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Similarity in qPCR Assay Parameters Validates the Reliability of Two Microtube Brands

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ABSTRACT

***Background*:** Quantitative PCR (qPCR) is a powerful molecular biology technique widely used for gene transcription analysis, pathogen detection, food and environmental contamination monitoring, due to its high sensitivity, specificity and accuracy when compared to immunological and microbiological assays. It enables real-time monitoring of DNA amplification playing a critical role in medical diagnostics, vaccine development and cancer research. This makes qPCR an essential technique in agriculture, forensics and environmental monitoring, solidifying its position as one of the most versatile tools in modern science. However, the cost of this molecular method remains a significant limitation, as expenses related to equipment and consumables (such as pipette tips, enzymes and reaction tubes) make the technique relatively expensive, particularly for high-throughput applications or laboratories with limited budgets.

***Materials, Methods & Results*:** The experiments were conducted using 2 very well characterized constitutive genes from *Rhipicephalus microplus*, *40s ribosomal protein* and *cyclophilin A*, allowing a consistent and reliable analysis of qPCR assay performance. These genes were selected due to their stable transcription across different biological conditions, ensuring minimal variability in amplification efficiency. Statistical analysis was performed using Mann-Whitney test, based on the mean and standard deviation of the Ct values, with a significance threshold set at $P < 0.01$. No significant difference ($P = 0.99$) was observed between the microtubes evaluated in this assay, indicating that, despite being from different manufacturers and price categories, the tubes presented comparable performance, in terms of amplification efficiency and reproducibility. This consistency was observed even when testing different reference genes and biological samples, further validating the robustness of the findings. Notable, the most cost-effective microtube, evaluated in this work, offered over 70% savings compared to the alternative, making it a highly attractive option for laboratories seeking to reduce expenses without sacrificing data quality.

***Discussion*:** Various alternatives have been explored to reduce the cost of the qPCR assay while maintaining reliability. These include the use of more economical thermal cyclers, minimizing consumable wastes, adopting portable qPCR system, simplifying nucleic acid extraction protocols, and optimizing reaction conditions to reduce the impact of inhibitors. Among consumables, approaches such as the reuse of pipette tips (where feasible) and reducing the volume of each reagent in the reactions have been reported. However, studies systematically evaluating the performance of different microtube brands in qPCR applications remain scarce. Here, 2 microtubes with identical physical specifications, but differing in brands and values, were tested in qPCR assays using constitutive tick genes. The results demonstrated no significant differences in reaction efficiency or reproducibility between the tubes, confirming that cost-saving alternatives can be implemented without affecting experimental outcomes. Therefore, the lower-cost microtube represents a viable and practical alternative to reduce expenses in qPCR assays while maintaining high performance standards. Future studies could expand this evaluation to include additional brands, tube materials, and reaction conditions to further validate these findings and optimize cost-efficiency in molecular biology workflows.

Keywords: qPCR; microtubes; performance, molecular assay, tick, cost-efficiency.

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INTRODUCTION

Quantitative real time polymerase chain reaction (qPCR) has been used routinely in research and applied in laboratories for pathogen identification, gene transcription analysis and detection of genetic mutations [11,16,23,26,27]. Specifically, in veterinary medicine [28], qPCR has been successfully used to detect feline leukemia virus [5,6,10], *Microsporum* sp. in cats [19], canine distemper virus [29], *Leishmania* sp. [21] and *Dirofilaria* sp. in dogs [14], and hemoparasites in cattle, such as *Babesia* sp. Also, in animal-derived foods, this technique has been applied to ensure that these products are free from microorganisms that could compromise product quality and consumer health [1,15,31].

However, a limiting factor is the high cost of these molecular assays, since there is a need to use microtubes, primers, tips, reagents and thermocyclers [7,24], making this technique usually more costly than other diagnostic methods, such as serological tests [20]. As an alternative, conventional PCR tests have been used, as they are less expensive compared to qPCR assays for pathogen diagnosis. However, PCR is primarily qualitative, meaning it detects the presence or absence of a target DNA sequence, but it does not provide a precise measure of the amount [4].

To improve the price-performance ratio of a qPCR test, laboratories can adopt cost-saving strategies while maintaining quality. Adopting similar quality consumables can reduce costs without compromising reliability. Therefore, with this cost-reduction focus, this study aims to compare microtubes from different brands, evaluating their performance to identify potential savings without compromising experimental reliability.

MATERIALS AND METHODS

Experimental area, animals and sample collections

Hereford cattle housed in a tick-free area at Faculdade de Veterinária of Universidade Federal do Rio Grande do Sul (UFRGS, Porto Alegre, RS, Brazil) were infested with 20,000 10-day-old *Rhipicephalus microplus* larvae [22]. After 21 days, fully engorged females were collected. This study was conducted in compliance with ethical and methodological guidelines, following the International and National Directives and Regulations.

Tissue dissection

Ovaries (OV), salivary glands (SG) and fat bodies (FB) were dissected from 9 fully engorged *R. microplus* females. A pooled sample of each tissue was prepared by combining tissues from 3 ticks. The dissection was conducted using RNase-free materials, such as tweezers, scissors, Petri dishes and a stereoscope. All materials were decontaminated with RNase away1 prior to dissection. Three pools of 3 ticks were used for each dissected tissue: OV, SG, and FB. The collected tissues were washed with PBS containing diethyl pyrocarbonate1. Approximately, 100 mg of each tissue was macerated with a pestle, stored in 1 mL of TRIzol reagent1 and stocked at -70°C until total RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. The RNA was quantified, the purity and contamination were assessed using a spectrophotometer measuring the 260/280 absorbance ratio. The integrity of the extracted genetic material was verified by agarose gel electrophoresis. The total RNA was treated with DNase1 and cDNA synthesis was performed using SuperScript™ III reverse transcriptase2 and oligo(dT) primers3. PCR assays targeting the actin gene (Table 1) [24] were performed using 100 ng of each Cdna4 (OV, SG and FB) as templates, following the manufacturer's instructions. The PCR conditions included 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min. Negative controls, in which no template was added and positive controls (previously synthesized cDNA from ovaries of fully engorged *R. microplus* females) were included in all analyses.

Microtubes

Microtubes brand A and B were selected for the experiments to compare and evaluate their performance in qPCR assays. A price quotation was conducted for qPCR microtubes from 2 brands with different prices.

qPCR Assays

Microtubes brand A and B were used for qPCR assays targeting the *40s ribosomal protein* (*40s*) and *cyclophilin A* (*cyc*) *R. microplus* genes (Table 1) [13,23,32], with 100 ng of cDNA from OV, SG and FB of fully engorged *R. microplus* females as templates.

Briefly, 35 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s were performed using a Rotor-Gene Q thermal cycler5 (Qiagen). The reactions were conducted in duplicate, using both microtubes brand A and B simultaneously in the same assays and the average of the cycle thresholds (Ct) of these reactions was performed. Negative controls, in which no template was added, were included in all analyses.

Statistical analysis

Statistical analysis was performed using the non-parametric Mann-Whitney U test (pMWU) via GraphPad Prism 8.0. Parameters for each reaction, based on Ct values and amplification curves, such as mean and standard deviation, were compared between the 2 independent groups (microtubes brand A and B). *P*-values < 0.01 were considered statistically significant.

RESULTS

RNA and cDNA

Total RNA was extracted from OV, FB and SG samples and, showing 2 distinct bands in the 18S and 28S regions (Figure 1A & B). cDNA synthesis was verified by agarose gel electrophoresis (Figure 2) and amplified products were observed at approximately 200 base pairs (bp), according to the primer sets used (Table 1).

Microtube performance

The Ct values obtained from qPCR (Table 2), in reactions using cDNA from R. microplus tissues with the *40s ribosomal protein* and *cyc* genes, were used for comparative analysis between the evaluated microtubes (Figure 3). No significant difference ($P = 0.99$) was observed between microtubes brand A and B when assessing qPCR parameters for OV, SG and FB tissues individually, not even when comparing the *cyc* and *40s ribosomal protein* results (Figure 3A, B & C). Similarly, no significant difference was identified when parameters such as mean and standard deviation were collectively analyzed for the tissues (Figure 3D). Thus, the performance of microtubes brand A and B in qPCR assays targeting the *40s ribosomal protein* and cyc *R. microplus* genes was similar.

Analysis of Ct values (Table 2) revealed no statistically significant difference between the 2 tubes brands, whether evaluating individual cDNAs or all tissues combined, as well as when using different primer sets for Rhipicephalus microplus genes.

As for the mean and standard deviation of Ct values (Table 3), no statistically significant difference was observed, either when evaluating the cDNAs individually or together, and even when using different housekeeping genes.

Price of microtubes

On April 22, 2024 boxes containing 1,000 microtubes from 2 different brands were priced differently. A box of microtubes from brand A was priced at USD 469.40, whereas a box of microtubes from brand B cost USD 134.60 per box, representing a difference of over 70% in value. The microtubes are designed for reactions between 10 and 50 µL. Both brands share identical specifications: each consists of strips containing 4 tubes and 4 caps, with 250 strips per box. Additionally, they are DNase/RNase-free, compatible with qPCR thermal cycler instrument, made of propylene, and have thin and transparent walls.

Table 1. Sequences of the *40s ribosomal protein (40S)*, *actin* and *cyclophilin A* primers from *Rhipicephalus microplus*, along with the size of the PCR product and their efficiency.

Target gene	Accession number	Primer sequence (5' - 3')	PCR product size	References	PCR efficiency
40s ribosomal protein	EW679928	F: GACCGATGGCTACCTCCTCCG R: CGAACCTGGTTGTGCTGAGCG	99	[32]	2.15
Actin	AY255624.1	F: GTACATGGTGGTGCCGCCG R: TCAGGTCATCACCATCGGCAAC	184	[23]	2.0
Cyclophilin A	CV445080	F: ATGCTGGCCCCAACACTAAT	104	[13]	1.9

Table 2. Ct values obtained using microtubes brand A and B in a qPCR reaction targeting the *40s ribosomal protein* (*40s*) and *cyclophilin A (cyc)* genes, with cDNA templates from ovaries, salivary glands and fat bodies from *Rhipicephalus microplus*.

Brand A				Brand B			
Gene	Tissue			Gene	Tissue		
	OV[a]	SG[b]	FB[c]		OV	SG	FB
40s	20.98	19.16	17.84	*40s*	20.73	19.54	17.69
	20.87	19.3	17.83		20.7	19.76	17.76
	20.46	19.16	17.8		20.5	19	17.62
	21.42	19.02	17.81		20.83	19.12	17.6
Cyc	20.82	19.4	18.63	*Cyc*	20.48	19.53	18.44
	20.74	19.5	18.49		20.37	19.52	18.32
	20.87	19.15	18.5		20.39	19.08	18.2
	20.53	19.11	18.38		20.34	19.09	18.11

[a]Ovary; [b]salivary glands; [c]fat body.

Table 3. Mean (average ± standard deviation) of Ct values of the assays using microtubes brand A and B in a qPCR with cyclophilin A (cyc) and *40s ribosomal protein* (40s) from *Rhipicephalus microplus*, with ovaries, salivary glands, fat bodies and all tissues combined.

	Brand A					Brand B				
	Gene	Tissue				Gene	Tissue			
		OV[a]	SG[b]	FB[c]	All tissues		OV	SG	FB	All tissues
Ct value	*40s*	20.93 ± 0.4	19.16 ± 0.2	17.82 ± 0.0	19.30 ± 1.3	*40s*	20.69 ± 0.1	19.36 ± 0.3	17.67 ± 0.1	19.23 ± 1.3
	Cyc	20.74 ± 0.1	19.29 ± 0.1	18.5 ± 0.1	19.51 ± 1.0	*Cyc*	20.4 ± 0.06	19.31 ± 0.2	18.27 ± 0.1	19.32 ± 0.9

[a]Ovary; [b]salivary glands; [c]fat body.

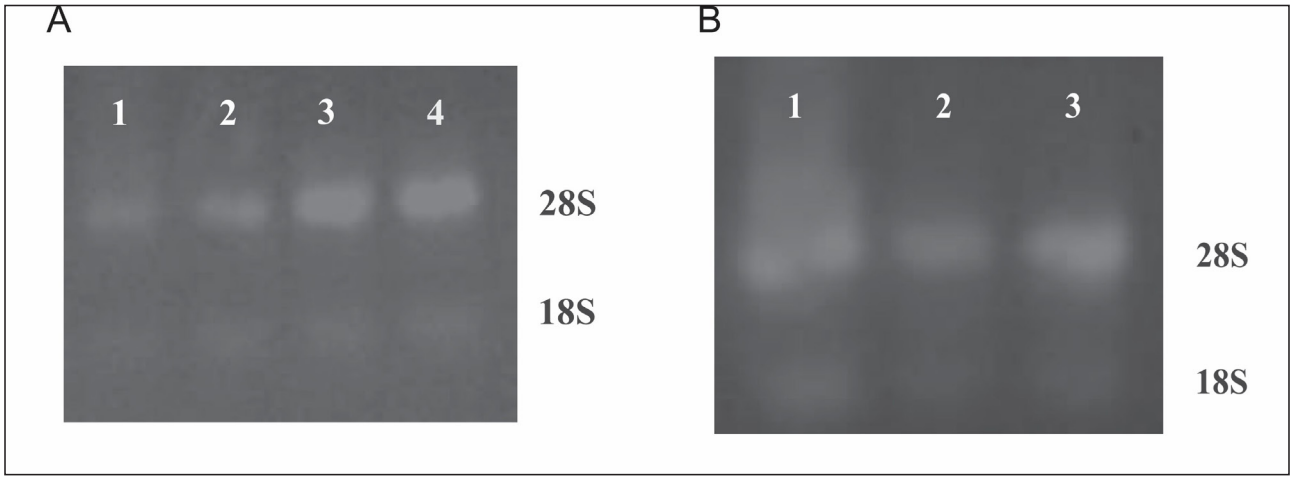

Figure 1. Agarose gel electrophoresis of *Rhipicephalus microplus* RNA. A- Lines 1 and 2: RNA of salivary glands; lines 3 and 4: RNA of ovaries from fully engorged females. B- Lines 1, 2 and 3: RNA of fat bodies from fully engorged females.

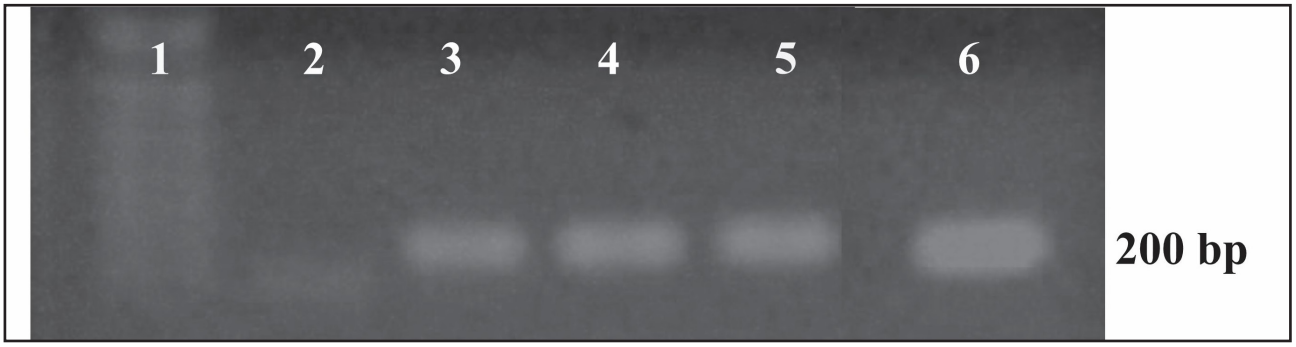

Figure 2. Agarose gel electrophoresis of *Rhipicephalus microplus* cDNA. 1) molecular weight marker; 2) negative control (no template was added); 3) positive control (previously synthesized cDNA of ovaries from fully engorged females); 4) ovaries; 5) salivary glands; 6) fat bodies from fully engorged females.

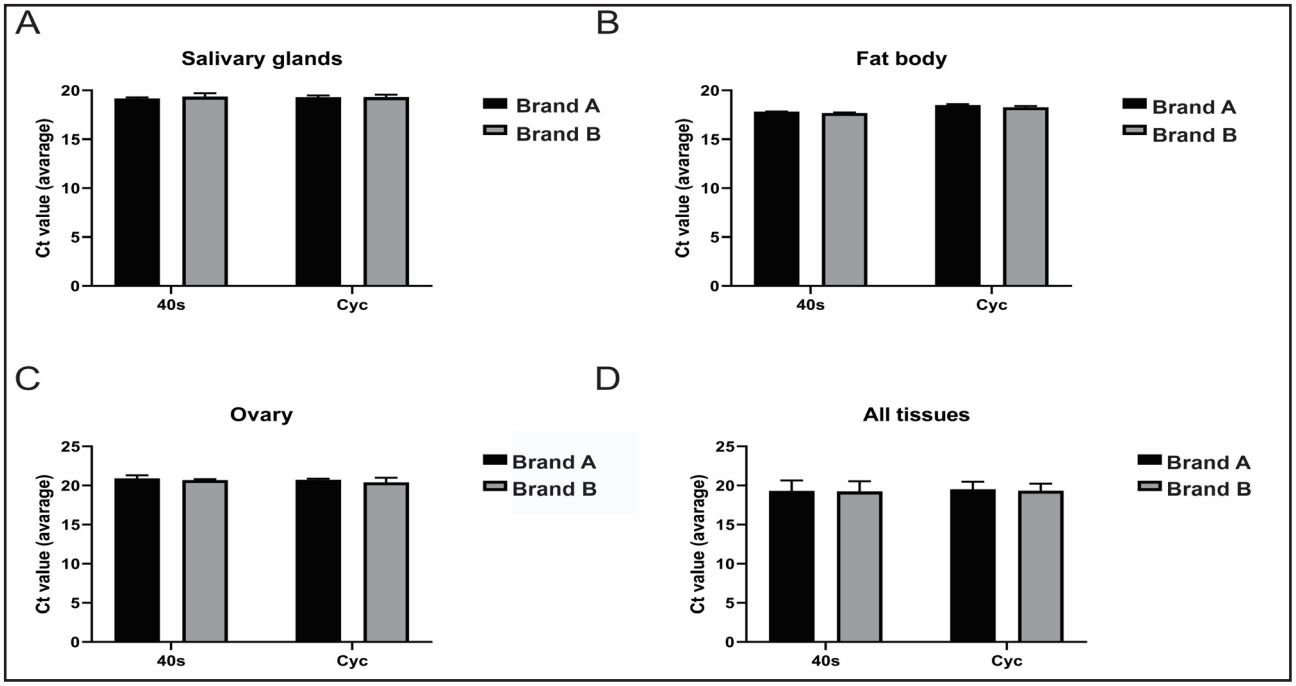

Figure 3. Ct values (average) in qPCR reactions employing *40s ribosomal protein* (*40s*) and *cyclophilin A* (*cyc*) from *Rhipicephalus microplus* using microtubes brand A and B, with cDNA from different tissues. A- Salivary glands; B- Fat body; C- Ovary; D- All tissues combined.

DISCUSSION

Different methods to reduce qPCR costs by using more economical thermal cyclers to perform the assay have been reported [25]. However, there is a lack of studies comparing different microtubes in qPCR assays to assess their performance. Thus, this study aimed to evaluate the performance of microtubes from 2 different brands, making qPCR tests more accessible. Here, cDNA from OV, SG and FB from fully engorged females of *R. microplus* were used in a qPCR assay of the constitutive genes, *cyc* and *40s ribosomal protein* and performed with 2 microtubes from different brands. Other experiments have also focused on making qPCR economically viable in clinical and laboratory routines through alternative approaches, such as saving on consumables and reducing costs [2].

The search for the development of new qPCR devices that are more practical, portable, economical and

that deviate from traditional thermocyclers is ongoing in research routines [25], such as a qPCR device based on a 3D-printed polypropylene chip, incorporating a water-based cooling system. This system demonstrated faster and more efficient cooling during the annealing phase of qPCR cycles compared to traditional thermocyclers that use fans or natural cooling, achieving up to 13 times faster cooling and reducing the total assay time from 90 to 70 minutes. However, this device exhibited higher Ct values, indicating lower amplification efficiency despite reducing the overall cost of the technique [25]. Similarly, a portable qPCR thermocycler was recently developed using a 3D-printed chassis and commercially available electronic components, significantly reducing manufacturing costs. This device was estimated to cost approximately USD 193.00, whereas conventional qPCR thermocyclers are priced around USD 24,000.00. However, like the previously mentioned model, the portable thermocycler also exhibited higher Ct values, suggesting lower amplification efficiency compared to traditional devices [17]. Another study used a multi-chamber chip made of polydimethylsiloxane, comparing its performance with conventional qPCR devices [12]. This chip was designed to accommodate small volumes of reagents and samples in each chamber, thereby reducing reagent consumption during reactions. The same study also evaluated amplification curves, reporting a slight variation in Ct values, with higher Ct values compared to conventional qPCR instruments. However, they were still effective and contributed to a significant reduction in the overall cost of the technique. Here, the assays using microtubes from two brands showed no significant difference ($P = 0.99$) based on the mean and standard deviation of Ct values when analyzed separately or in combination with cDNA from all *R. microplus* samples, even when different reference genes were used. Thus, the performance between microtubes brand A and brand B was not different indicating that both microtubes are effective and exhibit similar amplification pattern.

Moreover, a method for decontaminating residual DNA in disposable pipette tips was proposed using the Opentrons OT-2 liquid handling robot. The goal was to enable the reuse of these materials without compromising subsequent analyses, thereby reducing laboratory routine expenses. Thus, it was shown that decontaminated pipette tips were as effective as new ones, without interfering with subsequent reactions, resulting in a significant reduction in costs associated with these consumables [3]. Reduction in the use of reagents in the preparation of the amplification solutions by half, in relation to the standard assay was also evaluated [2]. This test proved to be effective, as it was as specific and sensitive as the standard method. In addition, it is also economical, when compared to the latter, as it reduces the cost of reagents, making it possible to analyze twice as many samples when using this test that uses fewer consumables. Thus, other studies have achieved long-term cost reductions in the technique, either by reusing pipette tips or modifying other consumables to achieve performance comparable to TaqMan detection at a lower cost.

CONCLUSION

In this work it was shown that the use of microtubes from different brands presented similar performance, allowing a reduction of more than 70% in the costs associated with specific qPCR microtubes. Further studies could validate these findings by testing samples from different organisms and other consumables.

MANUFACTURERS

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Conceived and designed the experiments: LSA, JW and ISV;

Performed the experiments: LSA and JW;

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Drafting the article: LSA, JW and ISV;

Critical revision of the article: LSA, JW and ISV.

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***Ethical approval*.** This study was conducted in compliance with ethical and methodological guidelines, following the International and National Directives and Regulations, as approved by the Animal Experimentation Ethics Committee of the UFRGS, project number 27559.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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