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New sensitive real-time PCR targeting *p28* gene for detection of *Ehrlichia canis* in blood samples from dogs

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ABSTRACT: *This study aims to describe a new detection method of a quantitative real-time polymerase chain reaction (qPCR) targeting the 28 kDa outer membrane protein gene (p28) as well as to compare this method with a conventional PCR (cPCR), which targets the same gene, in order to evaluate the performance of the technique designed in this study in detecting Ehrlichia canis (E. canis). Optimum oligonucleotides concentrations were reached, and the analytical sensitivity and specificity of the qPCR were performed. A total of 218 dogs' whole blood samples were conventionally collected for this study. The DNA was extracted from each sample. Subsequently, the samples were tested by an established cPCR and the new qPCR to compare each technique's performances. This new qPCR method for the molecular detection of E. canis presented a detection limit of ten copies of the fragment and was considered specific for E. canis according to analytical specificity analyses performed in vitro and in silico. The standard curve revealed 100% efficiency and a coefficient of determination (R^2) equivalent to 99.8%. Among the samples examined by qPCR, 24.31% were considered positive, significantly greater than those detected by cPCR (15.13%). The qPCR technique reached a higher sensitivity than the cPCR when targeting the p28 gene in detecting E. canis. The qPCR standardized in this study is an efficient method for confirming canine monocytic ehrlichiosis (CME) diagnosis and might provide the parasitemia monitoring during the disease treatment.*
Key words: *canine monocytic ehrlichiosis, molecular detection, diagnosis, hemoparasite.*

Novo PCR em tempo real sensível que visa o gene *p28* para a detecção de *Ehrlichia canis* em amostras de sangue de cães

RESUMO: *Este estudo tem como objetivo descrever um novo método de detecção de uma reação em cadeia da polimerase quantitativa em tempo real (qPCR) visando o gene da proteína da membrana externa de 28 kDa (p28), bem como comparar este método com um PCR convencional (cPCR), que visa o mesmo gene, a fim de avaliar o desempenho da técnica desenhada neste estudo na detecção de Ehrlichia canis (E. canis). As concentrações ideais de oligonucleotídeos foram alcançadas e a sensibilidade analítica e a especificidade do qPCR foram determinadas. Um total de 218 amostras de sangue total de cães foram coletadas convencionalmente para este estudo. O DNA foi extraído de cada amostra. Posteriormente, as amostras foram testadas por um cPCR estabelecido e o novo qPCR para comparar os desempenhos entre cada técnica. A curva padrão revelou 100% de eficiência e coeficiente de determinação (R^2) equivalente a 99,8%. Dentre as amostras examinadas por qPCR, 24,31% foram consideradas positivas, percentual significativamente maior do que as detectadas por cPCR (15,13%). A técnica qPCR atingiu uma sensibilidade maior do que a cPCR na detecção de E. canis. A qPCR padronizada neste estudo é um método eficiente para a confirmação do diagnóstico de erliquiose monocítica canina (EMC) e pode fornecer o monitoramento de níveis de parasitemia ao longo do tratamento da doença.*
Palavras-chave: *Ehrliquiose monocítica canina, detecção molecular, diagnóstico, hemoparasita.*

INTRODUCTION

Ehrlichia canis is an obligatory intracellular hemoparasite that possesses major significance in veterinary medicine, namely in the tropical countries where this infection occurs with more frequency (AGUIAR et al., 2020). The vector *Rhipicephalus sanguineus* sensu lato (s.l.) must actively contribute toward raising the infection rates in tropical areas (VIEIRA et al., 2011). Moreover, this pathogen has zoonotic potential, representing a public health concern (NICHOLSON, 2010).

The disease produced by *E. canis* is called canine monocytic ehrlichiosis (CME). According to epidemiological and experimental studies, CME's clinical presentation results in acute, chronic, or

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subclinical phases (WANER et al., 1997; DE CASTRO et al., 2004; MYLONAKIS et al., 2004). However, these phase differences are not explicit in dogs with naturally occurring disease (HARRUS et al., 2012).

In the subclinical phase, dogs usually show mild to non-existent thrombocytopenia or do not show clinical signs (WANER et al., 1997). During this phase, E. *canis* presents low parasitemia, and it is not frequently present in the peripheral blood, which complicates pathogen detection. In addition to these factors, E. *canis* might be only found in determined organs. For these reasons, it is possible to obtain false-negative results in PCR on blood samples (RODRÍGUEZ-ALARCÓN et al. 2020). Despite this detection bias, many studies have shown that molecular techniques present fewer false-negative results for ehrlichiosis diagnosis than other methods (RAMOS et al., 2009; NAKAGHI et al., 2010).

Several PCR modalities were designed to improve the sensitivity of *E. canis* detection in laboratory methods (DOYLE et al., 2005; NAKAGHI et al., 2010). Nested PCR (nPCR) has been used routinely to detect *E. canis* to increase analytical sensitivity over cPCR (SALLES et al., 2015; VELOSO et al., 2018; AYAN et al., 2020). Notwithstanding, this method has a high contamination risk that may result in unexpected cross-amplifications (DOYLE et al., 2005). The quantitative real-time polymerase chain reaction (qPCR) has been widely used to confirm the CME diagnosis (BUNRODDITH et al., 2018). This method is used frequently due to the various advantages it provides, such as specificity, sensibility, reproducibility, low risk of contamination, elimination of the post-amplification process, a consequent reduction in the time required for the assay to obtain the result, and the possibility of estimating the absolute number of target copies in the sample (DOYLE et al., 2005; PAULINO et al., 2018).

Besides the diversity of PCR techniques, various molecular markers have been used to confirm CME diagnosis (QUROLLO et al., 2017). Sequences coding ribosomal DNA (rDNA) are highly conserved, and it is the most used molecular marker for *E. canis* detection. Nevertheless, discrimination between species should use moderately conserved genes rather than highly conserved, such as rDNA (LYMBERY & THOMPSON, 2012).

DA COSTA et al. (2019) have reported that the *28 kDa surface-exposed antigen protein* (p28) gene shows a significant degree of conservation among the Brazilian, American, and Asian samples (99%), and also stated 79% homology with the closest organism, *Ehrlichia chaffeensis*. These results reveal that the p28 gene is moderately conserved among *Ehrlichia* species and highly conserved in the species *E. canis*, which are ideal characteristics for species discrimination using molecular methods. NAKAGHI et al. (2010) have already designed a conventional PCR highly specific targeting the *p28* gene. However, it presents low sensitivity due to the large size of the amplification product.

The present study aims to develop a sensitive qPCR method targeting the *p28* gene that may be used for specific detection of *E. canis* in clinical samples from naturally infected dogs as well as to compare this qPCR with a conventional PCR for targeting the *p28* gene of *E. canis,* as previously described by NAKAGHI et al. (2010).

MATERIALS AND METHODS

Animal and sampling procedures

A total of 218 whole blood samples were obtained by cephalic venipuncture in dogs from Small Animals Veterinarian Hospital at the Federal Rural University of Rio de Janeiro (HVPA-UFRRJ) from July 2017 to December 2017. These animals arrive at the hospital for various purposes. Some dogs were visiting to do check-ups or be vaccinated. Others presented non-specific clinical signs, for instance, lethargy, fever, pale mucous membranes, epistaxis, and hematological and biochemical disturbances.

The collected blood (2-3 mL) was stored in sterile tubes containing an anticoagulant (ethylenediaminetetraacetic acid) in the ultra-freezer (-80 ºC). The performing and interpreting of the molecular assays and statistical analyses were conducted using the double-blinded method. These samples were used first to evaluate qPCR's diagnostic yield and compare the results with the cPCR, which targeted the *p28* gene.

DNA extraction

Deoxyribonucleic acid (DNA) extraction was performed using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) and following the manufacturer's instructions. Subsequently, each DNA sample was quantified by the spectrophotometer Nanodrop ND-2000® (Thermo Scientific, Wilmington, DE, USA); the concentration was standardized between samples at 50 ng/µL.

Reference DNA controls

The DNA sample used as a standard positive control was collected from a sick dog presenting unspecific clinical manifestations. The cytological

exam of the patient's blood was performed, and it presented a suggestive *Ehrlichia*-positive result due to intracellular basophilic inclusions in monocytic cells observed by microscopy. The confirmation of the CME diagnosis was concluded following the determination of the positive results by a cPCR that targeted the *glycoprotein 19 kDa* (*gp19*) gene (HSIEH et al., 2010). The obtained amplicons were purified using a Clean Sweep kit (Applied Biosystems®) and were sequenced using the Sanger method to confirm the result. The sequence showed 100% similarity with *E. canis* sequences from GenBank and was deposited with the number of accessions 'MG584542'.

The negative standard DNA control was extracted from a naïve dog raised in experimental conditions with an ectoparasites-free environment. Subsequently, the sample was tested by two different molecular methods to increase the confidence margin. The applied cPCR used the *gp19* gene (HSIEH et al., 2010) and the *dsb* gene as the target (DOYLE et al., 2005). The molecular reactions provided negative results. Nuclease-free water (Ambion®) was used as a negative amplification control.

Optimization of qPCR

Primers and probes were designed using Primer Express® 3.0 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The oligonucleotides were tested using available software (Oligo Explorer 1.2, Hawthorne, NY, USA) for annealing temperature, self-annealing, and dimerizing, among additional specific functions.

The primers p28F (5'-GGGTGGCCCAAGAATAGA-3') and p28R (5'-GTTACTTGCGGAGGACATG-3') were designed to amplify a 143 bp fragment of the *E. canis-p28* gene. The hydrolysis probe chosen for use in this study was p28P (5'-VIC-TGCTTTATCTCATCATAGTTC-MGB-3'). A concentration primer test was performed to determine the required, ideal primer concentration to obtain the lowest cycle quantification (Cq) with a maximum fluorescence signal according to the baseline (ΔRn) in the absence of non-specific points of dissociation temperature. For this purpose, according to the manufacturer's description, an experiment was conducted with three replicates of each of the sixteen conditions established for the concentration test (200 nM to 800 nM). Reactions were performed at the StepOnePlusTM Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a total volume of 12 µL, comprising 1x SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and approximately 100 copies of *E. canis*-positive DNA as a template. The following thermocycling conditions were used: polymerase activation 95 °C for 10 minutes, 40 cycles were run with 20 seconds denaturation at 95 °C, 30 seconds annealing at 55 °C, and 30 seconds extension at 72 °C. A dissociation curve was produced to confirm the specificity of the amplification.

After standardizing the primer concentrations, the optimum probe concentration was determined. Probe assays were run in three replicates for each concentration (50 nM, 100 nM, 150 nM, 200 nM, and 250 nM) in a final volume of 12 µL, comprising 1x TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 200 nM of each primer, approximately 100 copies of *E. canis*-positive DNA as a template, and the respective probe concentration. During the qPCR assay analysis, it was possible to determine the ideal probe concentration based on the lowest Cq and the maximum ΔRn.

Reference cPCR

The cPCR previously described by NAKAGHI et al. (2010) using the oligonucleotides ECp28-F (5′-ATGAATTGCAAAAAAATTCTTATA-3′) and ECp28-R (5′-TTAGAAGTTAAATCTTCCTCC-3′) was chosen as the reference method for technique comparison considering both PCRs target the p28 gene. The reaction mix was set in a final volume of 25 µL containing 1× PCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl), 0.2 mM each dNTP, 2.5 mM MgCl2, 500 nM each primer, 1.25 U of Taq DNA polymerase, and 150 ng of sample DNA per reaction. The thermocycling conditions consisted of 95 °C for 5 minutes, 40 cycles at 95 °C for 30 seconds, the annealing temperature of 52 °C for one minute and 72 °C for two minutes, followed by a final extension at 72 °C for 5 minutes. The analytical sensitivity was evaluated based on the detection limit obtained through a tenfold dilution of the amplicon. The PCR products were submitted to electrophoresis and were run in a 2% agarose gel. The electrophoresis run was performed at 5 V/cm, and the gel staining was executed with ethidium bromide (0.4 mg/mL). The fragments were observed under ultraviolet light using the E-Gel Imager system (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). Samples were determined positives by the presence of a single band at the 823 bp fragment height.

Analytical sensitivity

Two standard curves were created using serial decimal dilutions of amplicons obtained in

cPCR for targeting the *E. canis p28* gene to provide the analytical sensitivity of qPCR. However, one of the curves was spiked, in each of the dilution points, with 20 ng of naïve dog's DNA from the reference negative DNA control to verify alteration in the qPCR performance. All amplicons obtained by this cPCR were purified with the Wizard® Genomic DNA Purification kit (Promega®, Madison, WI, USA) and quantified using Qubit® (Thermo Fisher Scientific, Wilmington, DE, USA). The purified cPCR product obtained a concentration of 50.5 ng/µL and this value was applied to calculate copy number using the following equation: copy number = (6.02 x 10^{23} (copies per mole) x DNA concentration (g)) / (target size (base pairs) x 660 (g /mol/bp)). The number of copies of the *p28* gene ranged from 1 to 10^6 per µL, with seven separate dilutions performed in triplicate. The linear regression along with the coefficient of determination (r^2) for each point of the curve was used to evaluate the efficiency of qPCR reactions, which was determined by considering the slope of the standard curve using the following formula: [Efficiency = 10 (-1 / slope) -1] (SVEC et al., 2015).

Analytical specificity

The analytical specificity was evaluated through *in silico* analysis and *in vitro* assay. The *in-silico* analysis was performed to investigate the oligonucleotides' conservancy between the *E. canis* sequences and variable regions between *Ehrlichia* species. Sequences of the *p28* gene and orthologs from *Ehrlichia* spp. deposited in GenBank were aligned using the algorithm ClustalW from the Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) for the bigger datasets (KUMAR et al., 2016). The oligonucleotides' specificity to the *E. canis p28* gene sequences was also confirmed through the primerBLAST Algorithm (NCBI, Bethesda, DM, USA).

The *in vitro* assay was performed using various DNA pathogens frequently found in dogs from Brazil, such as *Anaplasma platys*, *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *Babesia vogeli*, *Hepatozoon canis*, and *Rangelia vitalii*; the pathogens were obtained from blood samples of naturally infected dogs (DOYLE et al., 2005; OTRANTO et al., 2011; SANTOS et al., 2013; PAULINO et al., 2018; SOARES et al., 2011; SILVA et al., 2016). Additionally, the assay contained other pathogens' DNA, for example, *Leishmania infantum* from cellular cultures, *Babesia caballi*, *Theileria equi*, and *Neorickettsia risticii* from horses, indicating suggestive clinical signs (BHOORA et al., 2010; KIM et al., 2008; PUSTERLA et al., 2009), and *Babesia bovis* and *Anaplasma marginale* from naturally infected cattle (DE ECHAIDE et al., 1998; LINHARES et al., 2002). These animals had high parasitemia (in the acute phase), and their infections were detected by microscopy and confirmed by a specific molecular assay.

Analysis of the presence of inhibitors

A cPCR previously described by BRINKHOF et al. (2006) that targets a highly conserved gene of dogs (the Beta-actin protein) was performed, and the quality of DNA extraction was assessed through the spectrophotometer Nanodrop ND-2000® (Thermo Fisher Scientific, Wilmington, DE, USA). An inhibitory analysis was performed to verify if the negative samples were truly negative or if the amplification was undermined because of the inhibitors' presence. The test was executed, adding ten copies of the *p28* gene fragment from *E. canis* in all negative sample aliquots presented in this study.

Statistical analysis

In order to predict the number of copies from the Cq value, a simple linear regression analysis was performed for standard curves with and without the addition of dog DNA. The two standard curves were compared, applying Student's t-test to verify if there is a difference between the regression coefficients (slope value) and y-intercept between the linear regression analyzes performed for each curve.

The results of the real-time PCR and conventional PCR, targeting the *p28* gene of *E. canis*, were evaluated by the McNemar Test at a 5% significance level using BioEstat 5.0 software. This analysis aimed toward measuring the proportion of disagreement between qPCR and cPCR for *E. canis* detection in dog blood samples.

RESULTS

The qPCR designed in this study presented 24.31% (n = 53/218) of positive samples. By contrast, the cPCR presented 15.13% (n = 33/218). All the positive samples of cPCR were tested positively by qPCR.

The designed oligonucleotides targeting the *p28* gene of *E. canis* proved to be specific in the *in vitro* analysis when tested against pathogens commonly found in dogs in Brazil, including *E. chaffeensis* (the closest species).

The primers' optimal concentration was set at 200 nM for both forward and reverse settings,

which achieved a Cq value of 26.08 cycles, an ΔRn of 0.867, and no sign of dimerization using a standard sample with 100 copies of the *E. canis p28* gene as a template. The optimal probe concentration was 250 nM, reaching the minimum Cq value of 29.95 with the higher ΔRn 0.900 as the positive control.

The optimized reaction conditions developed in this study was a final volume of 12 µL, comprising 1x TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 200 nM of each primer, 250nM of the probe, and 150 ng of DNA sample.

The standard curve (without the dog's DNA) presents six dilution points with the number of copies in the log scale plotted against the quantification cycle values (Figure 1). The achieved determination coefficient (R^2) was 99.8%, the mean Cq values and standard error were from 19.12 ± 0.06 in the first dilution point (10^6 copies per µL), 22.28 ± 0.09 in the second point (10^5 copies per µL), 25.70 ± 0.04 in the third point (10^4 copies per µL), 29.32 ± 0.04 in the fourth point (10^3 copies per µL), 32.89 ± 0.09 in the fifth point (10^2 copies per µL), to 35.73 ± 0.35 in the last dilution point (ten copies per µL) (Figure 1). An efficiency of 100% was reached in the standard curve built without adding dog DNA (Figure 1).

The standard curve (with the dog's DNA) presents six dilution points with the number of copies in the log scale plotted against the quantification cycle values (Figure 2). The achieved determination coefficient (R^2) was 99%. An efficiency of 98.84% was reached in the standard curve with the dog's DNA (Figure 2).

The Student's *t*-test resulted in no significant difference between the linear regression analyzes of standard curves with and without the addition of dog DNA.

The analytical sensitivity of qPCR showed a detection limit of ten copies of the *E. canis p28* gene per µL in both standard curves.

All these results demonstrate that the developed qPCR method was efficient, specific, and sensitive for detecting *E. canis* DNA in blood from naturally infected dogs. In contrast, the analytical sensitivity of cPCR presented a detection limit of 100 copies per µL. The quantification range of the *E. canis p28* gene found in the studied samples was ten to 28.183 copies per µL.

The comparison between techniques revealed that qPCR presented a significantly higher number of positive samples in contrast with cPCR, the former showing 24.31% (n = 53/218) and the latter showing 15.13% (n = 33/218). The discordant

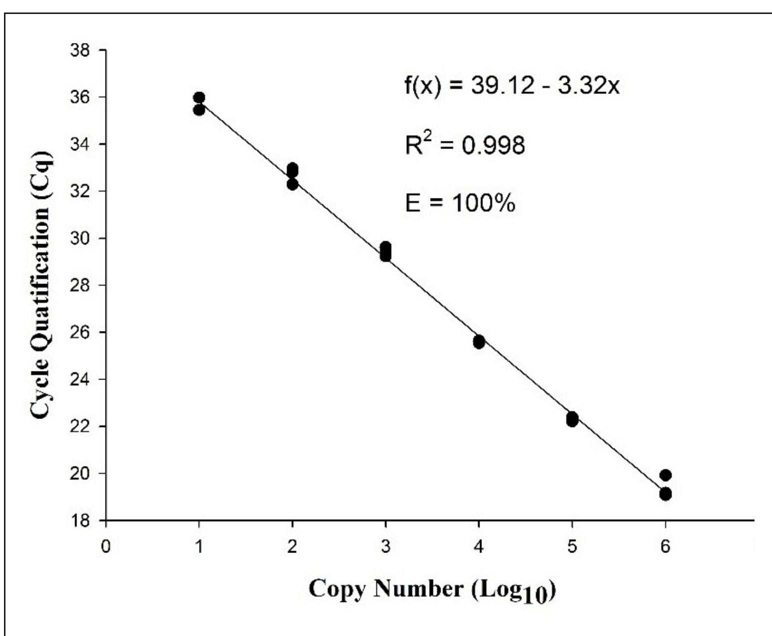

Figure 1 - Standard curve plotted from serial decimal dilutions of amplicon containing the *Ehrlichia canis p28* gene. The quantification cycle (Cq) value obtained by a real-time polymerase chain reaction using TaqMan technology was plotted as a function of the initial number of target copies.

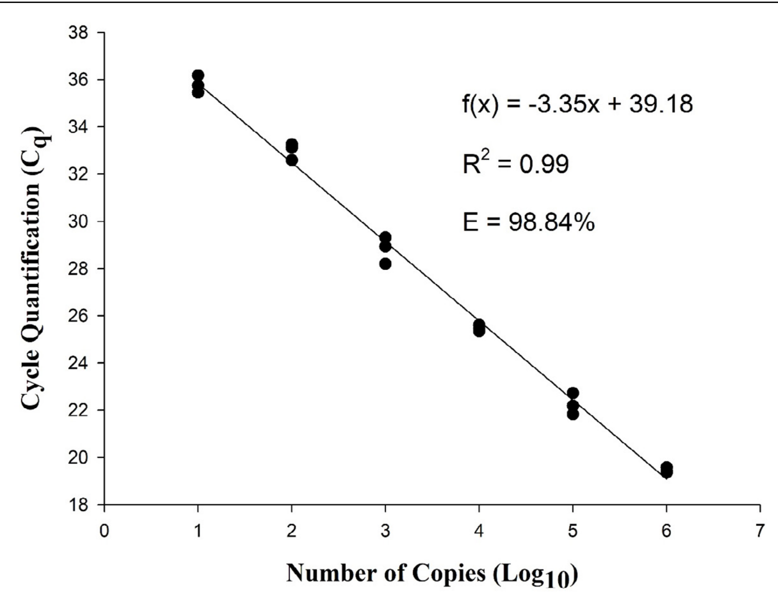

Figure 2 - Standard spiked curve plotted from serial decimal dilutions of amplicon containing the *Ehrlichia canis p28* gene. Each dilution point was spiked with 20 ng of standard negative DNA control. The quantification cycle (Cq) value obtained by a real-time polymerase chain reaction using TaqMan technology was plotted as a function of the initial.

pairs (twenty samples) tested positively for qPCR and negatively for cPCR presented Cq values ranging from 37 to 39 cycles. The statistical analysis showed a significant difference ($p < 0.001$) between the methods and favored the qPCR (Table 1).

DISCUSSION

Several real-time PCR protocols to confirm CME's diagnosis have been described during the last twenty years. DOYLE et al. (2005) described the multicolor qPCR as an assay capable of detecting and discriminating between *E. chaffeensis*, *E. canis*, and *E. ewingii* in a single reaction by amplifying a fragment of the *dsb* gene as well as detecting co-infections within the same sample. Although this technique is frequently used, the reported analytical sensibility is 50 copies, which is higher than the present study's detection limit (ten copies). PELEG et al. (2010) design a probe-qPCR targeting the 16S rDNA sequence. This qPCR assay exhibits the same detection limit found in the current study, albeit it does not present the same specificity level considering it reports primers with high conservancy between *Ehrlichia* species, E. *canis*.

QUROLLO et al. (2014) designed a qPCR assay displaying a detection limit of only five

Table 1 - Analysis of the disagreement between the real-time polymerase chain reaction (qPCR) and a conventional polymerase chain reaction (cPCR). A comparison of the results was obtained when samples were subjected to the detection of *Ehrlichia canis.*

cPCR	--------------------------qPCR-----------------------------		Total
	Positive	Negative	
Positive	33	0*	33
Negative	20*	165	185
Total	53	165	218

* $P < 0.001$ indicates significant disagreement between *p28*-cPCR and *p28*-qPCR by McNemar test.

copies. Meanwhile, it also displays a cross-reaction with *A. phagocytophilum* (SHEN et al., 2018). The qPCR technique developed in the present study did not cross-reacted with *A. phagocytophilum* in either specificity test.

Other hydrolysis probe-qPCR assays have been described within the existing literature (BANETH et al., 2009; THOMPSON et al., 2018). The qPCR assay described by BANETH et al. (2009) targets the *E. canis*-16S rDNA sequence. Unlike other bacteria with multiple copies of 16S rDNA, *E. canis* has only one copy of this molecular marker (MAVROMATIS et al., 2006). The gene that encodes the outer membrane 28 kDa protein has multiple copies in the *E. canis* genome (MCBRIDE et al., 1999). Therefore, when *p28* is targeted, the chances of *E. canis* detection occurring are increased. Regarding the qPCR assay designed by THOMPSON et al. (2018), the technique targets the *gltA* gene, and it aims at *Ehrlichia* genus-detection displaying a lower specificity level when compared to the current study.

The qPCR targeting the *p28* gene developed in this research proved to be suited to *E. canis* detection according to MIQE Guidelines (BUSTIN et al., 2009), displaying an adequate efficiency even upon a challenge (E= 98.84−100%) with no statistical difference between the values, and an excellent determination coefficient (99−99.8%). A limitation to the *p28*-qPCR developed in this study, which is an expected drawback to all DNA-based detection methods, regards the possibility of the appearance of mutations in the sequences targeted by the primers and probes due to the emergence of new divergent pathogen strains. The present study attempted to confront this limitation by choosing conserved regions of *p28* gene sequences of *E. canis*. However, this concern cannot be dismissed.

Considering the comparison of techniques performed in this study, the McNemar test has shown a significantly higher qPCR sensitivity over cPCR ($p < 0.05$). The samples included in the discordant pair group—meaning the samples tested positively by qPCR and negatively by cPCR— had many copies with a lower detection limit than the detection limit of cPCR. The cPCR uses agarose gels to reveal the results, which are not as precise as qPCR due to a low sensibility, revealing changes of about tenfold (TRIPATHI, 2010). On the other hand, qPCR is a very sensitive method because the applied fluorescence detection systems enable the capturing of minimal signs of fluorescence, detecting as little as a twofold change (TRIPATHI, 2010).

CONCLUSION

Due to CME's veterinary importance, the process for detecting *E. canis* must be improved. Furthermore, reliable and accurate techniques for detecting parasites are essential to creating a therapeutic plan and monitoring both the parasitemia and the infection during treatment (SILVA et al., 2016). The qPCR targeting the *p28* gene developed in this study proved to be suited to *E. canis* detection, achieving high sensitivity and specificity compared to other methods described previously in the literature. The qPCR may also be applied routinely within any laboratory without the need for post-PCR assays and can release fast and reliable feedback to veterinarians. It can also provide a quantitative measure of parasites even in samples with low copy numbers, indicating that it is useful in detecting clinical and subclinical patients. This characteristic allows clinicians to monitor the efficiency of different therapeutic protocols for ehrlichiosis.

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BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

The research ethics committee approved these procedures of the Universidade Federal Rural do Rio de Janeiro (UFRRJ) (protocol number: 3915240616).

DECLARATION OF CONFLICT OF INTEREST

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

AUTHORS' CONTRIBUTIONS

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

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