIF or MIT) were considered positive for the rabies virus (Table 1).

We selected positive samples isolated between 2011 and 2022 and negative samples isolated between 2020 and 2022. We deliberately choose samples with a positive result in only one of the techniques (21 negatives in DIF and positives in MIT and 13 positives in DIF and negatives in MIT) to assess the sensitivity of the technique in cases of samples with misdiagnosis for some reason, such as low viral load or brain deterioration.

Extraction of RNA RABV

A pool suspension of the central nervous system at 20% in buffered saline and antibiotics was prepared with fragments of Ammon's horn, cerebellum, and cortex, adding the spinal cord in cases of farm animals in the same way as is used for MIT (KOPROWSKI, 1996). The supernatant used in RT-qPCR was prepared immediately before the RNA extraction procedure.

Table 1 - Characterization of selected samples based on DIF and MIT diagnoses.

Group	Species	Number of positive samples*	Number of negative samples
Farm animals	Bovine	79	16
	Equine	16	14
	Ovine	4	2
	Swine	0	1
Pets	Dog	0	52
	Felid	1	39
Chiropters	*Artibeus lituratus*	17	11
	Chiroderma sp.	1	1
	Desmodus rotundus	1	3
	Eumops sp	0	9
	Lasiurus blossevillii	0	1
	Lonchorhina aurita	0	1
	Molossus sp.	0	20
	Myotis sp.	2	0
	Platyrrhinus lineatus	0	1
	Promops sp.	0	1
	Phyllostomidae family	0	6
	Vespertilionidae family	0	1
	Unidentified bat	0	7
Others	Non-human primate	0	6
	Bush dog	0	2
	Opossum	0	1
Total		121	195

*Samples with at least one of the positive techniques (DIF or MIT) were considered positive for the rabies virus.

The extraction of nucleic acids was performed using commercial kits for this purpose, following the manufacturer's instructions. We used Trizol Reagent®, Magmax Thermofisher®, or BioGene® Viral DNA/RNA Extraction kits. All extracted materials were stored at -80 °C.

RT-qPCR

RT-qPCR was performed as described by RUPPRECHT et al. (2019) with some modifications. The technique changes proposed in this article are 40 amplification cycles instead of 45; RNA extracted from the CVS virus strain employed as a positive control and synthetic positive control unused. More on that, the technique used in this article consists of a duplex reaction for simultaneous detection of the rabies virus genome (LN34 probe) and the host cell genome (β-actin probe). The virus detection was performed in only one well of the plate.

In each RNA extraction batch, DNase and RNase-free water were used as non-template control. In each RT-PCR reaction, a well was added as a negative control, using water in the same volume as the samples. Two forward and one reverse primer were employed to detect the rabies virus genome, and a pair of primers to detect the β-actin gene. The primers LN34 forward 2, LN34 reverse, and the probe LN34 have degenerate nucleotides (Table 2).

We performed the RT-qPCR reaction with the commercial AgPath-ID One-Step RT-PCR reagents kit (Applied Biosystems), heeding the manufacturer's recommendations. 2 µL of extracted RNA was used, and the final reaction volume was 25 µL. Samples with LN34 Ct value lower than 35 and β-actin lower than 33 were considered positive, following the criteria for reading the results recommended by RUPPRECHT et al. (2019).

To evaluate the best combination of primer concentrations, real-time PCR was performed in

Table 2 - Primer and hydrolysis probes used in RT-PCR. LN34 relative to rabies virus genome and β-actin relative to the host cell.

Name	Sequence	Position[a]
LN34 Forward 01	ACGCTTAACAACCAGATCAAAGAA	1 - 24
LN34 Forward 02	ACGCTTAACAACAAAATCADAGAAG	1 - 25
LN34 Reverse	CMGGGTAYTTRTAYTCATAYTGRTC	140 - 164
β-actin Forward	CGATGAAGATCAAGATCATTGC	-
β-actin Reverse	AAGCATTTGCGGTGGAC	-
Probe LN34	(FAM) AAC ACC YCT ACA ATG GA (QSY)	59 - 75
Probe β-actin	(VIC) - TCC ACC TTC CAG CAG ATG TGG ATC A - (QSY)	-

[a]The primer and probe positions are given relative to the *Lyssavirus* full genome.

triplicate, combining different final concentrations of sense and reverse primers (final concentration of primers: 200, 400, and 600 mM, totaling nine dilutions). In the case of the two LN34 sense primers, the sum of the primers was considered the final concentration.

After testing the best concentration of primers, the concentration of probes (60 nM, 120 nM, and 200 nM) was experimented in triplicate. Subsequently, three different annealing temperatures were tested (56 °C, 58 °C, and 60 °C).

The amplification protocol was: 1) reverse transcription: 45 °C for 10 min; 2) reverse transcription inactivation: 95 °C for 10 min; 3) amplification: 40 cycles at 95 °C for 15 s (denaturation) and then at 56 °C for 45 s (annealing and extension).

Comparison of mean values of Ct (threshold cycle) and ΔRn (normalized Reporter delta - fluorescence variation) for different concentrations of primer and probe was performed by analysis of variance (ANOVA) using the F statistic followed by minimum significant difference (MSD) values calculated by Tukey's test, with a significance level of 5%.

Sixteen rabies-positive and sixteen negative samples were tested using the Singleplex protocol proposed by RUPPRECHT et al. (2019) and the Duplex method proposed in this article. Ct and ΔRn values of paired samples were compared using Student's t-test.

Verification of method performance

The efficiency of the reaction for each of the targets (LN34 and β-actin) was calculated using the Software QuantStudio™ Desing & Analysis v. 1.5.2 (Applied Biosystems) based on the linear regression data of the RT-qPCR standard curve. RNA extracted from a CVS virus sample was employed at 5 dilution points at a factor of 1:5, and RT-qPCR was performed in triplicate.

A CVS virus with a titer of $10^{4.25}$ LD_{50}/30µl for mice was used to calculate the RT-qPCR detection limit. The CVS, diluted to 2.56 x 10^{-7}, was carried out in a 20% suspension of healthy mouse brains. Extractions in TRIzol Reagent were conducted with 300µL of this suspension for each dilution. For the highest dilutions (1.6 x 10^{-6}, 6.40 x 10^{-6}, and 2.56 x 10^{-7}), 21 replicates were processed, from extraction to real-time PCR. The detection limit was considered the highest dilution in which the LN34 marker was detectable in all replicates.

We used fourteen samples: seven positives and seven negatives for the presence of the rabies virus. The samples were processed in triplicate by two different analysts, who performed the procedure, from RNA extraction to reading by RT-qPCR. We repeated this procedure three times on several days. The method performance calculations evaluated measurement uncertainty, repeatability, and reproducibility, following the previous recommendations in BRASIL (2015).

Analysis of accuracy, sensitivity, and specificity of RT-qPCR

Aiming for accuracy, sensitivity, and specificity calculations, we individually analyzed the diagnostic techniques (RT-qPCR, DIF, and MIT). We related them to established true positives and negatives (WOAH, 2023b). The samples with rabies virus detected by DIF and/or MIT were considered "true positive" ones. The "true negatives" were the samples of which rabies virus was undetected in both techniques.

Samples showing divergent results were submitted to the conventional PCR technique to confront the diagnoses. Conventional

RT-PCR was performed as described by ORCIARI et al. (2001), using primers 504 (5' TATACTCGAATCATGATGAATGAGGTCGACT 3' - sense, position relative to the rabies virus genome 1286 -1317) and 304 (5' TTGACGAAGATCTTGCTCAT 3' - reverse, position 1514-1533).

Analysis of Ct values concerning species, DIF, and MIT

We grouped Ct values of the LN34 marker according to the host species and stratified it according to the results found in the DIF and MIT techniques. Then, we calculated means, medians, and minimum and maximum values. The results were analyzed by ANOVA, followed by Tukey's test. β-actin Ct values were evaluated for maximum, minimum, and average values among all samples.

Time to obtain the diagnosis by inoculation in mice

The average number of days between sample entry in the laboratory and the completion of the diagnosis of rabies in MIT was obtained from the laboratory records (2010 to 2022).

Evaluation of the cost of executing the techniques

A cost analysis was performed by sampling the main inputs used in RT-qPCR compared to the ones used in MIT. The number of samples considered for the calculation was the average of the last five years in the laboratory, 611 samples.

To calculate the cost of the molecular technique, we stipulated that extraction and RT-qPCR would be performed once a week (52 weeks), with an average of 11.75 samples per week. We also included the positive control and NTC (in the control template) in each test run.

The inputs included in the RT-qPCR cost calculation were the plastic consumables (microtubes, tips, strips), the extraction kit, the one-step RT-PCR kit, primers, and probes. To calculate the cost of MIT, the values of the last contracts for the acquisition of inputs, firmed in 2021, were used for the annual maintenance for raising mice and for inoculation (feed, wood shavings, insulin syringes, and disinfectant solutions) and the average of samples received. To assess the cost of human resources, we analyzed the time needed to complete the diagnostic protocol in one sample.

RESULTS

Standardization of RT-qPCR

Different combinations of the final concentration of sense/reverse primers and the final concentration of hydrolysis probes showed significant differences for both Ct and ΔRn values. The Ct values of the LN34 marker, in the different concentrations of sense and reverse primer, were: the lowest Ct was in the 200/400 concentration (Ct=23.23), the highest was in the 600/200 concentration (Ct=24.56) to mean Ct for all measurements was 23.62 (ANOVA P = 0.000899, and Tukey test MSD=1.1131).

The ΔRn values of the LN34 marker, in the different concentrations of sense and reverse primer, were: the highest ΔRn was in the 400/200 concentration (ΔRn=2.08), the lowest was in the 600/400 concentration (ΔRn=1.39), and the mean for all measurements was 1.74 (ANOVA P = 0.000878548, and Tukey test MSD=0.480035).

The most efficient values for Ct and ΔRn were found in the reactions with the highest concentration of probes. The Ct values for the 200 nM, 120 nM, and 60 nM probe concentrations were 21.78, 22.88, and 24.56 (ANOVA P = 0.000118544 and Tukey Test MSD=0.79961) respectively. The ΔRn values for the 200 nM, 120 nM, and 60 nM probe concentrations were 2.57, 1.45, and 0.58 (ANOVA P = 6.21061E-9 and Tukey Test MSD=0.1077), respectively. The three proposed annealing temperatures detected the LN34 and β-actin genes of the selected samples. However, the 56 ºC one showed the lowest Ct.

The mean Ct values of the LN34 marker showed no significant difference between the singleplex and duplex reactions (mean Ct LN34: Duplex = 22.0; Singleplex = 21.98; P-value = 0.64). The average Ct of β-actin in the duplex reaction was slightly lower than in the singleplex reaction (Ct β-actin Duplex = 23.81; Singleplex = 24.15; P = 0.002). The mean ΔRn values of the LN34 marker were slightly higher in the duplex reaction compared to the singleplex reaction (mean ΔRn LN34: Duplex = 1.73; Singleplex = 1.60; p-value = 0.015). The mean ΔRn of β-actin in the duplex reaction was lower than in the singleplex reaction (ΔRn β-actin Duplex = 4.77; Singleplex = 6.50; p = 3.001E-09).

After the initial standardization tests, the protocol chosen for the other stages of the work using the AgPath-ID™ one-step RT-PCR kit (Applied Biosystems™) were: primers 400 nM, probes 200 nM, annealing temperatures 56 °C and 40 cycles of amplification. Primers at an initial concentration of 10 µM and 5 µM probes were used, and the mixture performed as follows: 3.5µL water, 0.5µL LN34 forward 1 primer, 0.5µL LN34 forward 2 primer, 1.0µL LN34 reverse primer, 1.0µL β-actin forward primer, 1.0µL β-actin reverse primer, 1.0 µL LN34 probe, 1.0 µL β-actin probe, 12.50 µL RT-PCR Buffer,

and 1.0 µL 25X Enzyme Mix and 2 µL template RNA, totaling 25 µL per reaction. Samples with a Ct of the LN34 probe less than 35 and β-actin less than 33 were considered positive in the PCR.

Method performance

The reaction efficiency calculated for the LN34 target was 101.47% (slope: -3.287, correlation coefficient R^2: 0.976 and Y-intercept: 22.015). For the β-actin target, the efficiency was 96.22% (slope: -3.416, correlation coefficient R^2: 0.948 and Y-intercept: 26.746).

In the limit of detection analysis, the undiluted CVS virus had a Ct of 10.31. The last dilution in which all replicates detected the LN34 marker was 6.4×10^{-6}, with a mean Ct of 31.75.

In the quantitative analysis of the method, the expanded uncertainty (U) for the Ct LN34 values was 0.55 ($0.47 \leq U \leq 0.64$, 95% confidence interval). The measurement variation from repeatability and reproducibility, considering two analysts and different trials, was %R&R = % repeatability variance (3.67) + % analyst reproducibility variance (0.0) + % reproducibility trials variance (1.07) = 4.74.

Considering the qualitative result of the test, i.e., only positive or negative, all RT-qPCR results were consistent with conventional techniques. The results acquired by both analysts were indistinguishable, resulting in 100% sensitivity and reproducibility in RT-qPCR among the tested samples.

Accuracy, sensitivity, and specificity

When performing real-time PCR we considered DIF and MIT as "true" results. From the 121 positive samples, 120 were confirmed positive, and one was false-negative, while from the 195 negatives, 194 were confirmed negative and one false-positive (Figure 1). The accuracy, sensitivity, and specificity were 99.37%, 99.17%, and 99.49%, respectively.

The samples with the diverging outcomes were submitted to conventional PCR, and the results matched the ones obtained by real-time PCR.

Analysis of Ct values

LN34 Ct values and their means varied between species (ANOVA p=1.56861E-10). The lowest average was found in chiropterans, followed by ovine, bovine, feline, and equine species (Table 3). In the Tukey test, significant differences were reported in the CT values between Cattle and Horses (DMS=3.34), Cattle and Chiropterans (DMS=3.06), Equines and Chiropterans (DMS=4.06), and between Horses and Sheep (DMS=6.93).

		DIF and MIT results		
		PS	**NS**	**Total**
Result of RT-qPCR	+	120 (TP)	1 (FP)	121
	-	1 (FN)	194 (TN)	195
Total		121	195	316
		Diagnostic sensitivity TP/(TP+FN) 99.2%	Diagnostic specificity TN/(TN+FP) 99.5%	

Figure 1 - Diagnostic sensitivity and specificity estimates. The numbers were calculated considering samples with positive results in DIF and/or MIT as true positives, and samples with negative results in both techniques as true negatives.

PS - Positive sample; NS – Negative sample; TP – True positive; FP – False positive; TN – True negative; FN – False negative.

The mean LN34 Cts grouped by DIF and MIT results showed a significant difference (ANOVA p=2.0999E-13). The mean value of LN34 Ct, whose results were positive DIF and positive MIT (++), was equal to 19.1 (n=87); Positive DIF and Negative MIT (+-) was 24.07 (n=12); Negative DIF and Positive MIT (-+) was 27.89 (n=21) and the only sample with negative results in DIF and MIT that was positive in PCR was 33.63. Tukey's test showed a significant difference between samples with ++ and +-, ++ and -+ results. There was no significant difference between the Cts of the +- and -+ samples.

For all samples, the lowest β-actin Ct was 14.09, the highest was 32.8, and the mean was 22.82.

Time to obtain the diagnosis by inoculation in mice

To be considered negative in MIT, the inoculated mice must be observed for 30 days and remain healthy throughout the period. On average, the MIT indicated positive in 9.64 days. The average

Table 3 - Minimum, maximum, mean, and median LN34 Cts values found in positive samples in RT-qPCR in all samples analyzed and in different species.

		----------Ct RT-qPCR Values----------			
Species	DIF/MIT	Min	Max	Mean	Median
All samples	Positives (n=121)	11.15	34.95	21.24	20.66
	+/+ (n=87)	11.15	31.79	19.11	19.21
	+/- (n=12)	16.,37	31.94	24.07	23.35
	-/+ (n=21)	15.94	34.95	27.89	28.99
	-/- (n=1)	33.63	33.63	33.63	33.63
Cattle	Positives (n=78)	14.2	31.94	21.47	20.79
	+/+ (n=58)	14.2	27.46	19.81	19.89
	+/- (n=8)	20.79	31.94	25.46	25.74
	-/+ (n=12)	15.94	31.46	26.79	28.73
	-/- (n=0)	-	-	-	-
Equines	Positives (n=17)	20.55	34.95	27.45	27.44
	+/+ (n=5)	20.85	31.79	24.08	22.46
	+/- (n=3)	20.55	25.66	22.94	22.62
	-/+ (n=8)	27.15	34.95	30.49	30.79
	-/- (n=1)	33.63	33.63	33.63	33.63
Sheep	Positives (n=4)	14.7	20.22	17.16	16.86
	+/+ (n=3)	14.7	17.32	16.14	16.39
	+/- (n=0)	-	-	-	-
	-/+ (n=1)	20.22	20.22	20.22	20.22
	-/- (n=0)	-	-	-	-
Bats	Positives (n=21)	11.15	25.64	15.92	14.64
	+/+ (n=20)	11.15	25.64	15.90	14.61
	+/- (n=1)	16.37	16.37	16.37	16.37
	-/+ (n=0)	-	-	-	-
	-/- (n=0)	-		-	-
Cat	Positives (n=1)	26.29	26.29	26.29	26.29
	+/+ (n=1)	26.29	26.29	26.29	26.29
	+/- (n=0)	-	-	-	-
	-/+ (n=0)	-	-	-	-
	-/- (n=0)	-		-	-

Positives: Positive DIF and/or MIT; +/+: Positive DIF and Positive MIT; +/-: Positive DIF and Negative MIT; -/+: Negative DIF and Positive MIT; -/-: Negative DIF and Negative MIT.

time for cases in which DIF was negative and MIT was positive, was 15 days.

Cost of executing the techniques

The cost of each technique per sample was US$ 7.24 (seven dollars and 24 cents) for RT-qPCR and US$ 8,80 (eight dollars and eighty cents) for MIT (Table 4).

Regarding the working time required in each diagnostic technique for each sample, one day was necessary for the full performance of the RT-qPCR technique, while for the development of the MIT technique, daily monitoring of the inoculated animals was required up to 30 days in the case of negative sample for the rabies virus.

DISCUSSION

The present research evaluated the feasibility of replacing MIT with the RT-qPCR technique in a rabies diagnostic laboratory routine. The results demonstrated that this technique correctly identifies the rabies virus in the samples. We conducted a few modifications to the RT-qPCR technique proposed by RUPPRECHT et al. (2019). The most important were using one duplex reaction instead of two singleplex reactions per sample, and testing samples only once instead of in triplicate. Thus, in each well of the PCR plate, the genome of the rabies virus and the β-actin of the host cell were identified simultaneously, reducing costs and work.

A duplex RT-qPCR was also described by MINOZZO et al. (2022), presenting a satisfactory result for the diagnosis of rabies. However, differing from MINOZZO et al. (2022), we used the three LN34 primers recommended by RUPPRECHT et al. (2019), while MINOZZO et al. (2022) used only two of these primers (LN34 forward 01 and reverse) for detecting the rabies virus genome. The LN34 forward 02 primer has degenerate nucleotides and differed size-wise if compared to the LN34 forward 01. A degenerate primer increases the technique's sensitivity because it can anneal with different nucleotide sequences. Even though MINOZZO et al. (2022) pointed out that using two primers was satisfactory, we maintained three LN34 primers to cover a wide range of rabies virus variants. This decision does not impact the costs of the diagnosis.

RUPPRECHT et al. (2019) proposed the following parameters for RT-qPCR: final concentration of sense/reverse primers at 400/400 nM, final probe concentration at 200 nM, and annealing temperature at 56 °C. To assess whether the change from a singleplex reaction to a duplex one changes the behavior of the reactions, we tested the proposed technique for different final concentrations of primers, probes, and annealing temperatures. The RT-qPCRs with the lowest Ct value and the highest ΔRn value were considered the best results.

No significant differences were found between the best results obtained with the parameters proposed by RUPPRECHT et al. (2019), and the efficiency of the PCR reaction was close to 100% (101.1% for the LN34 target and 96.22 for the β-actin target). According to BRASIL (2015), the efficiency of the reaction should preferably be between 90 and 110%. Efficiency values above 100% may indicate poor sample quality or a pipetting problem.

In the standardization phase, the technique demonstrated robustness. When varying the

Table 4 - Cost of consumables (US$) per sample in MIT and RT-qPCR techniques.

Diagnostic technique	Consumables	US$/sample*	Total per sample*
RT-qPCR	Plastic materials	1.75	7.2
	Extraction Kit	2.72	
	Kit one-step RT-qPCR	2.42	
	Primers	0.012	
	Probes	0.35	
MIT	Feed	7.07	8.8
	Shavings	0.74	
	Disinfectant solutions	0.63	
	Syringe	0.36	

*Conversion values R$ x US$ on 08/03/2023.

concentration of primers and probes roughly three times, the results remained similar, even though there was variation in the values of Ct or ΔRn for the different parameters tested. According to MAGNUSSON & ÖRNEMARK (2014), the robustness of a technique indicates the method's reliability in its practical use.

Regarding the rabies-positive samples analyzed in this study, almost all came from bats or livestock animals (cattle, horses, and sheep). There was one sample from a cat, but this sample was typified as variant 3, commonly found in bats *Desmodus rotundus* (VIEIRA et al., 2010). Therefore, we have not analyzed characteristic variants of the rabies cycle in dogs and cats. Although not tested in this experiment, authors, who also used the LN34 primers, reported the detection of samples positive for rabies by RT-qPCR from domestic and wild canids (GIGANTE et al., 2018; WADHWA et al., 2017).

Among the negative samples for rabies, there were different host species: dog, cat, wild canid, wild feline, opossum, non-human primate, bovine, equine, sheep, and bats. The absence of virus samples from the urban rabies cycle is justified by the epidemiological profile of rabies change in Brazil due to the recent success of mass vaccination campaigns for dogs and cats. According to data from the Ministry of Health (BRASIL, 2022a), in the Southeastern Region of Brazil, where the state of Espírito Santo is located, between 2015 and 2022, the presence of variants 1 and 2 of the rabies virus was not recorded. In Espírito Santo, the last case of rabies diagnosed in a companion animal was a feline with variant 3, included in this study.

Considering rabies diagnoses by DIF and MIT as a reference, two samples showed divergent results. The sample (Rav104/22), which showed a negative RT-qPCR result and positive DIF, also did not detect the presence of the rabies virus by MIT and conventional PCR techniques. We can question whether the case would not be false-positive in DIF instead of being false-negative in RT-qPCR. SHARADA & KAVITHA (2020) reported several situations in which nonspecific fluorescence may appear in the DIF technique, such as drying of the conjugate, precipitation of soluble antigen, edge effect (fluorescence visible outside of tissue or dried at the edge) or tissue-related problems (impressions or smears too thick, deteriorated or samples proteins denatured due to heat or chemicals).

The sample (Rav113/22) with a positive result for rabies in RT-qPCR and negative in DIF and MIT was also submitted to the conventional PCR technique with detection of the rabies virus, corroborating the result acquired in RT-qPCR. Considering that the assessment targeted the brain of an euthanized horse, one can suspect that it stands out as a true positive. We repeated the entire procedure to remove the doubt of cross-contamination during the sample processing inside the laboratory, i.e., starting by extracting the original sample and, finally, the PCR. We found the same results, using primers for different regions of the nucleoprotein gene in conventional PCR compared to real-time PCR, a fact that reinforces that there was indeed rabies virus present in the sample. However, the possibility of contamination of samples before entering the laboratory still exists, considering that conventional disinfection techniques do not completely eliminate the presence of the rabies virus genome in necropsy instruments (AIELLO et al., 2016).

When compared to samples from cattle, those from horses are more challenging to diagnose by DIF and take longer to confirm the diagnosis of rabies by MIT [average of 1.8 days more in horses than in cattle in the laboratory of the Gedlab/IDAF(data not shown)]. In general, brains from equine specimens show a markedly lower amount of fluorescence when analyzed under a microscope, likely due to a lower viral load. Accordingly, these samples had the highest mean Ct value among species, indicating less virus in the brains. Moreover, we demonstrated that samples with divergent results between DIF and MIT presented higher Ct than samples with both DIF and MIT positive.

The Rav113/22 sample had a Ct of 33.63, which suggested that the detection limit was exceeded for conventional techniques. Conversely, it was possible to detect the presence of the virus in RT-qPCR and conventional PCR.

Given these remarks, we considered that the RT-qPCR technique correctly detects the presence or absence of the rabies virus in 100% of the samples analyzed in this study.

Even considering the failure of the RT-qPCR technique for detecting two samples with discordant results, this technique had a high sensitivity (99.17%). The highest sensitivity of RT-qPCR was reported by WADHWA et al. (2017) and MINOZZO et al. (2022). ROBARDET et al. (2021) reported sensitivities for the RT-PCR (99.3%), DIF (99.1%), and RT-qPCR (98.7%) techniques. In their study, the central nervous system samples came from inoculated animals, not naturally infected individuals with the rabies virus.

Among the 316 samples analyzed, 21 samples showed negative results in DIF and positive

in MIT, and 13 positives in DIF and negative in MIT. There was only one case of a positive sample in DIF and negative in MIT, in which the presence of the rabies virus was undetected in RT-qPCR. RT-qPCR detected the virus in all others.

Out of the 121 samples with a positive result in conventional techniques, at least six samples showed an advanced stage of decomposition. The detection of rabies virus by molecular techniques in decomposed samples has also been reported in previous works (WHITBY et al., 1997; APPLER et al., 2019).

When analyzing the costs of the RT-qPCR and MIT techniques, the use of mice showed a cost of 17.7% higher than the molecular technique. In addition, we observed that the molecular technique is less laborious. The vivarium for rearing and tracking inoculated mice requires daily monitoring, regardless of the number of samples arriving at the laboratory. Conversely, it is possible to concentrate the execution of RT-qPCR according to the receipt of samples.

The routine in a rabies diagnostic laboratory is unpredictable, as the samples that arrive at the laboratory come from suspicious animals or those that died with clinical signs of rabies, causing the frequency of receiving samples to vary. Therefore, it is unfeasible to accurately foresee the number of mice routinely used in the laboratory.

The time for releasing the result of mice inoculation is 30 days for negative samples. In cases of positive ones, the time will depend on the development of the disease in the mice. On average, the results were released in 9.64 days, but for cases where the DIF is negative, this time increases to 15 days. Using RT-qPCR in the laboratory routine, for situations where only rabies virus surveillance is carried out, it is possible to accumulate a few samples to process concurrently, saving inputs and work time. However, in cases where the suspected animal is involved in aggression towards a human or other animal, it is possible to carry out the diagnosis on the same day.

CONCLUSION

Replacing the MIT technique with RT-qPCR for diagnosing rabies proved feasible in a routine laboratory. The molecular procedure proved to be less laborious, presented greater sensitivity, shorter response time, and lower cost per sample when compared to MIT. The technique is robust regarding variations in reagents and annealing temperature and is repeatable among different operators.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. Founding sponsors had no role in the design of the study, data collection, analyses, interpretation of data, the writing of the manuscript, or in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the manuscript. All authors critically revised the manuscript and approved the final version.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

The animal study protocol was approved by the Ethics Committee of Universitdade de Vila Velha (UVV) (protocol code 639/2022 on August 1, 2022).

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