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Protocols

Development of a simple genotyping method based on indel mutations to rapidly screen SARS-CoV-2 circulating variants: Delta, Omicron BA.1 and BA.2

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ABSTRACT

The high need of rapid and flexible tools that facilitate the identification of circulating SARS-CoV-2 Variants of Concern (VOCs) remains crucial for public health system monitoring.

Here, we develop allele-specific (AS)-qPCR assays targeting three recurrent indel mutations, ΔEF156–157, Ins214EPE and ΔLPP24–26, in spike (S) gene to identify the Delta VOC and the Omicron sublineages BA.1 and BA.2, respectively.

After verification of the analytical specificity of each primer set, two duplex qPCR assays with melting curve analysis were performed to screen 129 COVID-19 cases confirmed between December 31, 2021 and February 01, 2022 in Sfax, Tunisia.

The first duplex assay targeting ΔEF156–157 and Ins214EPE mutations successfully detected the Delta VOC in 39 cases and Omicron BA.1 in 83 cases. All the remaining cases (n = 7) were identified as Omicron BA.2, by the second duplex assay targeting Ins214EPE and ΔLPP24–26 mutations. The results of the screening method were in perfect concordance with those of S gene partial sequencing. In conclusion, our findings provide a simple and flexible screening method for more rapid and reliable monitoring of circulating VOCs. We highly recommend its implementation to guide public health policies.

1. Introduction

Several SARS-CoV-2 Variants of Concern (VOCs) have emerged since the beginning of the COVID-19 pandemic and continuous tracking of their circulation around the world remains of utmost importance (Johnson et al., 2022; Salles et al., 2022). The VOC Delta (B.1.617.2), first identified in India, is characterized by higher viral infectivity allowing it to rapidly replace pre-existing lineages and to become the most common variant throughout the world since mid-2021 (Cherian et al., 2021; Ren et al., 2022). The Delta variant harbors a distinct mutation profile in S protein characterized especially by L452R and T478K substitutions in the Receptor Binding Domain (RBD) and a deletion of two amino acids (aa) at positions 156–157 (ΔEF156–157) in the N-Terminal Domain (NTD) (Ferreira et al., 2021; Mishra et al., 2022; Mittal et al., 2022). On November 26, 2021, the VOC Omicron (B.1.1.529) was first reported in South Africa and has since been linked to a sharp increase in confirmed cases of COVID-19 (Mohapatra et al., 2022; Thakur and Ratho, 2022). Compared to prior VOCs, Omicron was considered a product of extensive evolution and polymorphism pattern because it carries 46 substitutions, three deletions (ΔHV 69–70; ΔVYY 143–145 and ΔN 211), and an insertion of three aa (ins214EPE) (Viana et al., 2022). Untel recently, Omicron has yielded three major sublineages: BA.1, BA.2 and BA.3, with a predominance of the BA.1 subvariant that has overtaken the VOC Delta (Bruel et al., 2022). Subsequently, the spread of BA.2 has increased in many parts of the world. Two additional major sublineages, BA.4 and BA.5, were then detected in South Africa in January and February 2022, respectively, and since then they have become the dominant variants there.

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Compared to BA.1, BA.2 has 28 distinct mutations, notably the presence of a deletion of three aa at positions 24, 25 and 26 (ΔLPP24–26) and the absence of 214EPE insertion (Majumdar and Sarkar, 2022; Saxena et al., 2022).

Monitoring of SARS-CoV-2 lineages is generally based on the sequencing of the full viral genome or of partial relevant regions (Ko et al., 2022; Oude Munnink et al., 2020). However, this approach requires expensive resources, technical expertize, and longer data processing time, which can lead to a late response. In Tunisia, few laboratories have specialized facilities and expertize limiting the genomic surveillance of variants. In this context, the detection of distinct and recurrent small indel mutations encompassing the S gene could serve as useful potential targets for screening strategies. Here, we develop an alternative genotyping method based on recurrent small indel mutations to rapidly screen and distinguish Delta (B.1.617.2), Omicron BA.1 and BA.2 variants. Partial sequencing was performed to validate the screening strategy.

2. Materials and methods

2.1. Samples

Overall, 129 SARS-CoV-2 positive samples collected between December 31, 2021 to February 01, 2022 were analyzed in the laboratory of microbiology, Habib Bourguiba University Hospital, Sfax, Tunisia. Viral RNA was extracted from nasopharyngeal swabs using Chemagic Viral DNA/RNA kit special H96 (PerkinElmer, Inc.) on the Chemagic™ 360 instrument. SARS-CoV-2 infection was confirmed using different commercial real-time RT-PCR kits targeting ORF1ab and N genes (Wondfo 2019-nCoV Real-Time RT-PCR Assay, Guangzhou Wondfo Biotech Co., Ltd. and Wantai SARS-CoV-2 RT-PCR Kit, Beijing Wantai Biological Pharmacy Enterprise Co., Ltd). The amplification reaction was performed on the Applied Biosystems QuantStudio 5 instrument (Applied Biosystems™, ThermoFisher scientific). All selected samples had ORF1ab and N quantification cycles (Cq) $\leq$ 33. Among them, ten clinical samples having Cq values $<$ 16 were selected for the evaluation of the analytical specificity of the screening method.

2.2. Design of the screening method

Our screening method was focused on three genomic regions located in the NTD of the S gene to examine the recurrent small indel mutations ΔEF156–157, ins214EPE and ΔLPP24–26 using an allele-specific (AS)-qPCR approach. For each genomic region, two different Forward primers at their 3' end (WT-F and 1Δ-F) and a common Reverse primer were designed for the detection of wild-type and mutant strains (Table 1).

First, the reactivity of each of the six primer pairs was assessed in the group of ten selected clinical samples having low Cq values reflecting a high viral load to verify a possible cross-reactivity when using such screening approach. It was conducted using non-diluted and diluted cDNA products as templates for qPCR reactions. Through validation of the analytical specificity of each primer set using simplex qPCR, we established two duplex qPCR assays that each target two mutated regions easily distinguishable in a single reaction with melting temperature. According to the epidemiological status of SARS-CoV-2, the first duplex that was subjected for all samples targeted both ΔEF156–157 and ins214EPE mutations to detect the Delta and Omicron BA.1 VOC, respectively. The corresponding VOC was defined according to the melting curve analysis of PCR products showing a single specific peak at 76 °C for the Delta VOC or at 79 °C for the Omicron BA.1 VOC. The undetected cases were then subjected to a second duplex assay targeting both ins214EPE and ΔLPP24–26 mutations to identify Omicron sublineages BA.1 or BA.2 according to their specific peaks at 79 °C or 76.5 °C, respectively (Fig. 1). Additionally, a subset of positive Omicron BA.1 (n = 13) and Delta cases (n = 10) identified by the first assay was reassessed to verify the specificity of the second duplex assay.

2.3. RT and qPCR reactions of the screening method

Reverse transcription (RT) reaction was performed on 4 µl of RNA input in a final reaction volume of 20 µl containing 0.2 pmol of each of the three specific reverse primers (24–26-R, 156–157-R and 213-R; Tables 1), 1× reaction buffer, 20 nmol of dNTP, and 100 U of SCRIPT Reverse Transcriptase (Jena Bioscience). The reaction was incubated for 15 min at 50 °C and finally stopped by heating for 15 min at 70 °C. The qPCR reaction mix included 1 µl of non-diluted or diluted cDNA product, 2 pmol of each primer pair (Table 1), and 10 µl of 2 × TB Green Premix Ex Taq (TAKARA). The amplification conditions performed in Biorad-CFX96 were as follows: 95 °C for 30 s, then 40 cycles of 94 °C for 10 s and 60 °C for 30 s. The specificity of PCR products was confirmed by melting curve analysis from 65° to 95°C, with a heating rate of 0.5 °C/s. In addition, the PCR amplification efficiency of the three primer pairs targeting mutated sequences was calculated according to the standard curve method using the equation E % = (10 −1/slope − 1) × 100 by serial dilutions.

2.4. RT-PCR amplification for sequencing

RT-PCR was performed with Forward (FW) and Reverse (R2.1) primers to obtain a fragment of 1111 bp, encoding a sequence from codon 339 to codon 694 (Table 1). The target sequence was amplified in a 25 µl reaction volume containing 5 µl of each RNA, 0.3 µM of each primer, 200 µM dNTP, 5 µl Tp5X and 1 unit of the One Step Enzyme Mix

Table 1
Primers used for SARS-CoV-2 VOCs detection by AS-qPCR and partial sequencing.

	aa position in Spike gene	VOC	Name	Sequence (5'−3')	Amplicon size (Tm °C)
AS-qPCR	24–26	Omicron BA.2	WT24–26-F	AACCAGAACTCAATTACCCC	108 bp (77.5)
			Δ 24–26-F	CTTATAACCAGAACTCAATCATACA	104 bp (76.5)
			24–26-R	AACAAGTCCTGAGTTGAATG	
	156–157	Delta	WT156–157-F	TTGGATGGAAAGTGAGTTC	88 bp (76.5)
			Δ 156–157-F	AAGTTGGATGGAAAGTGGAG	83 bp (76)
			156–157-R	CAAGGTCCATAAGAAAAGGC	
	Ins 214	Omicron BA.1	WT214-F	TTAGTGCGTGATCTCCCTC	141 bp (78.5)
			Ins214-F	ATAGTGCGTGAGCCAGAAG	150 bp (79)
			214-R	TCCAACCTGAAGAAGAATCACC	
RT-PCR, semi-nested PCR and sequencing			FW	ATTACAAACTTGTGCCCTTTT	1111 pb
			FwN	TTAGAGGTGATGAAGTCAGA	900 pb
			R2.1	CTGCACCAAGTGACATAGTG	

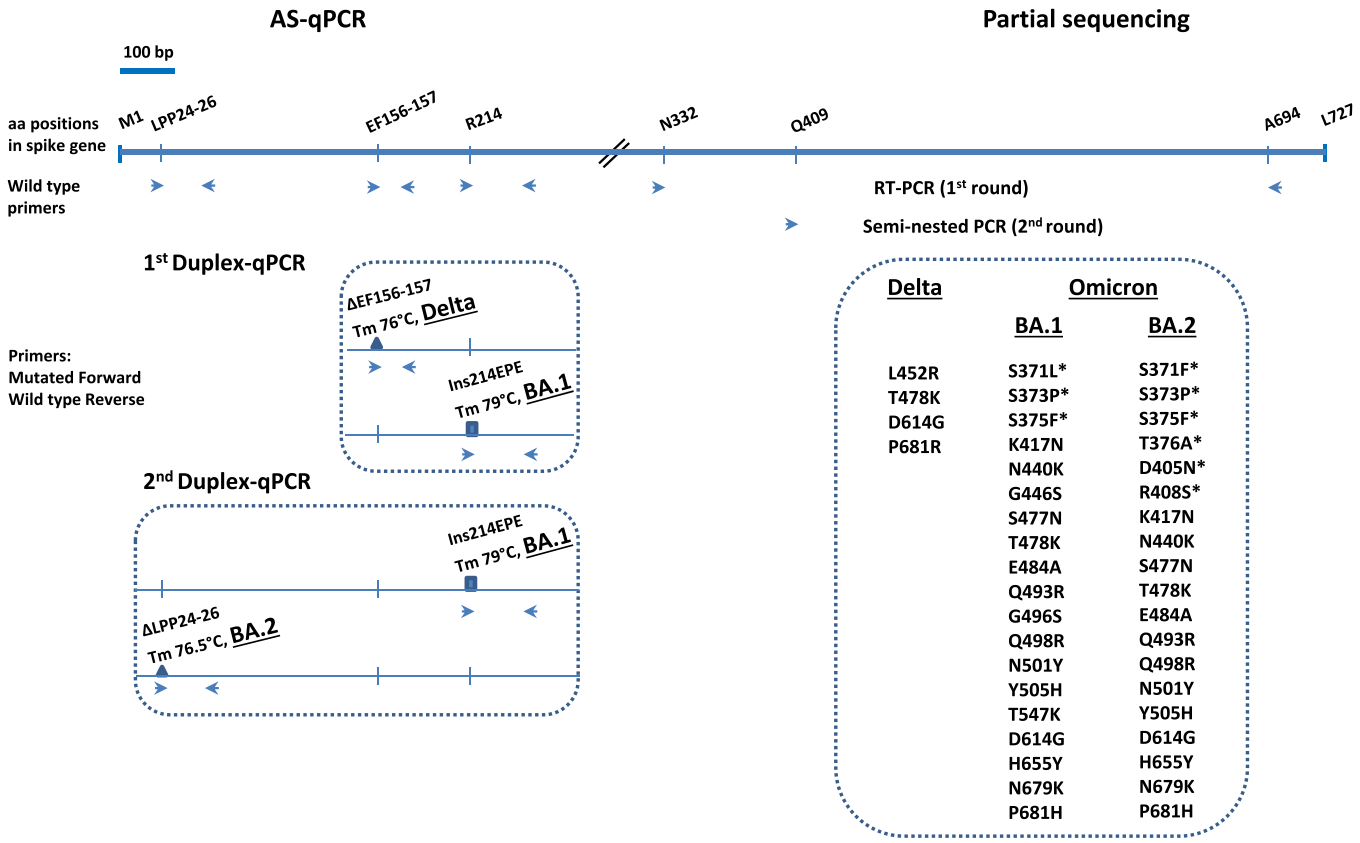

Fig. 1. Schematic representation which indicates the position of all primer sets used for AS-qPCR assays and partial sequencing. Two duplex qPCR were designed to differentiate Omicron BA.1 from Delta cases (1st Duplex) and Omicron BA.2 from BA.1 cases (2nd Duplex). The validation of the discrimination assay using partial sequencing was based on mutation panels characterizing each of the three VOCs. * Mutations not found by semi-nested PCR sequencing.

(Qiagen OneStep RT-PCR kit, Qiagen, Hilden). Amplification conditions consisted of 30 min at 50 °C for reverse transcription, 15 min at 94 °C for activation of the HotStarTaq DNA Polymerase, 40 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 57 °C, extending for 1 min at 72 °C and a final cycle of 10 min at 72 °C. Amplification was carried out on the Applied Biosystems™ SimpliAmp™, ThermoFisher Scientific. PCR products of the first round with a weak amplification signal were subjected to a second round of semi-nested PCR using the internal forward primer (FwN) to obtain a fragment of 900 bp, encoding a sequence ranging from codon 409 to codon 694. Semi-Nested PCR reaction was performed in a 25 µl reaction volume containing 3 µl of each PCR product, 0.4 µM of FwN primer, 0.3 µM of R2 primer and 12.5 µl of Emerald Amp® GT PCR Master Mix (Takara Bio Inc). Amplification was carried out on the BioRad T100™ thermal cycler with an initial denaturation at 94 °C for 5 min, 40 cycles of 15 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C followed by a final cycle of 10 min at 72 °C.

2.5. DNA sequencing and analysis

The purification of PCR amplification products was performed with HT Exo SAP-IT (Thermo Fisher Scientific). The sequencing reaction was carried out using the BigDye™ Terminator V.3.1 Cycle Sequencing kit (Applied Biosystems). Sequencing products were then purified using Invitrogen™ Dynabeads™ Magnetic Beads (ThermoFisher Scientific). The purified products were analyzed on the ABI 3500 Genetic Analyzer (Applied Biosystems). Raw sequences data were edited, aligned and assembled using the BioEdit Sequence Alignment Editor, Version 7.2. Delta, Omicron BA.1 and Omicron BA.2 variants were identified according to the presence of specific mutations as described in Fig. 1.

3. Results

3.1. Analytical performance of AS-qPCR

As shown in Table 2, the analytical specificity of each AS qPCR assay was performed on the selected group (n = 10) to distinguish between mutant and wild-type sequences. Perfect discrimination for each of the six tested primer pairs was obtained with no detectable cross-reactivity, especially when using the 10^{-2} diluted cDNA as a template. Based on simplex qPCR results, the corresponding VOCs were identified as follows: Delta (n = 3), Omicron BA.1 (n = 4) and Omicron BA.2 (n = 3). Similar results were also observed when the assays were run in duplex formats with ΔEF156–157 and ins214EPE mutations to identify Delta or Omicron BA.1, or ins214EPE and ΔLPP24–26 to differentiate BA.1 from BA.2 sublineage.

In addition, the amplification efficiencies of the three mutated DNA regions containing ΔLPP24–26, ΔEF156–157, and ins214EPE were investigated by testing 10-fold dilution of cDNA samples; they were 92.26%, 95.19% and 93.81%, respectively. Although the non-diluted cDNA samples showed lower Cq values for the respective genotype (15.4–19.4 *vs* 20.4–26.5 for diluted cDNAs), the opposite genotype was detected with Cq values > 35 in some samples, reflecting the presence of cross-reactivity. To avoid this issue, samples with Cq values < 20 in routine RT-PCR diagnosis were subjected to 10^{-2} cDNA dilution prior to investigation by duplex q PCR assays.

3.2. Delta and Omicron screening

Using the ΔEF156–157/ins214EPE duplex qPCR, amplification was successfully achieved in 122 cases (94.5%) with Cq values ranging from 20.4 to 38.7. The VOC Delta was identified in 30.2% of cases (n = 39),

Table 2
Performance of AS-qPCR assays (n = 10).

	Simplex qPCR assays						Duplex qPCR assays		
Samples[a]	WT24-26	Δ24-26	WT156-157	Δ156-157	WT214	Ins214	Δ156-157/ Ins214 (Tm °C)	Δ24-26/ Ins214 (Tm °C)	Identified VOCs
1	N	21.7	20.4	N	23.2	N	N	22.8 (76.5)	Omicron BA.2
2	N	23.1	22.1	N	24.9	N	N	23.7 (76.5)	Omicron BA.2
3	N	21.9	21.3	N	24.3	N	N	22.2 (76.5)	Omicron BA.2
4	21.7	N	21.5	N	N	24.4	22.8 (79)	23. (79)	Omicron BA.1
5	20.4	N	20.4	N	N	23.7	22.3 (79)	22.6 (79)	Omicron BA.1
6	25.9	N	26.5	N	N	25.4	25.8 (79)	25.5 (79)	Omicron BA.1
7	23.2	N	24.0	N	N	25.0	24.6 (79)	25.2 (79)	Omicron BA.1
8	26.2	N	N	24.4	23.5	N	24.9 (76)	N	Delta
9	24.3	N	N	23.9	24.0	N	24.1 (76)	N	Delta
10	26.5	N	N	25.2	25.1	N	25.1 (76)	N	Delta

[a] cDNA samples diluted at 10^{-2}; N: no detected signal after 40 cycles; xx: Cq values

whereas, 64.3% of the tested samples (n = 83) were identified as VOC Omicron BA.1. In the remaining 7 cases (5.4%), the amplification signal was not detected by any of the two primer sets. Then, the ins214EPE/ ΔLPP24–26 duplex qPCR was carried out for 30 cases, including the undetectable cases (n = 7), and a subset of Omicron BA.1 (n = 13) and Delta (n = 10) defined by the previous duplex. Indeed, all 13 cases of Omicron BA.1 were detected once again, while no amplification was observed for all Delta cases. In addition, the previously undetectable cases were identified as Omicron BA.2 by the second round of screening. Overall, the adopted screening assays showed the presence of Delta, Omicron BA.1 and BA.2 in 39, 83 and 7 clinical samples, respectively.

3.3. Screening validation by sequencing

The sequences of the S gene were obtained for 122 among 129 samples included in the present study. The distribution of detected variants was as follows: 35 Delta variants, 81 Omicron BA.1 and 6 Omicron BA.2 subtypes. This result showed a concordance of 100% with the screening method. In the remaining seven cases, low viral loads and/ or low-quality of RNA samples could explain the sequencing failure.

4. Discussion

The incessant emergence of novel SARS-CoV-2 VOCs has conferred additional challenges for their detection and screening worldwide. Since late 2021, multiple molecular assays based on mutation-specific qPCR approach have been developed using individual or combined genomic targets for the surveillance of VOCs circulation. However, the performance of these assays should be re-assessed for each emerging variant which could bring novel mutational characteristics. Therefore, the development of more appropriate molecular tests remains necessary. Here, we describe a flexible genotyping method based on recurrent small indel mutations to rapidly screen the currently circulating Delta and Omicron VOCs.

The use of small indel mutations as molecular targets for the establishment of SARS-CoV-2 screening tests could provide more efficient genotype discrimination with a cost-effective format. Such a test have been obtained by using the ΔHV69–70 mutation for the Alpha VOC detection (Abdel Sater et al., 2021; Bechtold et al., 2021; Lee et al., 2021; Vega-Magaña et al., 2021). The single-nucleotide substitution-based assays have also been successfully reported for N501Y and E484K screening, but using particular probe design types, notably locked nucleic acid (LNA) or sloppy molecular beacon (SMB) (Abdel Sater et al., 2021; Banada et al., 2021; Vega-Magaña et al., 2021). Our screening strategy was designed to detect Delta and Omicron BA.1 VOCs depending on the presence of either ΔEF156–157 or ins214EPE, respectively. The duplex assay successfully identified the circulating VOCs in 122 out of 129 clinical samples. Notably, the Omicron BA.1 sublineage became dominant in less than 4 weeks in our cohort (65% *vs* 30% for Delta). At the time of writing the manuscript, the selected target ins214EPE has also been successfully described by Phan et al., to detect Omicron (B.1.1.529) in clinical samples collected from mid-December 2021 to the first week of January 2022 (Phan et al., 2022).

With the emergence and the spread of Omicron BA.2 sublineage in many countries including Denmark, Nepal and the Philippines, it was important to monitor its introduction and local transmission, especially for the seven undetectable cases by the first screening assay (5.4%). BA.2 differs from BA.1 by several genetic variations such as the presence of ΔLPP24–26 and the absence of ins214EPE. Therefore, the small indel mutations were selected as molecular targets for a second duplex qPCR assay to discriminate between the Omicron BA.1 and BA.2 sublineages. Interestingly, the previously undetected samples (n = 7) were then identified as BA.2, with the first case dating back to late January 2022. Pending the availability of specific Omicron screening assays, Caputo et al. showed that the S-gene target failure (SGTF) based method was a very useful tool for tracking the Omicron VOC in Italy within three weeks of the first detection (Caputo et al., 2022). Although the SGTF is widely used to flag potential cases of Omicron BA.1, it is currently not recommended, as the circulating Omicron BA.2 sublineage lacks the Δ69–70 mutation. To track Omicron, a differential test has been designed based on the Δ31–33aa deletion that is present in the main genotype BA.1, as well as in BA.2 and BA.3 subtypes (Tsui et al., 2022).

Partial sequencing targeting the spike gene, encoding 339–694 aa, revealed a perfect concordance with our described screening method, supporting the efficiency of the design used. Indeed, our screening method proved to be accurate and robust, which highlights its use for rapid genomic surveillance of Delta and Omicron variants while waiting for sequencing results. Furthermore, it can be adjusted to include new emerging variants, as demonstrated by the second duplex qPCR which was designed to distinguish between BA.1 and BA.2 subtypes. The low cost and the ease of integration into laboratories make it an appealing strategy for the high-throughput screening of SARS-CoV-2 positive samples, especially in developing countries such as Tunisia. Of note, the use of a one-step RT-qPCR reaction to screen several target mutations could lead to the depletion of extracted RNA samples which was considered as a main limitation of this strategy (Chung et al., 2022; Neopane et al., 2021; Wang et al., 2021). Through this study, we recommend to generate the corresponding cDNAs in a single independent RT reaction that can be used to screen for all mutations of interest by qPCR.

Similar to other previous reports that evaluate the viral load required for mutation-specific qPCR approach, the performance of our screening assays was not evaluated in the case of a diagnostic RT-PCR Cq value greater than 33 (Sandoval Torrientes et al., 2021; Vega-Magaña et al., 2021; Wang et al., 2021). Therefore, the use of such an approach should be optimized for maximal specificity with higher reaction stringency.

In conclusion, we report here the development and the validation of a simple and flexible screening method based on recurrent small indel mutations to track SARS-CoV-2 Delta and Omicron BA.1 and BA.2 variants. This approach is easy to implement in the existing routine

diagnostic workflow and is suitable for high throughput VOCs monitoring settings.

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Informed consent statement

Patient consent was waived. According to the approval of the institutional review board and as samples were fully anonymized, the bioethics committee waived the requirement for informed consent to be able to manage the pandemic.

CRediT authorship contribution statement

Study conception and design: WA, LFB. Collection and verification of data: WA, AT, FS, RD, AC, AHS. Cleaning and analysis of data: HKH, AH, RMG, AG, HSG. Drafting of the manuscript: WA, AT, FS, SG, LFB. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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