Standardization of a SYBR Green Based Real-Time PCR System for Detection and Molecular Quantification of *Babesia bovis* and *B. bigemina* in Water Buffaloes (*Bubalus bubalis*)

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Abstract: Water buffalo (*Bubalus bubalis*) is a potential reservoir for *Babesia bovis* and *B. bigemina* in tropical regions, but the epidemiological evidence of their reservoir competence is limited, especially due to the lack of diagnostic tests capable of detecting and quantifying the low-level parasitemia present in the carrier animals. In this paper we present the standardization process of a SYBR Green based real-time PCR system (qPCR), consisting of two single qPCR assays, for the detection and quantification of *B. bovis* and/or *B. bigemina*. Both assays were optimized in similar protocols, including reagent concentrations and thermocycling parameters, so it is possible its use as a multiple qPCR in a single run. Both single assays showed a suitable analytical performance, especially by allowing detection of a greater number of carrier animals when compared with nested PCR assays (nPCR) against a reference panel of 60 DNA samples extracted from blood of both, infected- and non-infected buffaloes. Furthermore, a mathematical algorithm to convert the qPCR outcomes in percent of infected red blood cell was used, and was found that the estimated parasitemia in carrier buffaloes within the reference sample panels were close to those described in carrier cattle. This method could be a useful tool for epidemiological studies on the participation of the bubaline specie in the epidemic process of bovine babesiosis.

Keywords: *Babesia bovis*, *B. bigemina*, water buffalo, parasitemia, qPCR, standardization.

INTRODUCTION

Bovine babesiosis is one of the main constraints for livestock in tropical and subtropical regions of the world, caused by the tick-borne protozoa *Babesia bovis, B. bigemina* and *B. divergens* (Apicomplexa: Babesiidae). *B. bovis* and *B. bigemina* are widespread in Latin America, Africa, Australia, and Asia, and they generally occur as mixed infections, whereas *B. divergens* is only found in the northeast of Europe and Tunisia [1, 2]. The cattle is the maintenance host of *B. bovis* and *B. bigemina*; however the animals of the subspecie *Bos taurus taurus* are very susceptible, whereas those of *B. t. indicus* are more resistant and, generally, remain as carrier hosts for a long time, especially for *B. bovis* [3-5].

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Epidemiological studies in endemic areas showed that other ruminants may be carriers of *B. bovis* and *B. bigemina*, such as the white-tailed deer (*Odocoileus virginianus*) [6], the impala (*Aepyceros melampus*), the african buffalo (*Syncerus caffer*) [7], and the water buffalo (*Bubalus bubalis*) [8]. However, no evidence of infection maintenance and cross-species transmission (reservoir competence) have been found in these host populations, so that they could be incidental hosts [9]. Specifically, water buffaloes are a potential reservoir because of the frequent coexistence with cattle in livestock ecosystems [10,11] and as it has been demonstrated that these ruminants are able to sustain the complete tick life cycle of *R. microplus* [12, 13].

On the other hand, water buffalo is resistant to babesiosis, and when infected it will develop a subclinical form of the disease (carrier host) [11, 14], likely with low levels of parasitemia because they are undetectable by Giemsa-stained blood smears [8, 10].

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However, no quantitative evidence of parasitemia and infectiousness are found in buffaloes. In carrier cattle the parasitemia by *B. bovis* and *B. bigemina* remains below 0.01% IRBC/mL (Percent infected red blood cells per mL), which is sufficient to infect tick populations [2, 15].

Identifying the hosts that contribute most to the infection of vectors population is crucial for understanding the transmission dynamics of vector borne diseases, as well as for planning intervention strategies targeting the relevant infected host groups [16]. The parasitemia is an important factor of the host reservoir competence on vector borne pathogens, influencing their contribution (host infectiousness profile) to the infected vector population [17]. The Giemsa-stained blood smears is a conventional method used to determine parasitemia in clinically infected cattle, but this test has low analytical sensitivity and is ineffective in carrier animals [15, 18, 19].

The introduction of PCR method allows detection of carrier cattle infected with *B. bovis* and *B. bigemina*, especially nPCR assays with a lower limit of detection on 0.0000001% IRBC/mL [2, 15]. Subsequently, the real-time PCR (qPCR) techniques have improved the diagnosis of bovine babesiosis and allowed to quantify the infection levels in carrier animals, with high sensitivity, specificity and analytical accuracy [20-22].

However, the effectiveness of qPCR in the diagnosis and quantification of these protozoa in other host species has not been evaluated. In addition, the estimates of infection levels on carrier cattle are based on parasites/µL [21,23], which require an additional procedure enabling the transformation of qPCR outcomes into percent IRBC/mL. The present work was carried out with the objective of standardizing a SYBR Green based real-time PCR system for the detection and quantification of *B. bovis* and *B. bigemina* in carrier hosts, especially in water buffaloes.

MATERIALS AND METHODS

qPCR Assays

This work was conducted at the Laboratory of Animal Health, Embrapa Pecuária Sudeste, São Carlos, São Paulo, Brazil. A SYBR Green based real-time PCR system was used; it consisted of two single assays, one for *B. bovis* and the other for *B. bigemina*. The primers used were those described by Buling *et al*. [22], which amplify 88bp fragments of the mitochondrial cytochrome b gene (*mt-Cytb*). For *B. bovis*: cbosg forward. 5'-TGTTCCTGGAAGCGTTGATTC-3' and cbosg Reverse. 5'-AGCGTGAAAATAACGCATTGC-3'; for *B. bigemina*: cbisg forward. 5'-TGTTCCAGGAGA TGTTGATTC-3' and cbisg reverse. 5'-AGCATGGAAAT AACGAAGTGC-3'. Primer specificity was verified with the sequences available in GenBank by using the search tool BLASTn (http://blast.ncbi.nlm.nih.gov/ blastn).

The qPCR reactions were initially performed in a final volume of 15µL, with 7.5µL of the commercial mix (2X) Lumino SYBER® Green qPCR Ready Mix (Sigma), 1µL of each primer (10µM), 2µL of DNA, and nuclease-free water (Promega). The same amplification program was used for both assays: an initial cycle of 95ºC/5min, followed by 40 cycles of 95ºC/5seg, and 60ºC/45seg for hybridization and extension jointly. In addition, a melting curve was included to verify the specificity of the amplicons, between 65°C and 95°C with increments of 0.5°C/5seg. A thermocycler CFX Real-Time PCR Detection Systems (BioRad) and PCR Low-Profile 8-Tube strips (BioRad) were used.

Standard Test Method for Comparison

A nPCR system targeting the gene *18S rRNA* of *B. bovis and B. bigemina,* previously reported by Guerrero *et al*. [24] but with modifications, was used as reference (hereinafter "standard test"). Briefly, the PCR reactions were performed in 25µL, which contained 12.5µL of commercial mix (2X) Jump Start RED TaqReady Mix (Sigma Aldrich), 1.0µL (10µM) of each primer, 2µL of DNA, and nuclease-free water. Subsequently, the nPCR reactions were performed in 20µL, which contained 10µL of (2X) Jump Start RED TaqReady Mix (Sigma Aldrich), 1.0µL (10µM) of each primers, 1µL of the PCR product as template DNA, and nuclease-free water (Promega).

DNA for Positive and Negative Controls

Genomic DNA from ¨Jaboticabal¨ isolate of *B. bovis* and *B. bigemina* was used as a positive control (hereinafter "positive control"). The DNA was extracted with the commercial kit Ilustra Blood Genomic Prep Mini Spin (GE Healthcare) from 300µL of blood obtained from artificially infected splenectomized calves sampled at peak parasitemia, according to Machado *et al*. [25]. The genomic DNA from blood of newborn water buffalo calf free of *Babesia* spp. was used as the negative control.

Also, a reference sample panel (hereinafter "reference samples") was used for each assay for

comparing the outcomes with the standard test method, according to the recommendations by the World Organization for Animal Health [26]. Each panel had 60 DNA samples, 30 of them were nPCR-positive and 30 nPCR-negative. The DNA samples were extracted from the blood of water buffaloes randomly selected from farms in western Cuba, as described by Obregón *et al*. [8]. DNA extraction was performed from 300µL of blood by using the WIZARD Genomic DNA Purification Kit (Promega).

Standard Curves

Initial qPCR reactions with positive controls as target DNA (ten replicates) were performed for each assay, and the resulting amplicons (88pb) were used to construct the standard curve for each assay. The amplicons were directly purified from the qPCR product using the commercial kit Pure Link PCR Purification Kit (Invitrogen) and cloned into the plasmid pGEM®-T Easy Vector Systems (Promega) (3000pb) as described by Bilhassi *et al*. [23].

Afterwards, the concentration of the recombinant plasmid were determined using a Nanodrop 1000 v.3.5 spectrophotometer (Thermo Fisher Scientific), and the number of copies (NC) of template DNA (CN/µL) was estimated by the equation described by Ke *et al.* [27]: CN/µL= Conc. (g/µL) x N_A / MW (g/mol), where NA is Avogadro constant (6.022 x 10^{23} copy/mol) and MW is the molecular weight of each pair of nucleotides (660) multiplied by the molecular weight of the recombinant plasmid (3088bp). Subsequently, ten-fold serial dilutions (from 10^{-1} to 10^{-10}) were made.

Optimization of qPCR Reactions

The critical parameters were simultaneously optimized in the two assays. These were the hybridization temperature (63°C, 60°C, and 57°C), time of hybridization and extension (30seg, 45seg, and 60seg), concentration of primers (0.1µM, 0.2µM, 0.4µM, 0.6µM, and 0.8µM), and volume of template DNA (1µL, 2µL and 4µL). The 10^{-2}-10^{-8} dilutions of the standard curve were used as template DNA.

Linearity of the results of the qPCR assays was assessed from the efficiency of the PCR reactions of the standard curves and by the parameters: slope, y-intercept, and the regression coefficient (R^2). In addition, ten samples from carrier buffaloes for each hemoparasite were tested to verify that they presented the quantitative cycle (Cq) within the range of the standard curve, as recommended by Mueller *et al*. [28].

Evaluation of Analytical Performance

The analytical performance of the assays was evaluated following the methodology proposed by the OIE for standardization of molecular assays [29, 30]. The intra- e inter-assay repeatability was measured from the coefficient of variation (CV) of the Cq values in the standard curve. Ten trials of each assay, with each standard dilutions tested in triplicate, were included in the analysis. The analytical specificity (Asp) was determined according to the capacity of differentiating between *B. bovis* and *B. bigemina*, and host genomic DNA (exclusivity and selectivity), for which the 10^{-3} dilution was selected in each standard curve, and DNA negative control. For each assay, the three samples were simultaneously tested, and no-template reactions (NTC) were included in each trial as contamination control.

The lower limit of detection, referred as analytical sensitivity (Ase), was experimentally determined and used as the limit of quantification (cutoff) in each assay, according to Caraguel *et al*. [31]. For this, a nPCR-positive sample was selected for each hemoparasite, and, from each of them, seven serial dilutions, from 10^6 CN/µL to 10^{-1} CN/µL, were done. Five replicas of each dilution were amplified, and NTC were included in each trial. The cutoff was established in the Cq of the lowest dilution where more than 50% of the replicates amplified.

The results of the qPCR assays were compared with the standard test method (reference samples). The results were confronted in 2x2 contingency tables, and the agreement was measured using kappa coefficient (IC. 95%). Calculation of the kappa coefficients was performed with the on-line tool VassarStats (http://vassarstats.net). The level of agreement was classified following the scale described by Landis and Koch [32]: < 0.10= Poor agreement; 0.20- 0.39= Fair agreement; 0.40-0.59= Moderate agreement; 0.60-0.79 = Substantial agreement; 0.80-1.00= Almost perfect agreement.

Procedure to Estimate Parasitemia

The parasitemia was estimated in the reference samples that resulted positive by the qPCR, for each protozoan. The initial target DNA copy number in each sample (CN/µL) was calculated based on Cq value with the program Bio-Rad CFX96 Manager v. 3.1 (BioRad). Afterwards, the number of parasites per mL of blood was estimated with the formula proposed by Ros-

García *et al*. [33] with modifications: P= CN/µL (V_B/V_{EX}) (V_{EL}/V_T) (1/CN), where: P- is the number of parasites per mL of blood, V_B- volume of reference blood (1mL), V_{EX}- volume of blood for extracting DNA (200µl), V_{EL}= DNA elution volume (100µl), V_T-volume of DNA in the PCR reaction, and CN- the gene copy number. For CN, 100 copies of the gene *mt-Cytb* were considered, according to Salem *et al*. [34].

Subsequently, the percentage of infected erythrocytes per mL of blood (IRBC/mL) was calculated by the equation: IRBC/mL (%) = P/ (MI x RBC) x 100, where MI – number of merozoites in an erythrocyte, and RBC- average erythrocytes per mL of blood. For the MI, two parasites per cell were considered [5], and for RBC, a value of the reference in buffaloes of 7.06±0.07 X 10^6 E/µL was considered [35].

RESULTS AND DISCUSSION

The concentration of the recombinant plasmids was 479.6 ng/µL for *B. bovis* and 200.2 ng/µL for *B. bigemina*; therefore, the serial dilutions used in the standard curves (10^{-2} a 10^{-8}) showed a copy number of target DNA in the ranges 1.4x10^9 - 1.4x10^3 CN/µL for *B. bovis* and 5.8x10^8 - 5.8x10^2 CN/µL for *B. bigemina*.

Both qPCR reaction assays were more efficient with a hybridization temperature of 60ºC for 45 seg and primer concentrations between 0.4µM and 0.8µM; it was decided to use 0.4µM of each primer since this is the minimum effective concentration according to Bustin and Nolan [36]. Besides, both assays operated correctly with 2µL and 4µL of template DNA solution, but with 1µL, uniformity decreased in the replicas of the least concentrated dilutions, possibly due to the reduction of the probabilities to place the target DNA in the PCR reaction, a phenomena known as "monte carlos effect" [36]. Two microliters of template DNA were selected for both assays; however, up to 4µL can be used, what contributes to the assay accuracy at low levels of parasitemia in carrier hosts, especially for *B. bovis* [2, 9].

The two qPCR reaction assays showed a good linearity performance, according to the amplification of the standard curve (Figure **1**). In ten trials, the R^2 values remained between 98% and 100% in both assays, the slope remained at -3.28±0.15 in the *B. bovis* assay and -3.30±0.13 for *B. bigemina*, corresponding to efficiencies within a range of 95% - 100%, and the y-intercepts values were of 42±4 cycles for *B. bovis* and of 43±3 for *B. bigemina*. These results were considered as satisfactory since the ideal efficiency in a qPCR reaction is 100% (slope -3,32), but 90 -110% is acceptable [27, 35]; besides, the ten samples of carrier buffaloes examined for each assay showed the Cq between the 10^{-3} and 10^{-7} standard dilutions (data not shown).

During this standardization step, it was observed that the amplicons of the qPCR assays presented the melting peaks at 78.5ºC in *B. bigemina* and at 79.5ºC in *B. bovis*, which allowed differentiating the amplicons of unspecific bindings and primer dimers. These results were not in agreement with the results reported by Buling *et al*. [22], who recorded different values and 2ºC of difference between the melting peaks (74.15 ± 0.18ºC for *B. bigemina* and 76.4 ± 0.21ºC ± for *B.*

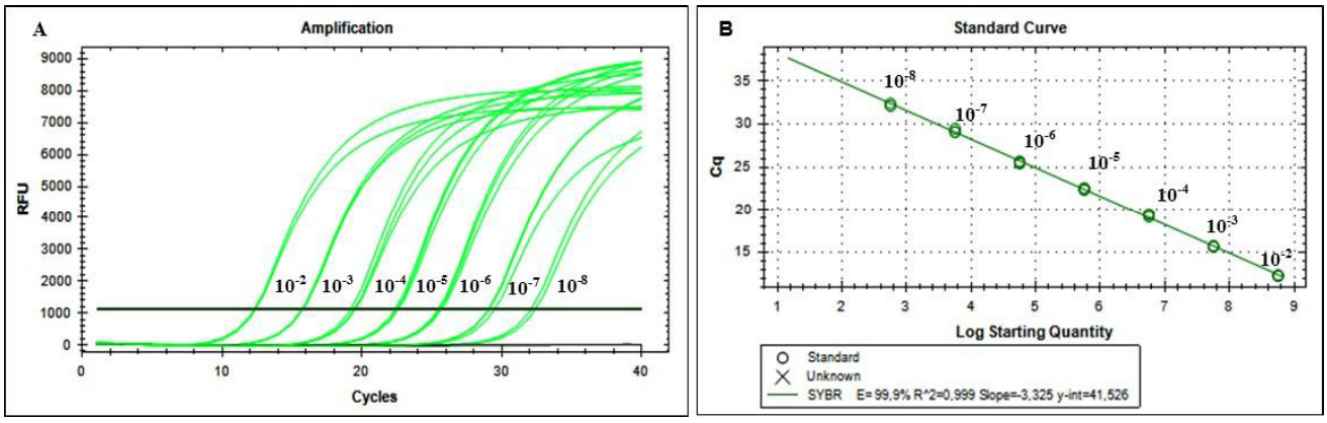

Figure 1: Linear range of the qPCR assay for *B. bovis*. **A**. Real-time PCR fluorescence curves derived from serially diluted standard concentration (plasmid copy number); each plot corresponds to seriated dilution in the standard curve, ranging from 10^{-2} to 10^{-8}, the y-axis indicates the fluorescence intensity, and the automatic threshold line (1000) indicates the threshold cycle (Cq) of each standard dilutions (in triplicate). **B**. Regression curve generated by plotting the mean Cq values as a function of the starting copy number (Log) of the standard dilutions, showing the PCR reaction efficiency (E), correlation coefficient (R^2), Slope and y-intercept. Automatically generated graphics by the software Bio-Rad CFX96 Manager v. 3.1 (BioRad).

Table 1: Intra- and Inter-Assay Repeatability of the qPCR Assays for *B. bovis* and *B. bigemina*

Standard curves dilutions	qPCR- *B. bovis*			qPCR- *B. bigemina*		
	Cq means	Inter-assays CV (%)	Intra-assays CV (%)	Cq	Inter-assays CV (%)	Intra-assays CV (%)
10^{-2}	12.4	2.3	0.4	11.2	1.6	0.7
10^{-3}	16.6	3.8	0.5	14.4	1.1	0.6
10^{-4}	19.6	2.8	0.8	17.9	1.7	0.8
10^{-5}	22.8	3.2	0.5	20.3	0.2	0.4
10^{-6}	26.2	3.2	0.3	24.6	0.6	0.2
10^{-7}	29.3	2.1	0.5	28.3	1.5	1.1
10^{-8}	32.1	2.1	1.1	31.0	1.5	1.6

bovis) of the amplicons with these primers, maybe because different platforms and qPCR commercial mix were used. Based on the low values of the coefficient of variation between the Cqs of the standard dilutions (Table **1**), the qPCR assays showed a high repeatability, which indicated that the optimization of the qPCR reactions was achieved [27].

The ASp of both assays was confirmed; especially, the melting curve analysis discriminated between *B. bovis* and *B. bigemina,* and those of the matrix components (host DNA) (Figure **2**). The melting temperature of the nucleic acid fragment is affected by its length, GC content, and the presence of base mismatches, among other factors. The melting-curve analysis is a straightforward way to ensure reaction specificity of the real-time PCR reactions and reduces the need for the time-consuming gel electrophoresis [36]. Furthermore, performing both assays with the same thermocycling parameters confirmed the feasibility of the system as multiple qPCR, allowing the specific diagnosis of the two protozoan parasites in several samples in a single qPCR run.

The ASe was similar in both assays, established in 20 DNA copies (CN/µL), so the cutoff was located in the Cq 38 (Figure **3**). This methodology to select the cutoff allows reducing the number of replicates to predict the minimum concentration of the target DNA in which more than 50% of the samples will be detected; specifically, five replicates per dilution allows to estimate with 95% confidence [31]. The results indicate that the assays allow detecting and quantifying even a parasite on the sample (300µL of blood) because the gene *mt-Cytb* can be found about 100 times in each parasite of *Babesia* spp. [34].

In the analysis of the diagnostic performance of the qPCR assays against samples diagnosed by nPCR, a kappa coefficient of 0.63 was obtained for *B. bovis* and k=0.80 for *B. bigemina* (Table **2**), so the agreement was evaluated as substantial. The differences were

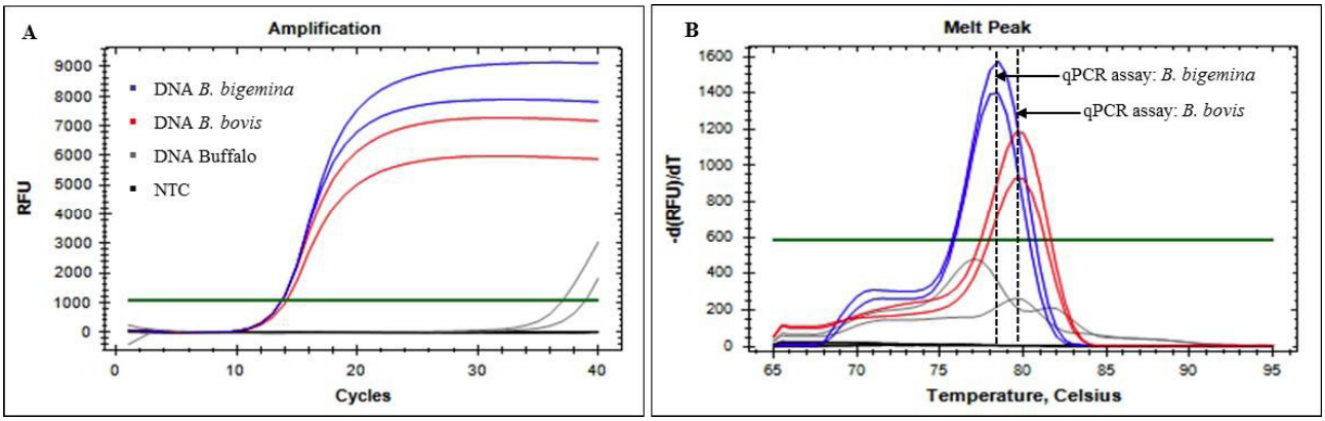

Figure 2: Analytical specificity of the qPCR assays. **A**. Real-time PCR fluorescence curves derived from DNA standard dilution (10^{-3}) in the standard curve of *B. bovis* and *B. bigemina* (positive control), water buffalo genomic DNA (negative control), NTC-no template control, each sample in duplicate. **B**. Melting curve analysis. *B. bigemina* and *B. bovis* amplicons (only obtained in the corresponding assay) are distinguished by the difference of approximately 1°C in their melting peak. Automatically generated graphics by the software Bio-Rad CFX96 Manager v. 3.1 (BioRad).

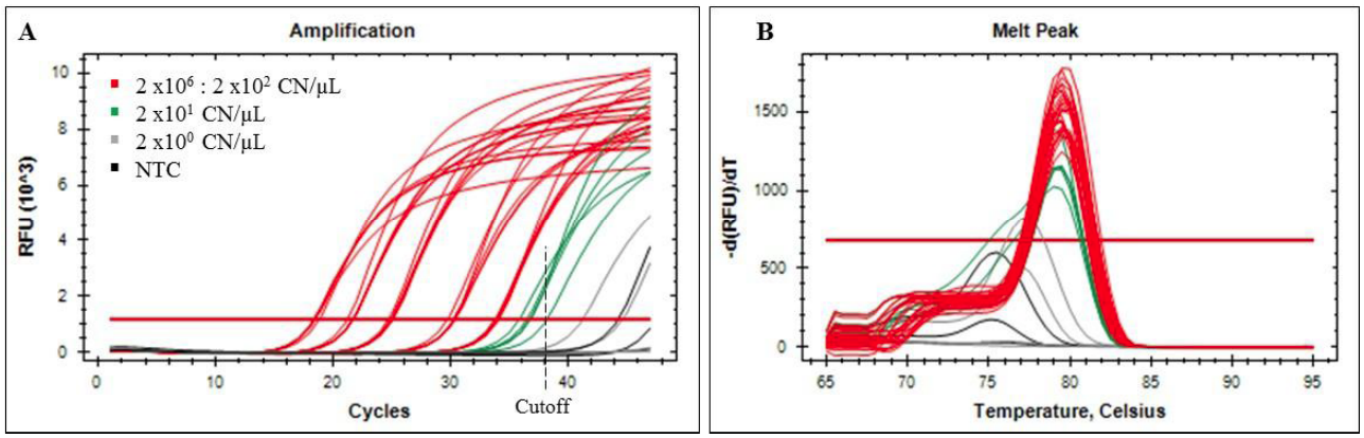

Figure 3: Analytical sensitivity and cutoff of the qPCR assay for *B. bovis*. **A**. Real-time PCR fluorescence curves derived from a serially diluted DNA reference sample; each plot corresponds to DNA dilution (in quintuplicate) in the range from 20 x10^6 to 2 CN/µL; NTC- no template control. The detection limit was established in 20 CN/µL (green), indicating the corresponding Cq cutoff (38). **B**. Melting curve analysis of resulting *B. bovis* amplicons. Automatically generated graphics by the software Bio-Rad CFX96 Manager v. 3.1 (BioRad).

essentially in the number of animals with positive diagnosis for both hemoparasites, in favor of the qPCR assays, so that 33% of the negative animal for *B. bovis* by nPCR resulted positive by qPCR, and 20% of the negative animals for *B. bigemina* by nPCR resulted positive by qPCR.

The differences could be a consequence of the number of copies of the target DNA since the nPCR assays were targeted to the gene *18S rRNA*, which presents three copies in the genome of *Babesia* spp., and the lower detection limit of these assays is equivalent to 14 parasites according to Guerrero *et al*. [24]. This result is independent to the fact that the qPCR and nPCR assays usually have similar Ase when they have the exact same target DNA [37, 38].

The increase of Ase of the extra chromosomal DNA based test with respect to the ribosomal DNA-based test in the diagnosis of *Babesia* spp. was previously reported by Salem *et al*. [34], who compared the conventional PCR assay and observed that the extra chromosomal DNA assays were 20% more sensitive. Additionally, Buling *et al*. [22] with qPCR assays similar to those used in this work, confirmed that the analytical sensitivity was 100% higher than that in the ribosomal DNA-based assay. In correspondence, Billhassi *et al*. [23], with qPCR, identified Nellore cattle infected with *B. bovis* that could not be previously detected by nPCR.

In the quantification by qPCR, all the positive samples to *B. bovis* showed the Cq between the cycles 23 and 32 (Figure **4**), corresponding to parasitemia values between 0.2 and 0.0000001% IRBC/mL, with a mean of 0.01% IRBC/mL. The samples positive to *B. bigemina* showed a Cq between 20 and 35, with a higher range of parasitemia values estimated between 5 and 0,000001% IRBC/mL, with a mean of 0.08% IRBC/mL. These parasitemia levels are characteristic of bovines carrying these hemoparasites in endemic areas [9, 39]; so they may be sufficient for infection of feeding ticks, enabling the biological transmission of these protozoa from carriers buffalos, however the infectiousness of these hosts should be analyzed in future research.

Table 2: Agreement between the Results of the qPCR and nPCR Assays

nPCR*	qPCR- *B. bovis*		qPCR- *B. bigemina*	
	(+)	(-)	(+)	(-)
(+) 30	29	1	30	0
(-) 30	10	20	6	24
Total	39	21	36	24
Kappa (IC.95%); SE	0.63 (0.44-0.82); 0.09		0.80 (0.65-0.94); 0.07	

*Identifies the panel of reference samples selected by the nPCR results.

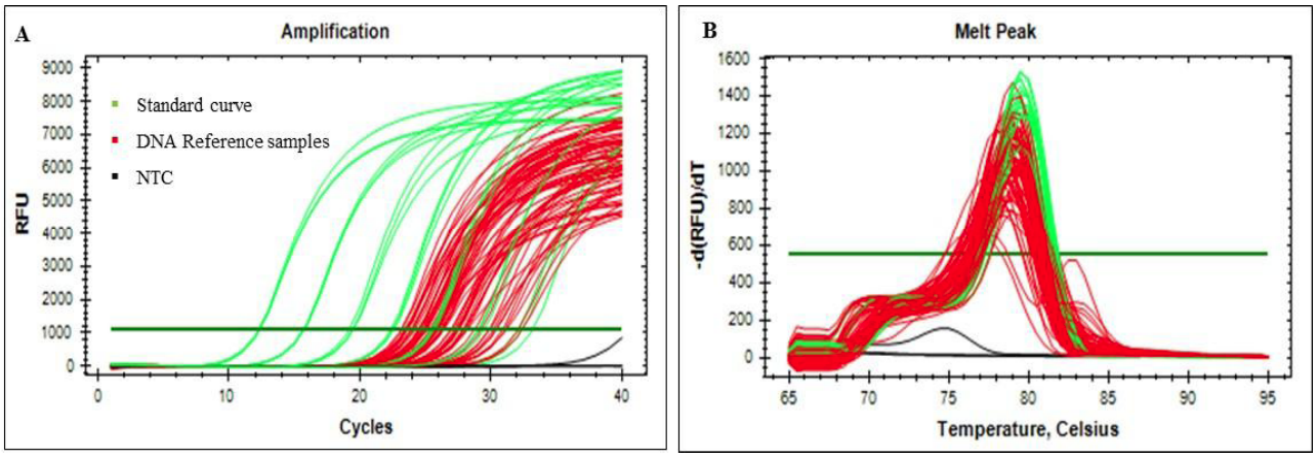

Figure 4: Analysis of the reference samples by qPCR assay for *B. bovis*. **A**. Real-time PCR fluorescence curves derived from a standard curve (green) and DNA reference sample (red), each sample and standard dilution in duplicate, NTC- no template control. **B**. Melting curve analysis of resulting *B. bovis* amplicons. Automatically generated graphics by the software Bio-Rad CFX96 Manager v. 3.1 (BioRad).

Low-level parasitemia in cattle characterizes the balanced interaction among hosts, hemoparasites and vectors, which assures the persistence in the hosts and the endemic stability in the herds. On the contrary, high parasitemia and clinical symptoms appear when there is no compensation between pathogen virulence and host resistance [3]. These results confirm that water buffalo offers natural resistance to *B. bovis* and *B. bigemina* [10, 40]. Further studies must be conducted to confirm the capacity of carrier buffaloes of infecting tick populations and their contribution to the epidemiological process of the bovine babesiosis.

CONCLUSIONS

The standardized SYBR Green based real-time PCR system, targeting the extra chromosomal *mt-Cytb* gene, allowed the sensitive detection and quantification of *B. bovis* and *B. bigemina*, and its usefulness under field conditions was confirmed with water buffalo blood samples. This qPCR system provides quantitative data of the parasitemia levels what should contribute to measure the host competence and reservoir capacity of water buffaloes for these protozoa.

CONFLICTS OF INTEREST

None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence the content of the paper.

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