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# The effect of q-RT-PCR analysis method on saline gargle samples in SARS-CoV-2 clinical diagnostic methods

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**ABSTRACT**

COVID-19 is a devastating disease, and its control is difficult due to its high transmissibility rate and a long incubation average period (6.4 days). Additionally, more than half of the infected patients were asymptomatic young people or children. The asymptomatic virus transmission is the actual challenge to controlling the disease. Because of limited treatment options, diagnosis techniques have been the first focus all over the world, involving q-RT-PCR as a gold standard, serological tests, point of care studies, or RT-LAMP. Generally, nasopharyngeal, and oropharyngeal samples are preferred clinically as sources. However, alternative sources are being researched, particularly for healthcare professionals who have difficulty taking samples, patients who are afraid of giving samples, and pediatric patients. Herein, physiological saline has been utilized to offer an alternative source besides the swab samples for use in q-RT-PCR. In this study, 212 randomly chosen patients' samples were studied, and we evaluated the concordance and accurate q-RT-PCR results in two different sources, obtained from swab and gargle samples of patients. Herein, physiological saline is utilized, which is widely used medically as a recommended irrigating and wound dressing solution. We obtained in our experiments with this method, the confidence interval determines 74.50% positivity when compared to the routine q-RT-PCR procedure as summarized. In addition, when only the gargle sampling method is studied in low-income countries, the cost of testing for COVID-19 will decrease significantly. Because this method does not require vNAT or VTM transport solution sterile swab sticks as shown. The plastic container with a lid in which the patient can gargle with SF and spit it out is an ideal method for this. Additionally, it provides a great cost-benefit in low-income countries.

**Keywords:** SARS-CoV-2, q-RT-PCR, saline, physiologic serum, gargle

## INTRODUCTION

Up to now, the COVID-19 pandemic has affected more than 588,757,628 individuals and caused more than 6,433,794 deaths worldwide [1,2]. The primary routes of disease transmission are direct or indirect contacts, respiratory droplets, and contaminated objects. Incubation time has been revised to 6.4 days in recent studies [3]. SARS-CoV-2 is enveloped with a positive-sense, single-stranded RNA genome and measures on average 30 kilobases. It belongs to the genus beta coronavirus and its virion contains four major structural proteins: the nucleocapsid (N) protein, transmembrane (M) protein, envelope (E) protein, spike (S) protein, additional membrane glycoprotein (HE), and 16 non-structural proteins (nsp1-16) [4]. The N protein plays a functional role in binding to the coronavirus RNA genome, creating the nucleocapsid, and replication of viral RNA and the host's cellular response to viral infection. On the other hand, to understand the transmission of the virus globally and also in mutation regions, the most important part is the S protein of SARS-CoV-2 [5]. The S glycoprotein is a type I membrane glycoprotein and consists of various functional domains near the amino (S1) and carboxy (S2) termini [6]. The S1 subunit is involved in receptor binding functions (RBD), whereas the S2 subunit is involved in viral and cellular membrane fusion [7]. Moreover, two of them are responsible for binding the host receptor as the host-cell interaction, human Angiotensin-converting enzyme 2 (ACE2) [8] as shown in **Figure 1**.

The high risks have been determined in the patient's mouth, nasopharynx, saliva, as well as exposure to even blood. Direct or indirect contact with droplet splatter and aerosols increases the contamination risks. The disease symptoms are reported as throat sores, fatigue, shortness of breath, fever,

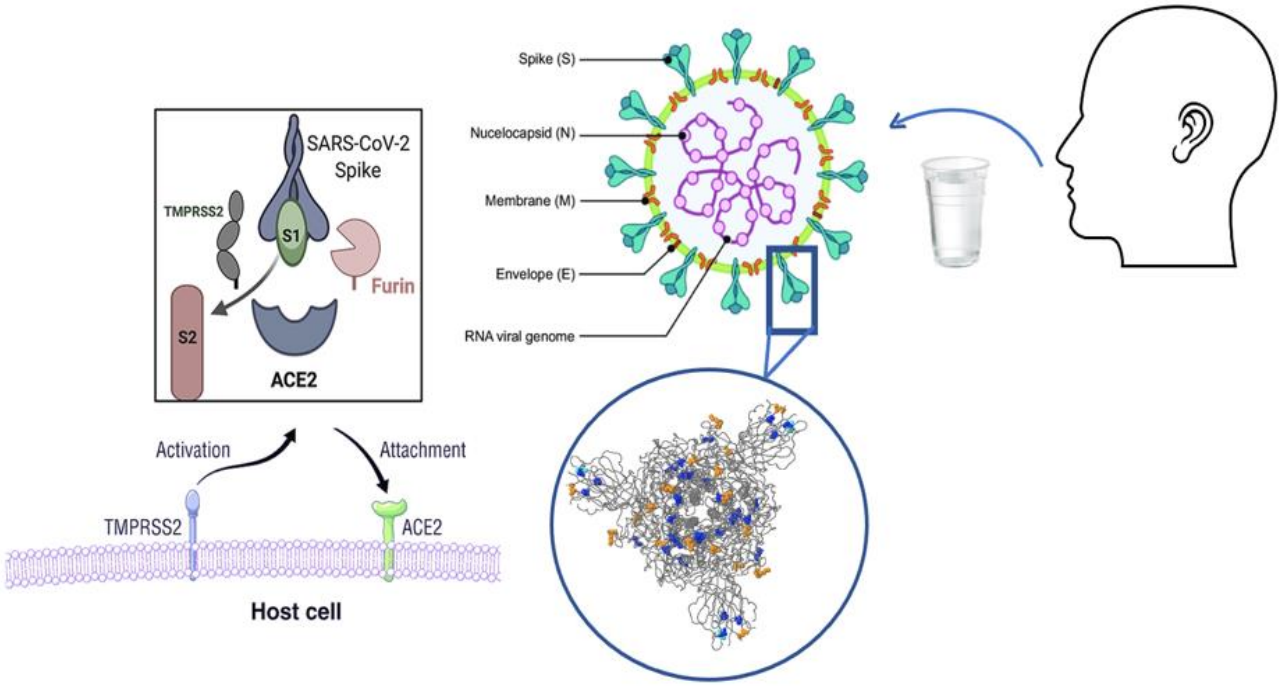



**Figure 1.** General structure of SARS-CoV-2 and host binding ability and gargles utilization

and cough [9]. To date, there is no effective and specific antiviral treatment for SARS-CoV-2. Generally, hydroxychloroquine, lopinavir-ritonavir combination, and remdesivir have been offered. However, clinical indications showed that these drugs are not completely useful for COVID-19 treatment [10].

Thus, reliable and accurate SARS-CoV-2 diagnosis is the cornerstone of public health interventions. Many diagnosis techniques are described, such as point of care (POC) devices, q-RT-PCR as a gold standard method, serological tests, and RT-LAMP [11]. The saliva samples of COVID-19 positive patients contain live viruses that lead to viral transmission from person-to-person. Additionally, the source for the tests is supplied from combined oropharyngeal and nasopharyngeal swabs (ONPS) [12]. However, the actual limitations of such sources are that they require specialist personnel and equipment for ONPS collection. Thus, alternative sample collection methods are thought of by researchers. Clinically, swabs of the upper respiratory tract and the self-collected saliva of patients are the actual sources of disease diagnosis. ACE2 is highly expressed by the oral mucosal epithelial cells [13]. The viral load of SARS-CoV-2 is related to the severity of COVID-19. Thus, gargle could be a promising aspect of an implemented strategy against SARS-CoV-2 infection.

Gargle samples have been reported with reliable results as a convenient, anautomous, and non-invasive sample collection method for the detection of SARS-CoV-2 [14,15]. In literature, some commercial gargles are described such as Oradex, Colgate Plax/Fruity Fresh, Thymol Mouthwash by Xepa, and Bactidol [16]. However, considering the low cost, we describe the physiological serum as effective as a source for the diagnosis with q-RT-PCR. Herein, the main objective of this study was to evaluate the concordance and accurate results in samples obtained from randomly chosen individuals for SARS-CoV-2 detection with the two-target; swab and gargle resources for q-RT-PCR.

## MATERIAL AND METHOD

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. This study protocol was reviewed and approved by 28.09.2021 at date of Ethics committee meeting numbered 09 of Uskudar University - Approval date: 30.09.2021 Subject No: 61351342/EYLÜL 2021-26 and Republic of Turkey, Ministry of Health, Covid-19 Scientific Research Studies Approval No: YakupArtik-2021-09-21T21_27_31.

### Sample Collection, Transportation, and Storage

For the diagnosis of COVID-19, nasopharyngeal swabs were collected by trained personnel and transferred to Kanuni Sultan Suleyman Training and Research Hospital, Istanbul in Viral Nucleic Acid Buffer (vNAT). 212 randomly selected samples were tested with the Bio-Speedy SARS-CoV-2 Emerging Plus-Bioeksen Kit (Bioeksen R&D Technologies, Istanbul, Turkey) and analyzed on the Biorad CFX96 (Bio-Rad Laboratories, Inc. USA) platform. All results (quantification cycle [CT] and Relative Fluorescence Units [RFU] values) were evaluated. Both serological serum (5 mL) and nasopharyngeal swab samples were collected from each patient. Samples were collected in the form of a gargle serological fluid (approximately 5 mL). Samples were collected in the form of a gargle with serological fluid. The nasopharyngeal swab was routinely taken from the nose using a swab stick. The samples were studied within 24 hours and both groups were stored at +4 °C.

### The Sample Preparations for q-RT-PCR Tests

The preferred kit does not require any extra RNA extraction steps due to the use of vNAT with nucleic acid extraction property. Vigorous vortexing of the vNAT solution was enough for RNA extraction. The transfer tube (vNAT) contains two mL of viral nucleic acid extractive and preservative liquid. It is used for the transport of nasopharyngeal, oropharyngeal, nasal, and

**Table 1.** Test interpretation of Bio-Speedy SARS-CoV-2 Emerging Plus-Bioeksen Kit (Bioeksen R&D Technologies, Istanbul, Turkey)

| Situation | FAM | ROX | Cy5 | Cy5.5 | Result |
|---|---|---|---|---|---|
| 1 | - | - | - | - | 1. SARS-CoV-2 negative |
| 2 | + | - | - | - | 1. SARS-CoV-2 positive<br>2. B.1.1.7, E484K, and L452R included variants negative |
| 3 | + | + | - | - | 1. SARS-CoV-2 positive<br>2. One of the variants containing E484K positive<br>3. L452R and B.1.1.7 included variants negative |
| 4 | + | + | + | - | 1. SARS-CoV-2 positive<br>2. B.1.1.7 positive carrying the E484K mutation<br>3. Variants containing L452R are negative |
| 5 | + | + | - | + | 1. SARS-CoV-2 positive<br>2. One of the variants containing both E484K and L452R is positive<br>3.B.1.1.7 is negative |
| 6 | + | - | + | - | 1. SARS-CoV-2 positive<br>2. B.1.1.7 positive<br>3. variants containing L452R and E484K are negative |
| 7 | + | - | - | + | 1. SARS-CoV-2 positive<br>2. One of the variants containing L452R positive<br>3. Variants containing E484K and B.1.1.7 are negative |
| 8 | + | - | - | + | 1. SARS-CoV-2 positive<br>2. B.1.1.7 positive carrying the L452R mutation<br>3) Variants containing E484K are negative |

**Table 2.** Comparison of positivity and negative results of both physiologic serum gargle and q-RT-PCR

| | | q-RT-PCR nasal swab result | | p |
|---|---|---|---|---|
| | | Negative | Positive | |
| Serum physiologic gargle q-RT-PCR result | Negative | 145 (98.0) | 20 (31.3) | 0.001* |
| | Positive | 3 (2.0) | 44 (68.8) | |

Note. *Chi-square test

oral/saliva swab samples to the laboratory, nucleic acid (NA) extraction, NA preservation, and pathogen inactivation.

All patients' samples were collected in both swab and gargle form. Physiological saline was used for the gargle sample preparation. The physiological saline was collected by shaking the mouths of the patients thoroughly. Samples were studied within 24 hours. For gargle samples, 50 µl of patient sample in saline and 50 µl of vNAT fluid were combined in an Eppendorf tube before q-RT-PCR was performed. Gargles were vortexed for at least 15 seconds and combined with the q-RT-PCR mix (2.5 µl sample-7.5 µl mix).

### Bio-Speedy SARS-CoV-2 Emerging Plus-Bioeksen Kit (Bioeksen R&D Technologies, Istanbul, Turkey)

FAM, HEX, ROX, Cy5, and Cy5.5 channels are utilized to investigate the SARS-CoV-2 variants. Channels refer to ORF1ab+N, RNaseP mRNA (IC), Spike (S) E484K mutation, Nucleocapsid (N) D3L mutation, and Spike (S) L452R mutation, respectively. According to the kit protocol, 2.5 µl patient samples with vNAT were added to a 7.5 µl ready kit mixture to achieve 10 µl PCR mixture in total. The kit detects the qualitative detection of SARS-CoV-2 and variants containing B.1.1.7, E484K, and L452R mutations.

The protocol takes 30 minutes. For routine positivity, in addition to Orf1ab and N gene regions found in all SARS-CoV-2, N D3L mutation for B.1.1.7 mutation, E484K mutation for detection of variants that are likely to evade immunity by antibody, and variants with higher contagiousness target the L452R mutation. Thermal cycle parameters of q-RT-PCR amplification were as follows: 52 °C for 3 min (1 cycle) for reverse transcription, 95 °C for 10 s for holding (1 cycle), then 5 cycles of 95 °C for 1 s, and 60 °C for 1s for denaturation, finally 35 cycles of 85 °C for 1 s, and 60 °C for 1 s; annealing, and extension, respectively.

### Test Interpretation, Bio-Speedy SARS-CoV-2 Emerging Plus-Bioeksen Kit (Bioeksen R&D Technologies, Istanbul, Turkey)

The threshold set was arranged as 200 according to the kit protocol for the Biorad CFX96 (Bio-Rad Laboratories, Inc. USA) platform. Hex channel is utilized as an internal control. If the FAM, ROX, and Cy5 CT values are smaller or equal to 33 (≤33), the result means positive, otherwise the result is considered negative. Additionally, the results are examined in **Table 1**.

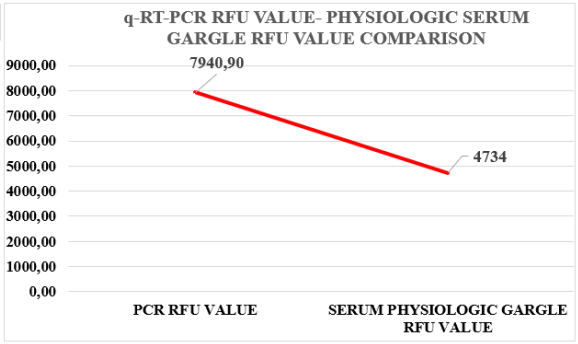
### Statistical Analyzes

The data was analyzed using the SPSS 25.0 package program. The distribution of the data was examined with the Kolmogorov-Smirnov test. While evaluating the study data, Student's t-test, one-way analysis of variance (ANOVA), $\chi^2$, and Fisher exact tests were used for parametric data as well as descriptive statistical methods (mean, standard deviation, and frequency). It was calculated in the 95% confidence interval when evaluating the study.

## RESULTS AND DISCUSSION

Herein, 212 randomly chosen patients' samples were studied. These samples were first studied according to the routine q-RT-PCR method. The 48 samples are negative, while 64 patient samples are positive. These samples were then studied with saline gargles. While 165 negative results were found in the same samples, positivity was observed in 47 samples. In addition, although three samples studied with saline were negative in routine q-RT-PCR processes, they were positive results when studied with this method, as shown in **Table 2**.

A

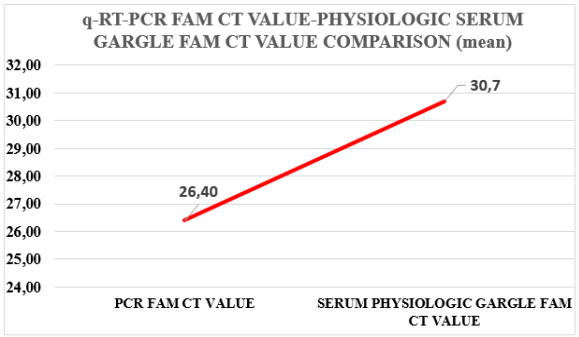



B

| | | Serum physiologic gargle RFU |
|---|---|---|
| q-RT-PCR RFU value | r | .081 |
| | p | .600* |
| | n | 44 |

**Figure 2.** A. RT-PCR RFU value and physiologic serum gargle RFU value comparison. B. Physiologic serum gargle RFU (*Pearson correlation)

A




B

| | | Serum physiologic gargle FAM CT value |
|---|---|---|
| q-RT-PCR FAM CT value | r | .241 |
| | p | .114* |
| | n | 44 |

**Figure 3.** A. RT-PCR FAM CT value-Phyiologic serum gargle FAM CT value comparison (mean). B. Physiologic serum gargle FAM CT value (*pearson correlation)

**Table 3.** q-RT-PCR and serum physiologic gargle mutation result

| | | q-RT-PCR mutation result | | p |
|---|---|---|---|---|
| | | ORF1ab+N | L452R | |
| Physiologic serum gargle mutation result | ORF1ab+N | 28 (96.6) | 6 (40.0) | 0.001* |
| | L452R | 1 (3.4) | 9 (60.0) | |

Note. *Chi-square test

**Table 4.** Mutation results of q-RTPCR and physiologic serum gargle

| | | n | % |
|---|---|---|---|
| q-RTPCR result | Negative | 148 | 69.8 |
| | Positive | 64 | 30.2 |
| q-RT-PCR mutation result | ORF1ab+N | 46 | 71.9 |
| | L452R | 18 | 28.1 |
| Physiologic serum gargle q-RT-PCR result | Negative | 165 | 77.8 |
| | Positive | 47 | 22.2 |
| Physiologic serum gargle mutation result | ORF1ab+N | 35 | 74.5 |
| | L452R | 12 | 25.5 |

When the RFU irradiation results obtained from the q-RT-PCR device of all samples were evaluated, there was no significant decrease. The RFU radiation limit value of these samples, which were analyzed in the Biorad CFX96 (Bio-Rad Laboratories, Inc. USA) device, was set to 200 according to the kit protocol. Samples above this value are considered positive. For this reason, it was concluded that there was no significant difference in the positivity sigmoid curves obtained with the average irradiance value of 7,940.90 RFU obtained in the routine procedure of the samples taken with gargles and the average of 4,734 as shown in **Figure 2**.

When the CT results obtained from the q-RT-PCR device of all samples were evaluated, no significant decrease was observed. The CT limit value of these samples is set at 33 according to the kit protocol. Samples below this value are evaluated as positive. Thus, there was no significant difference in the positivity sigmoid curves obtained with the average value of 26.40 CT obtained in the routine procedure of the samples taken with gargle and the average of 30.7 as summarized in **Figure 3**.

SARS-CoV-2 mutation types were detected according to the kit protocol. When the SARS-CoV-2 results of 44 patients who were positive according to the routine q-RT-PCR results were compared with the gargle, it was observed that the sample of 15 patients had the L452R mutation in the routine q-RT-PCR procedure. 29 patients did not have any mutations. When the positive results obtained in gargle are evaluated on the same samples, the results show that 10 patients out of 44 patients have the L452R mutation. The results are compared with the routine procedures performed in q-RT-PCR as shown in **Table 3**. The only patient who could not detect the mutation in the result (3.4%).

When all the 212 patients' results obtained in the experiment were evaluated, 69.8% of negative and 30.2% of positive results were obtained in routine q-RT-PCR procedures. In the method studied with gargle on the same samples, 77.8% negative and 22.2% positive results were obtained.

These rates are calculated as a percentage and the positive results are obtained in gargle at a rate of 74.50%, as shown in **Table 4**. Furthermore, 71.9% of the patients did not show any mutations and 28.1% showed mutation types according to the routine q-RT-PCR results. When these rates are compared based on gargle results, the patient sample without mutation was 74.5%, and with the mutation it was 25.5%.

The values greater than 200 RFU, less than 33 CT, and sigmoidal curve results are considered positive results according to the kit protocol. Herein, when the mean values of the CT and RFU results of the two methods were examined, significant variations did not occur. The RFU values were 7,375.0 in routine q-RT-PCR and 4,658.9 in gargles procedures.

**Table 5.** CT and RFU results of q-RTPCR and physiologic serum gargle

| | n | Minimum | Maximum | Mean | Standard deviation |
|---|---|---|---|---|---|
| q-RT-PCR RFU value | 64 | 900.0 | 25,000.0 | 7,375.0 | 4,754.3 |
| q-RT-PCR FAM CT value | 64 | 17.0 | 33.0 | 27.10 | 4.00 |
| Physiologic serum gargle RFU value | 47 | 200.0 | 17,000.0 | 4,648.9 | 4,844.7 |
| Physiologic serum gargle FAM CT value | 47 | 25.0 | 33.0 | 30.7 | 3.11 |

When the minimum and maximum values of these values are examined, there is no significant change for both methods (q-RT-PCR RFU value min: 900.0 max. 25,000, physiological gargle RFU value min. 200.0 max. 17,000). When all these samples were evaluated over the CT value, both methods were accepted as 33.0 according to the kit protocol, while the minimum values were obtained as 17.0 in the routine q-RT-PCR process and 25.5 in the gargle as shown in **Table 5**.

## CONCLUSION

Throughout history, many pandemics have been described. Since late December 2019, the world has been battling a highly contagious and devastating viral pandemic called SARS-CoV-2, caused by the COVID-19 disease. Coronaviruses are a group of single-stranded RNA viruses that are classified into four genera (α, β, γ, and δ). The phylogenetic analysis of SARS-CoV-2 has shown that it belongs to the genus β-CoV. It is also examined in chemical, biological, radiological, and nuclear defense (CBRN defense or CBRNE defense) members, which is a worldwide health concern [17].

Detecting the infected individuals and diagnosing them quickly is pivotal to controlling the pandemic. Therefore, many diagnosis techniques have been described, such as RT-LAMP, point of care (POC) systems, serological tests, and q-RT-PCR as a gold standard method [18]. The oropharynx or nazofarenks serves as a reservoir of SARS-CoV-2. In light of the tremendous need to increase the availability of diagnostic tests, gargles have been utilized promising alternative results [19]. The first mechanism of gargles to reduce a load of active viruses in the oropharynx is to disrupt or destroy the viral envelope. Although for the gargle usage, ethanol was chosen as the first chemical to disrupt the genetic material of the virus, it is effective only in inactive ingredients at concentrations below 25% [20]. Additionally, its usage is not appropriate for the oropharynx.

According to the literature, 20% ethanol has reduced the infectivity of SARS-CoV-2 by >87%, whereas 30% ethanol reduces the infectivity by >99.99% [21]. Challacombe et al. have described the povidone-iodine (PVP-I) gargle and nasal spray as effective in reducing SARS-CoV-2 in the dental setting [22]. Herein, physiological saline is utilized, which is widely used medically as a recommended irrigating and wound dressing solution. It is highly compatible with the human body and tissues chemically because of its antiseptic property [23]. Although 3 patient samples studied with routine q-RT-PCR were negative, they were also positive when studied with gargle (2%) as shown in **Table 1**. According to the mutation results, 1 patient result has been shown as a mutation in the gargle studied, however, it is negative with routine procedures performed in q-RT-PCR (3.4%).

Considering the pandemic situation, this rate is a serious public health problem L452R, and delta variants of COVID-19 threaten public health to a large extent [24]. This result is also of great importance for the detection of positive samples that cannot be detected in q-RT-PCR. Considering that the experimental results are repeated by comparing them with the public populations and results are obtained, positives and mutations that cannot be detected in the routine q-RT-PCR process are observed. This is not related to the kit or the device. It is related to the method of working and taking samples. In this case, our prediction in our research is to take samples from patients with both gargle and swab samples. Thus, q-RT-PCR can also work with gargle samples of emergency patients that cannot be detected in routine procedures. This can also be applied to healthcare professionals who have difficulties in taking samples, patients who have a phobia about giving samples, and pediatric patients. Out of 212 patients, we obtained in our experiments with this method, the confidence interval determines 74.50% positivity when compared to the routine q-RT-PCR procedure as summarized in **Table 4**. In addition, when only the gargle sampling method is studied in low-income countries, the cost of testing for COVID-19 will decrease significantly. Because this method does not require vNAT or VTM transport solution sterile swab sticks as shown in **Figure 1**. In addition, the amount of this solution usage will be minimized. The plastic container with a lid in which the patient can gargle with SF and spit it out is an ideal method for this. Additionally, it provides a great cost-benefit in low-income countries.

**Author contributions: YA & NPC:** developed the protocol, determined the method, conducted the experiments, analyzed the data, and created the administrative process, vouched for the entire experimental process, and wrote the article; **SZMK & CK:** provided support on academic consultancy for the research process; **SD:** collected samples; **YU:** wrote the revised article and vouched for it; **NH:** provided support for academic consultancy in the research process; **HS:** provided support on academic consultancy for the research process and calculated the statistics; & **ZAK:** calculated the statistics. All authors have agreed with the results and conclusions.

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**Declaration of interest:** No conflict of interest is declared by authors.

**Data sharing statement:** Data supporting the findings and conclusions are available upon request from the corresponding author.

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