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Original Article

Validation of RT-qPCR test for SARS-CoV-2 in saliva specimens

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ABSTRACT

Background: Saliva samples may be an easier, faster, safer, and cost-saving alternative to NPS samples, and can be self-collected by the patient. Whether SARS-CoV-2 RT-qPCR in saliva is more accurate than in nasopharyngeal swaps (NPS) is uncertain. We evaluated the accuracy of the RT-qPCR in both types of samples, assuming both approaches were imperfect.
Methods: We assessed the limit of detection (LoD) of RT-qPCR in each type of sample. We collected paired NPS and saliva samples and tested them using the Berlin Protocol to detect SARS-CoV-2 envelope protein (E). We used a Bayesian latent class analysis (BLCA) to estimate the sensitivity and specificity of each test, while accounting for their conditional dependence.
Results: The LoD were 10 copies/mL in saliva and 100 copies/mL in NPS. Paired samples of saliva and NPS were collected in 412 participants. Out of 68 infected cases, 14 were positive only in saliva. RT-qPCR sensitivity ranged from 82.7 % (95 % CrI: 54.8, 94.8) in NPS to 84.5 % (50.9, 96.5) in saliva. Corresponding specificities were 99.1 % (95 % CrI: 95.3, 99.8) and 98.4 %(95 % CrI: 92.8, 99.7).
Conclusions: SARS-CoV-2 RT-qPCR test in saliva specimens has a similar or better accuracy than RT-qPCR test in NPS. Saliva specimens may be ideal for surveillance in general population, particularly in children, and in healthcare or other personnel in need of serial testing.

Introduction

As of 22 April 2022, there had been 505.8 million confirmed cases and 6.2 million deaths from COVID-19 in the world [1]. The development of natural or vaccine immunity, advances in treatment, and the emergence and predominance of more transmissible but less pathogenic variants of SARS-CoV-2 have led to a strategy of pandemic control in most countries. Although many countries have significantly reduced or suspended COVID-19, testing and surveillance are still central to a life with SARS-CoV-2 in the foreseeable future, since a large fraction of the world population remains susceptible to SARS-CoV-2 infection [2].

Real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) in nasopharyngeal swab (NPS) or aspirate (NPA) is the gold standard to diagnose SARS-CoV-2 infection [3]. RT-qPCR testing is still needed during the pandemic control phase, to confirm infection in low prevalence settings, and for differential diagnosis in patients with respiratory symptoms. Unfortunately, false-negative tests could occur when viral load is below the limit of detection of the test, due to the natural course of the infection or inadequate collection of NPS [4]. In addition, collection of NPS is a relatively invasive and uncomfortable procedure, requires technical expertize and personal protective equipment, and could increase the risk of infection in healthcare personnel [4–6]. Thus, developing and using inexpensive, simple, acceptable, and reliable methods for the collection of

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specimens is of the essence, particularly in countries with limited resources.

Saliva samples may be an easier, faster, safer, and cost-saving alternative to NPS samples, and can be self-collected by the patient with slight guidance and supervision from healthcare personnel [7,8]. Despite its potential advantages, saliva testing is not used in many countries. This may be partly a consequence of uncertainty on the accuracy of RT-qPCR in saliva. Indeed, some paired-sample studies show that NPS outperforms saliva [9,10], while others suggest that saliva is equivalent or better than NPS [11–14]. This raises concerns about using RT-qPCR in NPS samples as a reference standard to evaluate the accuracy of tests in saliva [15,16]. In this study, we evaluated the sensitivity and specificity of RT-qPCR in both NPS and saliva samples using Bayesian latent class analysis (BLCA), which does not require the true infection status of each individual to be known [17,18]. In addition, we present findings from a surveillance program based on RT-qPCR testing in saliva, conducted in Bucaramanga, Colombia.

Materials and methods

Study design and sample

We quantified the limit of detection (LoD) of RT-qPCR to detect SARS-CoV-2 in saliva and NPS samples, and estimated the diagnostic sensitivity and specificity of the test in each type of sample. Individuals tested for the first time at the Central Research Laboratory (CRL), Universidad Industrial de Santander (UIS), Bucaramanga, Colombia, for the purpose of clinical care or epidemiologic surveillance were invited to participate and provide paired NPS and saliva samples. Written informed consent was obtained from all participants ≥ 18 years, parental consent and the child assent were obtained from those 7–17 years old, and only parental consent from those < 7 years old. The study was approved by the Research Ethics Institutional Review Board (IRB) of the Universidad Industrial de Santander (UIS).

Limit of detection

To quantify the LoD of RT-qPCR in saliva and NPS samples, we spiked quantified SARS-CoV-2 viral RNA, rather than infectious virus, into samples of saliva and NPS from non-infected individuals, prior to RNA extraction. We used a serial dilution of a positive control for SARS-CoV-2 gene E (2019-nCoV_E_positive control, IDT) in concentrations of 2.500 copies/mL, 500 copies/mL, 250 copies/mL, 100 copies/mL, 50 copies/mL, 25 copies/mL, and 10 copies/mL. These standard dilutions were prepared from a positive control stock standard with 2×10^8 copies/μL.

RNA extraction and RT-qPCR were conducted as described below. The LoD was determined by analyzing 20 replicates of each standard sample dilution. The lowest concentration (copies/mL) at which 95 % (19/20) of the 20 replicates tested positive was taken as the LoD [11,19]. The correlation between the copies of the SARS-CoV-2 RNA and the Ct value was also estimated.

Diagnostic sensitivity and specificity of RT-qPCR in saliva and NPS

We collected paired samples of NPS and saliva from all participants, from May 5th to June 4th, 2021. At that time, the Pango lineages B.1.621 and B.1.621.1, also called Mu, and designated by the World Health Organization as a variant of interest, was dominant in Colombia [20]. Paired samples were collected and transported, by trained health care providers, following national guidelines [21]. Participants restrained themselves from smoking, drinking, eating or brushing their teeth for 30 min before the collection of saliva samples.[22] Two mL of saliva were collected in sterile ≤ 30 mL urine collection containers with 2 mL of viral transport media based on VTM composed of fetal bovine serum (FBS), Hanks' Balanced Salt Solution (HBSS), antibiotics and antifungals, as well as phenol red [23]. NSP and saliva samples were refrigerated at 2–8 °C until testing. All paired samples were tested in parallel.

RNA was extracted using MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit (ThermoFisher Scientific), following standard manufacturing procedures, with some modifications. The plate shaker was set at 1650 r.p.m. in all steps, except the elution step, and the incubation in the lysis step lasted eight minutes at 65 °C. Two hundred μL of saliva or NPS samples were used for RNA extraction on a Microlab STARlet automation robot. Extracted RNA was stored at 4 °C if the RT-qPCR was done shortly after extraction. Otherwise, it was stored at minus 80 °C.

The RT-qPCR analysis was done following the Berlin Protocol [24], with primers and probes to detect SARS-CoV-2 envelope protein (E) and nucleocapsid (N) genes. The N gene was evaluated in the saliva sample when the E gene was positive or in both samples when the E gene results were discordant between saliva and NPS samples. We tested for the N gene in all saliva-positive samples and in both saliva and NPS samples if results were discordant. The RNAsa P gene was used as an internal control in all tests. Reverse transcription and quantitative PCR were performed in a step using the Luna® Universal One-Step RT-qPCR Kit (NEB). A 25-μL reaction was set up containing 5 μL of RNA, 12.5 μL of 2 × reaction buffer, 1 μL of enzyme, and 0.4 μL of a 50 mM magnesium sulfate solution. Thermal cycling was performed at 55 °C for 10 min for reverse transcription, followed by 95 °C for three minutes and then 45 cycles of 95 °C for 15 seconds, and 58 °C for 30 seconds in the StepOnePlus Real-Time PCR Systems (Applied Biosystems). We used 2021-nCoV_E_Positive Control (IDT, #10007725) as DNA-based positive control for the DNA amplification phase of the reaction. Extraction controls were included in each test. Tests were considered positive when the Ct value of the target was ≤ 40 cycles. A Ct value < 34 cycles was also used as cut points to define a positive test, because existing evidence suggest that individuals with high Ct-values may be less infective than those with lower values [25,26]. Test results were analyzed using StepOnePlus™ Software v2.3 (ThermoFisher).

Statistical analysis

We used means and proportions to describe relevant characteristics of the sample of participants. We calculated the sensitivity and the specificity of RT-qPCR in saliva, using the test in NPS as the standard. Bayesian 95 % credible intervals (95 % CrI) for sensitivity and specificity were calculated using uninformative Jeffreys' priors [27]. However, estimates of sensitivity and specificity may be incorrect if an inaccurate reference test were used as the gold standard test [28]. Therefore, we treated true disease status as a latent variable, and used this latent variable as the reference test [15,16]. We used a Bayesian Monte Carlo Markov chain approach to draw inferences about the accuracy of each test, while accounting for conditional dependence between the two tests [17,18]. Conditional dependence means that information on whether one test was positive conveys information on the likelihood of the other test being also positive. This was likely in our case, as we are using the same test, RT-qPCR, in two different types of samples (saliva and NPS). We calculated pooled sensitivity and specificity of both tests and their 95 % CrI across study groups, using a Bayesian latent class meta-analysis (BLCA) [29]. We pooled the estimates across groups with different expected prevalence of infection: participants with and without symptoms at the time of the test, and participants who were at high risk because they were contacts of confirmed cases or health care workers. The BLCA accounts for correlation between sensitivity and specificity of RT-qPCR in NPS and saliva samples. Analyzes were conducted using Stata® 16.1 software. The programs for the BLCA

were accessed through an app on R Shiny, version 1.5.0 available at https://bayesdta.shinyapps.io/meta-analysis/ [30]. Based on results from a previous meta-analysis, we selected a prior distribution for sensitivity and specificity corresponding to 95 % CrI of 74.7–91.4 % and 98.2–99.8 % for the test in saliva, and 76.8–92.4 % and 97.4–99.8 % for the test in NPS, respectively [30]. We assumed a true prevalence of infection of 15 %, which corresponded to average the proportion of positive tests reported in the city during the study period, and conditional dependence in positive and negative individuals. Three Gibbs sampling chains were used, with 1000 iterations each. Pooled estimates were obtained after 1 million iterations from each chain. Convergence was assessed by visual inspection of each Markov chain and Brooks-Gelman-Rubin statistics (R-hat < 1.1) [31].

SARS-CoV-2 infection surveillance using the validated RT-qPCR test in saliva samples

After completing the evaluation of the diagnostic performance of the SARS-CoV-2 RT-qPCR test in saliva, the approval for use was requested to and granted by the Colombian National Institute of Health. The test in saliva was then used for epidemiological surveillance in Bucaramanga, from September 6th to December 18th, 2021. Tests were provided at no cost at public testing sites across the city. Collection and testing of saliva samples were conducted under the same conditions used in the validation study. Following national policies, mandatory surveillance data were furnished to the Colombian Surveillance System, and test results were given to each person.

Results

Analytical sensitivity and limit of detection

The LoD of RT-qPCR was 10 copies/mL in saliva and 100 copies/mL in NPS. At those concentrations, RNA amplification was successful in 20 replications of tests in saliva (100 %) and 19 replications of tests in NPS (95 %). Negative, positive (E and N genes), and internal (RNAsa P) controls had Ct values within the expected range for the test.

Diagnostic performance of SARS-CoV-2 RT-qPCR in saliva specimens

Paired samples of saliva and NPS were collected in 412 participants. Their mean age was 38.3 years (95 % confidence interval – CI: 36.6–39.9 years), a fifth (n = 83) were health care workers, and 58 % (n = 239) were women (Table 1). In addition, 169 (41 %) were contacts of suspected or confirmed COVID-19 cases, and 110 (27 %) were symptomatic. In the subset of 99 symptomatic participants with available data on the onset of symptoms, 93 % were tested within a week of symptoms onset, and the average time to test was 3.9 days (95 % CI: 3.4, 4.3). In average, participants who were infected (n = 68) were tested 3.5 days after the start of symptoms (95 % CI: 2.9, 4.1).

Table 1
Demographic and clinical characteristics of the participants (n = 412).

Characteristic	Number (%)	
Age		
< 18 years old	29	(7.0)
18–60 years old	334	(81.1)
> 60 years old	49	(11.9)
Male gender	173	(42.0)
Health care worker	83	(21.0)
Contact with suspected/confirmed case	169	(41.7)
Symptomatic*	42	(62.7)
Days with symptoms (n = 63)[a]		
0–3	13	(20.6)
4–7	16	(25.4)
≥ 8	34	(54.0)

* Out of all positive for the E gen in saliva or nasopharyngeal swap (one with missing data).
[a] Up to the day when the person was tested (5 with missing data).

Out of 68 infected individuals, 14 were positive only in saliva (E and N genes). In these 14 individuals, Ct values in saliva ranged from 28.4 to 36.0 cycles for the E gene and from 29.6 to 36.8 cycles for the N gene. On the other hand, six samples that were E-positive in NSP samples (Ct values from 31.5 to 35.5 cycles) were E- and N-negative in saliva.

When the E gene in NPS was considered as the gold standard, using a Ct value < 40 as diagnostic cut-point, the sensitivity of saliva testing was 88.9 % (95 % CrI: 78.5, 95.2; Table 2), and the specificity was 96.1 % (95 % CrI: 93.7, 97.7). The sensitivity and specificity of saliva testing did not changed substantially when a Ct value < 34 was used as diagnostic cut-point (Table 2). Nevertheless, participants who were symptomatic had significantly lower Ct values for the E gene than those without symptoms, but the difference was significantly larger in NPS (− 11.1; 95 % CI: − 15.6, − 6.5) than in saliva (− 7.6; 95 % CI: − 9.9, − 5.3).

BLCA results when averaging parameters in symptomatic and asymptomatic participants, and in individuals with and without high risk, assuming conditional test dependence, indicated a sensitivity ranging from 82.7 % (95 % CrI: 54.8, 94.8) in NPS to 84.5 % (50.9, 96.5) in saliva (Table 3). Corresponding specificities were 99.1 (95 % CrI: 95.3, 99.8) and 98.4 (95 % CrI: 92.8, 99.7). In both analyzes sensitivity was slightly higher and specificity slightly lower in test in saliva.

Use of RT-qPCR for SARS-CoV-2 in saliva for surveillance

Between September and December 2021, 4932 individuals attended free access diagnostic points in Bucaramanga, in Colombia. Out of them, 57.2 % were female, 5.7 % were 2–10, 16.8 % were 11–19, 68.1 % were 20–59, and 9.4 % were 60–94 years old. In addition, 24.5 % were symptomatic. The overall positivity of the RT-qPCR in saliva samples was 2.04 % (95 % CrI: 1.68, 2.47). However, the positivity was 1.02 % (95 % CrI: 0.73, 1.38) and 5.2 % (95 % CrI: 4.06, 6.57) in asymptomatic and symptomatic individuals, respectively. The prevalence of infection changed with time, from 1.11 % (4/361) in September, to 1.08 % (7/648) in October, to 2.74 % (37/1352) in November and 2.02 % (52/2571) in December. Correspondingly, the proportion of infections in symptomatic patients increased up to 9.14 % in December but fluctuated from 0.24 % to 2.02 % in asymptomatic individuals (Table 4).

Discussion

In this study, we standardized and validated a RT-qPCR test for the SARS-CoV-2 E gene in saliva. The LoD of our tests, 10 copies/mL in saliva and 100 copies/mL in NPS, corresponded to that from tests with a high analytical sensitivity (around 100 copies/mL) [19] and were similar or lower than that observed in other studies [7,11,32,33]. The presence of SARS-CoV-2 in saliva has been ascertained in several studies [6,34], and is likely due to the binding of the virus to ACE-2 receptors in epithelial cells lining salivary gland ducts [35], and to contamination by nasopharyngeal secretions [36]. It is noteworthy that the LoD for the test in saliva was one order of magnitude below that in NPS, which would corresponds to a 13 % absolute increase in test sensitivity [19]. On the other hand, the LoD in saliva samples, was considerably lower than the LoD in untreated saliva samples (225 copies/mL) and in saliva samples treated with guanidinium (100 copies/mL), reported by Callahan et al. [11]. This could be attributed a better stability of the samples and a higher yield of RNA, due to the use of a different viral transport medium in our study. In addition, the higher positivity in saliva samples could

Table 2
Sensitivity and specificity of RT-qPCR for E gene testing in saliva samples (n = 412), compared to nasopharyngeal (NPS) samples as standard, by cycle threshold cut-point value (Ct-cut) and presence of symptoms.

Ct-cut	Standard	Symptoms	TP/FN/FP/TN*	Sensitivity (%)	Specificity (%)
40	E/NPS[a]	All	48/6/14/344	88.9 (78.5, 95.2)[b]	96.1 (93.7, 97.7)
		Yes	39/0/3/68	100.0 (93.8, 100.0)	95.8 (89.2, 98.8)
		No	8/6/11/267	57.1 (31.9, 79.7)	96.0 (93.3, 97.9)
34	E/NPS	All	44/5/12/351	89.8 (79.1, 96.0)	96.7 (94.5, 98.2)
		Yes	37/1/3/69	97.4 (88.4, 99.7)	95.8 (89.3, 98.8)
		No	6/4/9/273	60.0 (30.4, 84.7)	96.8 (94.3, 98.4)

* True positive/false negative/false positive/true negative.
[a] Gen E, in nasopharyngeal swap.
[b] 95 % credibility intervals.

be explained because we did RNA extraction from saliva specimens. In a meta-analysis, Lee et al. showed a significant rise of 29 % in the positivity of saliva specimens when the RNA extraction was done (without extraction 60 %, CI95 % 49–70 % vs with extraction 89 %, IC95 % 83–92 %) [37].

When the test in NPS was taken as the gold standard, the sensitivity of the saliva test was close to 90%, regardless of the Ct cut-point used to define a positive result. However, sensitivity was considerably higher in symptomatic than in asymptomatic participants (about 90 % vs 60 %). Specificity was above 95 % in all scenarios. Our overall sensitivity and specificity estimates, when NPS was taken as the reference test, were consistent with those from meta-analyses including thousands of participants [30,37]. On the other hand, when neither the test in saliva nor the test in NPS was taken as the standard, the sensitivity of test in saliva was at least 83% and reached up to 91%, depending on the model for test dependency. Moreover, the test in saliva detected 14 cases of infection that were not detected in NPS specimens, and the test in NPS detected only six cases that were not detected in saliva tests. The net difference of eight additional cases of infection explains the slightly higher sensitivity of tests in saliva observed in our study. This is consistent with previous studies showing that RT-qPCR tests in the saliva may be similar or better than in NPS samples [11,14,38,39].

A similar or better performance of saliva could be explained by the use of a test with high analytical sensitivity [11]. In addition, testing saliva with a high-analytical-sensitivity assay results in a higher sensitivity than testing NSP during the early phase of SARS-CoV-2 infection [14]. This is important, because detectable viral loads precede symptoms for several days in most cases [14,40]. Moreover, viral load in saliva seems to peak days before than in nasal swaps [13], and levels of SARS-CoV-2 RNA in saliva appear to be less variable than those in NPS [12]. All this suggest that selection of an optimal sampling site depends on the stage of the infection and that saliva could be useful for surveillance of SARS-CoV-2 infection in the general population, including those with high vaccination coverage. Also, saliva could be useful during periods and places where variants that result in asymptomatic or mild disease are predominant, such as Omicron [41], because viral RNA in saliva peaks sooner than in NPS. In consequence, due to its lower cost, easiness of use, acceptability, and potential for early detection of infections, RT-qPCR tests in the saliva could play an important role in controlling transmission in general populations, and particularly in children, and in healthcare or other personnel in need of serial testing.

Table 4
Prevalence of SARS-CoV-2 infection by month and symptomatic status. Bucaramanga, Colombia, 2021. (n = 4932).*

Month	Cases	Patients tested	Prevalence/1000 (95 % confidence interval**)
Symptomatic			
September	1	76	13.2 (1.4, 59.8)
October	6	223	26.9 (11.3, 54.6)
November	22	538	40.9 (26.5, 60.2)
December	34	372	91.4 (65.3, 123.9)
Total	63	1209	52.1 (40.6, 65.7)
Asymptomatic			
September	2	283	7.1 (1.5, 22.5)
October	1	420	2.4 (0.3, 11.1)
November	16	794	20.2 (12.0, 31.7)
December	18	2164	8.3 (5.1, 12.8)
Total	37	3661	10.1 (7.2, 13.7)

* 62 participants did not have data on presence of symptoms, and one of them was positive.
** Confidence intervals calculated using Jeffreys' correction.

Our short lasting surveillance program in Bucaramanga added to the evidence in support of a successful use of saliva sampling in mass population screening, in different countries and settings [42–44]. We avoided test errors due to contamination with blood, food, drinks, or smoke by collecting saliva samples at least 30 min after eating, drinking, brushing teeth, or smoking. This had little impact on the use of saliva samples, because the waiting period was already completed after commuting to the lab and completing the mandatory COVID-19 national surveillance form. Saliva testing could be particularly useful in school children, one of the targets in our surveillance program, because collection of saliva samples is better accepted by children. In addition, SARS-CoV-2 infections could be more often detected in saliva than NPS, probably due to difficulties in obtaining high quality NPS samples in children [42].

Table 3
Sensitivity and specificity of RT-qPCR for E gene testing in saliva and NPS samples (n = 412) from Bayesian latent class analysis, assuming both tests are inaccurate, by presence of symptoms and by expected risk of infection.

Group	Sample	Priors Sens/Spec	Median (95 % CrI) Sensitivity (%)	Specificity (%)
Symptoms				
	Saliva	75.0–92.0/98.0–99.8	84.5 (50.9, 96.5)	98.4 (92.8, 99.7)
	NPS	77.0–93.0/98.0–99.8	82.9 (51.4, 95.6)	99.0 (95.2, 99.8)
Risk[a]				
	Saliva	75.0–92.0/98.0–99.8	85.2 (57.0, 95.7)	98.7 (94.0, 99.8)
	NPS	77.0–93.0/98.0–99.8	82.7 (54.8, 94.8)	99.1 (95.3, 99.8)

*95 % Credible interval.
[a] Health care personnel and contacts with suspect of known positive case were considered at high risk of being infected.

Contrary to the majority of previous studies, that included already confirmed cases with unknown duration of infection, we conducted our study in participants without a previous diagnosis. This corresponds better to a real life setting, where testing is conducted in patients demanding attention or in contacts of known or suspected cases. In addition, we conducted a BLCA, instead of assuming that RT-qPCR in NPS was the reference test, which results in biased estimates of sensitivity and specificity [28], unless NPS were 100% accurate. BLCA is more appropriate when a reference test is inaccurate, and allowed us to obtain valid estimates of the sensitivity and specificity of RT-qPCR in both types of samples.

One limitation of our study was the small number of positive cases, which resulted in wide confidence intervals for the estimates of sensitivity. In spite of this, our findings provide support for saliva been similar or better test sample than NPS. We assumed equal accuracy in symptomatic and asymptomatic, and in high and at low-risk individuals. Although supported by some studies [3,45,46], this assumption should be evaluated in future studies. Our samples for diagnostic performance of saliva testing were obtained before the introduction of Omicron variants in Colombia and when vaccination coverage were still under 50% in adult population [47]. Therefore, we did not assess the performance of saliva testing in presence of more transmissible but milder variants.[48] However, saliva testing performance seems similar in the presence of Delta and Omicron SARS-CoV-2 variants. In spite of the potential usefulness of saliva tests, our findings cannot be directly extrapolated to other populations. In addition, the performance of saliva tests should be estimated in each lab, to account for differences in methods, experience, and viral transmission rates in the population.

Conclusion

Our results indicate that SARS-CoV-2 RT-qPCR test in saliva specimens has a similar or better accuracy than RT-qPCR test in NPS. Moreover, the SARS-CoV-2 RT-qPCR in saliva specimens are ideal for diagnosis and surveillance in general population, since it is easier, cheaper, and more acceptable to the population, especially children and high-risk populations in need of serial testing such as healthcare personnel. We suggest future studies to replicate our findings in participants with different prevalence of infection, to assess consistency of findings under different settings and scenarios and obtain evidence to support the use of saliva sampling as preferred surveillance method.

CRediT authorship contribution statement

Luis Miguel Sosa Ávila, Laura Andrea Rodríguez Villamizar, Myriam Oróstegui Arenas, Ruth Aralí Martínez-Vega, Lina María Vera Cala, and Leonelo E. Bautista formulated the hypothesis and designed the research. Martha Lucía Díaz Galvis, Mayra Alejandra Jaimes Campos, and Anyela Lozano-Parra performed the laboratory tests. Leonelo E. Bautista performed the statistical analysis. Luis Miguel Sosa Ávila drafted the manuscript. All authors revised manuscript critically for important intellectual content and approved the manuscript.

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Declarations of Interest

None.

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