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Article

PCR-based Detection of Typhoidal *Salmonella* from Human Clinical Samples and evaluation of Traditional PCR, Nested-PCR And Widal Test in Pune, India

Abas O. Hadi[1]*, Hussein Alburkat [2]*, Taisir Abdulelah Kadhim [3], Ammar M S Al-Helli[3], Mustafa Hamza Ghazi[6], Yasir Abdulameer N. Aldabbagh[7], Reem Numaan Talib [5], Sama Hussein Ali[3], Noor Hameed Imran[4], Ruaa Al-mohsen[9], Turki Muftin Saad[6], Pallavi Mandave[8]

[1] Department of optics, College of health and & medical Technology, Sawa University, samawah, Iraq. ; abbas.omran.a@sawa-un.edu.iq (A. O. H.)
[2] Emerging infections research group, Viral zoonoses research Unit, Faculty of Medicine, University of Helsinki; Hussein.alburkat@helsinki.fi (H. A.)
[3] College of Nursing, Al-Muthanna University; Iraq, Taisirak14@mu.edu.iq (T. A. K.); Samakarkosh@mu.edu.iq (S. H. A.); Ammar_alhelli@mu.edu.iq (A. M. S. A.)
[4] Department of Chemistry, College of Science, Al-Muthanna University; Iraq, noorhameed@mu.edu.iq (N. H. I.)
[5] Department of pathology and poultry diseases, College of veterinary medicine, Al-Muthanna University; Iraq; reemnumaan@mu.edu.iq (R. N. T.)
[6] Department of Medical Laboratory, College of health and & medical Technology, Sawa University, samawah, Iraq; mustafa.hamza@sawa-un.edu.iq (M. H. G.); turkey.muften@sawa-un.edu.iq (T. M. S.)
[7] Department of Health Administration, College of Health & Medical Technology, Sawa University, Samawah, Iraq, yasir.abdulameer@sawa-un.edu.iq (Y. A. N. A.)
[8] Yashavantrao Chavan Institute of Science, satara, Maharashtra, India; scipalsbiotech01@gmail.com (P. M.)
[9] Department of Public Health,Al-Muthanna Health Directorate, Samawah, Iraq, ruaaajel@gmail.com (R. A.)
* Correspondence; abbas.omran.a@sawa-un.edu.iq (A. O. H.); Tel.: +964 780 205 4626
hussein.alburkat@helsinki.fi (H. A.); +358451629995

Abstract: *Salmonella* is a foodborne pathogen associated with localized outbreaks that potentially pose a huge risk to the public health in some countries such as Africa and India, especially in the regions lacking health care measures. The focus of the present study is the molecular detection method of *salmonella*. The study was conducted to identify *Salmonella* strains by PCR targeting flagellin specific gene from suspected patient's clinical blood samples. Serologically, Widal test results showed that all serum samples except one patient (ID 7) were positive for O antigen. The serum of three patients (ID 4, 5 and 10) were found to be positive for H and O antigens while one patient (ID 9) showed agglutination for antigen H. The PCR approach used in this study was successful for fast and precise detection of clinically related Salmonella Typhi. Seven samples out of 10 were positive for *Salmonella Typhi* as fragments of 458-497bp were observed on the gel-agarose 0.8% corresponds to flagellin gene of *S. Typhi*. Relatively, smaller amplicons of 366-343bp were observed utilizing nested PCR as well, which seems more sensitive than the conventional PCR. Therefore, we recommend the PCR approach performed in this study to be used as a fast, cost-effective, and time-consuming tool for diagnostic purposes of *Salmonella*.

Keywords: PCR, Salmonella Typhi, Widal Test, BHI

1. Introduction

Salmonella typhi is a foodborne pathogen spreading worldwide and is the causative agent for typhoid fever. Typhoid fever affects around seventeen million people worldwide each year, resulting in 600,000 deaths [1, 2]. Typhoid fever is a major health problem in many developing countries. Overall, typhoid and paratyphoid fevers were responsible for 9·8 million (5·6–15·8) DALYs in 2017, down 43·0% (35·5–50·6) from 17·2 million (9·9–

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27·8) DALYs in 1990 [3]. Typhoid fever is highly endemic in countries where there is neither a safe water supply nor adequate sanitation [4 , 5]. Citizens of low-income countries, those in Southeast Asia, Africa, India, and Latin America seems to be most affected by typhoid [3]. The disease is an important public health problem in Indonesia as well [6].

The transmission of typhoid fever occurs through faecal contamination of water and food [4, 7, 8] either through typhoid fever patients or through carriers excreting *Salmonella*. Carriers such as food handlers are an important source of transmission [9, 10 , 11]. Case–control studies of risk factors for typhoid fever in endemic areas have been reported from several countries: Chile [12], Italy [6], Philippines [13], and Indonesia [14]. Infection risk is enhanced when a convalescent patient or a carrier is actively shedding the pathogen in their intestinal tract.

The clinical identification of typhoid fever has been a medical difficulty due to its similarities to other febrile infections [15]. A single carrier's bacterium can have several genotypes, making it difficult to identify the source of an outbreak [16]. However, routine detection methods, blood or stool cultures, as well as the Widal test, can be used to diagnose the causative of the infection. However, the latter methods have many limitations. After checking out malaria, diarrhea, or pneumonia in less developed nations, chloramphenicol is usually used as a treatment trial, while waiting for the results of the Widal test and blood and stool cultures [17]. Antigen suspensions of *Salmonella* can be utilized in slide and tube procedures. By using the blood cultures methods, results are hampered by past antibiotic use and a low bacterial load [18] but it is time consuming. The generally used serological test, the Widal test, is only positive in the early stages of the disease. In later stages when the IgG antibodies developed, Widal test could be used to determine the antigens. PCR is a sensitive and specific tool to detect the genetic material of microbes in the early stage of infection. Furthermore, its application in endemic areas is fraught with complications [19]. Many studies attempted to identify *S. Typhi* using several genes by PCR, such as O antigen somatic genes [20], H antigen gene (fliC-d) [21], and Vi capsular antigen gene (viaB) [22]. However, these genes cannot be used to diagnose *S. Typhi* because they are not specific and were detected in different *Salmonella* serotypes. The lack of specificity in these target genes in *S. Typhi* inforce the scientific community to find a solution for *S. Typhi* diagnosing. A combination of different pairs of primers using nested PCR is needed to increase the sensitivity and specificity of the PCR diagnostic test.

In this study, we have evaluated two different approaches to diagnose *S. Typhi* from clinical samples that have been suggested as successful approaches to diagnose S. Typhi [15, 21]. The conventional PCR was successful in our study and showed high sensitivity and specificity. It can be a helpful technique during the treatment of typhoid fever since it can be utilized even if antibiotic therapy has been started if the pathogen load is very low [15]. The detection was more sensitive with a nested PCR. Molecular detection performed in this study can be used as an alternative approach to traditional diagnostic methods as we attempted to optimize the nested PCR for more accurate and precise diagnostics.

2. Materials and Methods

2.1 Patients enrollment and sample collection

Ten blood samples were collected from septicemia patients hospitalized in Bharati Hospital and Government General Hospital, Pune, India. The consents were obtained from all participants prior to the study according to the ethical SciPals Biotech committee board guidelines (SPB/019/2017-2018). The samples were collected at 12th of April 2018. The enrolled individuals were between ages 12-48 years with clinical diagnosis of typhoid fever were included in the study. In addition, 10 blood samples of healthy individuals were involved in the study as healthy control group. Serum was separated and used for Widal

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test. The plasma and peripheral blood mononuclear cells (PBMC) were separated from blood and used for DNA isolation.

The age and gender distribution of the patients and healthy individuals are given in Table 1.

Table 1. Age and gender of enrolled patient and healthy individuals in the clinical study

Age (years)	Title 2 Male/Female		Septicemia group (n=10) Male/Female	
Less than 20	01	01	01	01
21-30	00	02	00	02
31-40	02	01	02	01
41-50	02	01	02	01

2.2 Bacterial strains

Seven *Salmonella* strains were obtained from the American Type Culture Collection (ATCC) (Table 2) and *Salmonella typhi* (ATCC 19430) was used as a positive reference.

Table 2: Selected *Salmonella* strains from the American Type Culture Collection (ATCC)

Sr. No.	Species	Source
1.	*Salmonella typhi*	ATCC 19430
2.	*Salmonella paratyphi*	ATCC 11511
3.	*Salmonella muenchen*	ATCC 8388
4.	*Salmonella hirschfeldii*	ATCC 13428
5.	*Salmonella ententidis*	ATCC 13076
6.	*Salmonella typhimunium*	ATCC 13311
7.	*Salmonella choleraesuis*	ATCC 6958

2.3 Widal test from serum samples

H and O antigens of *Salmonella Typhi* and H antigen of *S. Paratyphi* were assigned as the main antigens for the test. The Widal test was performed within 24 h of collection of samples. A dilution of >1 in 80 for O antigen and >1 in 160 for H antigen considered positive of typhoid fever.

2.4 Isolation of Salmonella from blood samples

About 5 mL of blood was aseptically injected into a sterile bottle containing 50 mL of sterile Brainheart Infusion Broth (BHI) and incubated at 37° C. Blood cultures were regularly inspected to check for turbidity and color changes that indicate microbial growth. The culture was incubated for at least 7 days (to confirm negative result). Subcultures were performed as follows: From each positive blood bottle, one loopful isolated culture was plated with MacConkey agar and Salmonella-Shigella agar (S.S. agar). The agar was streaked and incubated at 37° C for 24 hours. The isolate was stained with gram stain and examined under light microscope.

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2.5 DNA extraction from the isolated colonies and the reference strains

PBMC was used for DNA isolation. DNA was extracted from the culture by the colony PCR method [23]. A single colony of the organism was suspended in 100µl of Milli-Q water in a micro centrifuge tube and boiled at 100°C for 5 min. Then after, it was centrifuged at 9,500 g for 5 min. The supernatant was decanted and used as a template for PCR [23]. Another technique has been used to extract DNA from the reference strains and the blood samples according to the manufacturer's instructions (DNA Isolation Kit for Mammalian Blood, Cat no.: 11667327001). The purity and concentration of the extracted DNA were determined using Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA). The supernatants transferred to new tubes and stored at -20°C.

2.6 Gene specific primers

Table 3 represents primers used in the conventional and nested PCR [15, 21], respectively. ST1 and ST2 were used and predicted to produce approximately 450-500 bp amplicon size, while NST1 and NST 2 expected to produce 350 bp amplicon size.

Table 3: List of primers targeting *S. Typhi* specific genes for the development PCR assays

Sr. No.	Primer name	Sequence 5′- 3′	Product size	Corresponded to nucleotides
Conventional PCR				
1	ST1-F*	TAT GCC GCT ACA TAT GAT GAG	497 bp	1024 - 1044
2	ST1-R**	TTA ACG CAG TAA AGA GAG		1504 - 1521
3	ST2-F	ACT GCT AAA ACC ACT ACT	458bp	1072 - 1089
4	ST2-R	TTA ACG CAG TAA AGA GAG		1513 – 1530
Nested PCR				
5	NST1-F	ACT GCT AAA ACC ACT ACT	366 bp	1060-1077
6	NST1-R	TGG AGA CTT CGG TTG CGT AG		1407-1426
7	NST2-F	AGA TGG TAC TGG CGT TGC TC	343-bp	1092 – 1111
8	NST2-R	TGG AGA CTT CGG TCG CGT AG		1416 - 1435

The flagellin gene of *S. typhi*; * F: Forward; **R: Reverse

2.7. Detection of specific genes using ST1, ST2, NST1 and NST2 by PCR amplification

For conventional PCR, a 25 µl amplification mixture containing 10µl of commercial master mix (Bangalore Genei, Bangalore), 7 µl of MilliQ water, 2 µl Primers (Forward and reverse primers, ST1 or ST2) with 4 µl of the extracted DNA were used. The reaction was carried out in an Eppendorf Mastercycler (Eppendorf, Germany). PCR was performed using the following conditions: Initial denaturation at 94°C for 2 min; annealing at 57°C for 1 min 15 sec; extension at 72°C for 1 min, repeated for 40 times, and final extension at 72°C for 7 min 40 cycles of 2 min each at 94°C for denaturation followed by annealing at 57°C for 1 min 15 sec and extension at 72°C for 1 min. A final extension for 7 min was done at 72°C.

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For nested PCR, a 25 µl amplification mixture containing 10 µl of commercial master mix (Bangalore Genei, Bangalore), 7 µl of MilliQ water, 2 µl Primers (Forward and reverse primers, NST1 or NST2) with 4 µl of the 1 in 5 diluted amplified products from the regular PCR was used. Amplification conditions were similar to the first round PCR with the exception of annealing was done at a higher temperature at 68°C for 1 min 15 sec. The PCR amplification was observed on the 0.8% agarose gel

3. Results

3.1 Widal test flagellar (H) and somatic (O) antigens

The Widal tests results showed that all serum samples [2], except serum of patient 7, were positive test for the somatic O antigen as indicator of acute infection. For H antigen, as an indicator of persistent infection and an effective factor to identify enteric fever, sixteen [24] serum samples were positive, while patients 4, 5, 9 and 10 were negative. Serum samples of patients 4, 5 and 10 were found to be positive for antigen AH while patient 9 showed agglutination for antigen BH only. Interestingly, all serum samples showed agglutination with at least two antigens using the Widal test. Subsequently, the Widal test results revealed that all patients are having *Salmonella typhi* infection. Table 3 shows the result of Widal test of enrolled patients.

Table 4: Results of the serological response with Widal agglutinin of all patients

Patient	Typhi O	Typhi H	Paratyphi AH	Paratyphi BH
1	80±0.1	90±0.3	-	-
2	20±0.4	160±2.5	-	-
3	50±2.4	50±2.6	-	-
4	110±1.5	-	20±0.2	-
5	160±2.3	-	50±0.8	-
6	40±1.9	>350±2.1	-	-
7	-	160±3.0	-	-
8	30±0.2	150±3.1	-	-
9	150±0.9	-	-	30±1.0
10	175±1.5	-	30±0.4	-

3.2 Isolation of DNA from Bacteria and blood Samples

Various strains of *Salmonella* were subjected to DNA isolation as a positive indicator. The bacterial DNA isolation from various strains of *Salmonella typhi* was successful from all reference strains 1-7. *Salmonella typhi* ATCC 19430 was used as a positive reference. All

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the strains showed more than 5kb DNA. However, we used the DNA isolation kit to ensure successful isolation of the bacterial DNA. The DNA isolation was performed from the blood samples using the same DNA isolation kit, it was successful. Patients and healthy samples showed maximum amount of isolated DNA which was further used for the gene specific amplification.

3.3 PCR amplification of

3.3.1. Amplification of S. Typhi flagellin gene using ST1 and ST2 primers

Using ST1 and ST2 primers targeting flagellin gene, 497-458bp fragments were obtained of the amplification PCR. Figure 1 depicts the PCR amplified product of ST1 and ST2 flagellin gene (458bp). Patient samples 2, 4, 5, 6, 8, 9 and 10 showed amplification of flagellin gene. Patient samples 1, 3 and 7 along with healthy control didn't show any amplification of flagellin gene. Although the patients with typhoid symptoms, their PCR test was negative.

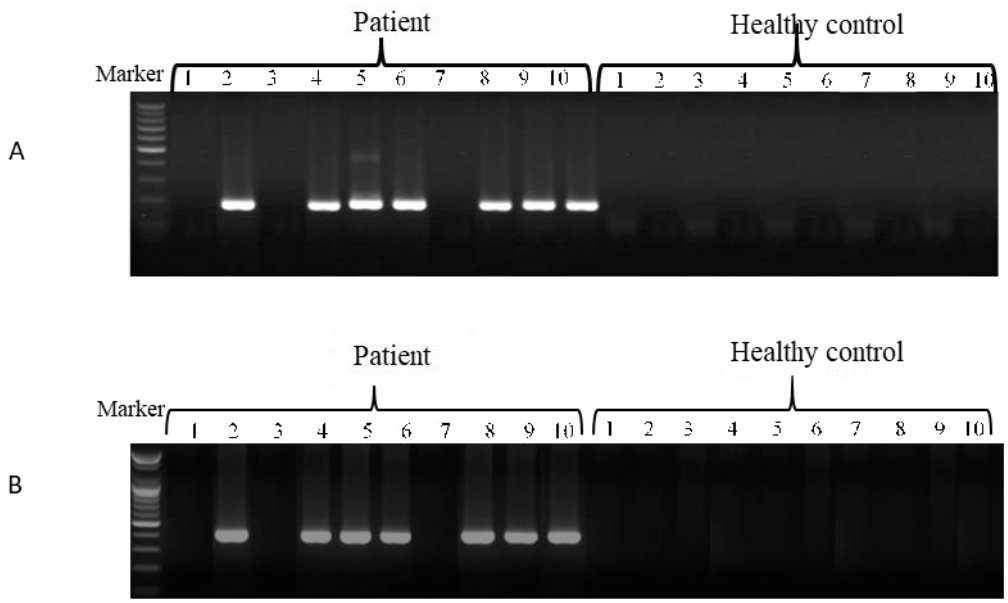

Figure 1: Flagellin genes ST1 and ST2 detection in patients and healthy controls. Marker: 1Kb DNA marker; Patient samples 2, 4, 5, 6, 8, 9 and 10 showed amplification of flagellin gene.

3.3.2. Amplification of flagellin gene using NST1 and NST2 primers

Using primers targeting interested regions of flagellin gene, fragments of 366-343bp were observed on the gel-agarose. Figure 2 depicts the PCR amplified products using NST1 and NST2 primers.

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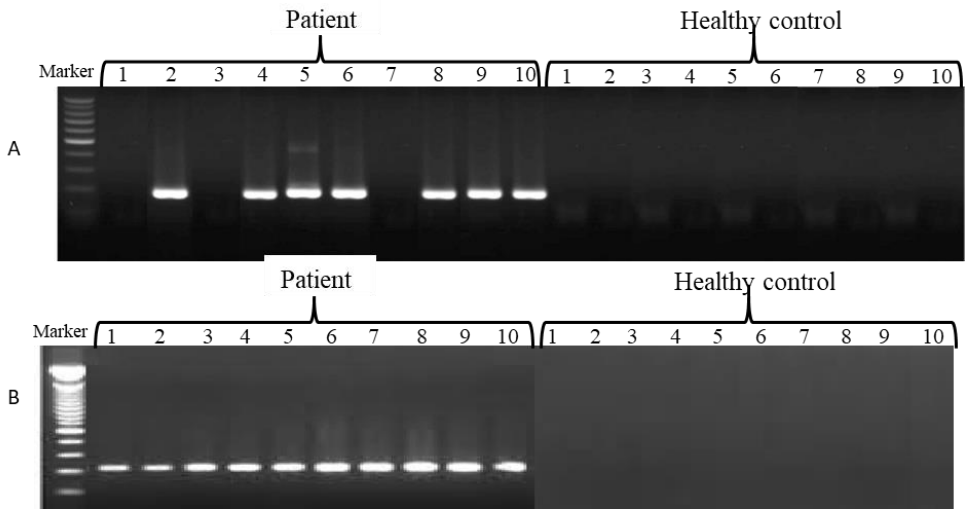

Figure 2: Flagellin gene NST1 and NST2 detection in enrolled patients and healthy controls. Marker: 5Kb DNA marker; About 343 bp amplification was observed on 0.8% agarose gel.

4. Discussion

Typhoid fever is a bacterial infection caused by Salmonella Typhi results in mild to severe symptoms [36] depending on the serotype and usually appear after 6-30 days of exposure [25]. A high fever usually develops gradually over several days. Some people might get the infection, but they are asymptomatic, yet they can still spread the disease to others. Typhoid fever, like paratyphoid fever, is an intestinal fever [36]. The length of the *S. typhi* flagellar antigen gene (Hl-d) is approximately 1.53kb [26, 24]. Although antigen d is found in many Salmonella species other than *S. typhi* [27, 28]. The flagellin gene of *S. typhi* possesses unique nucleotide sequences in the hypervariable region of the gene [24]. The nucleotide sequences and predicted amino acid sequences of region VI (corresponding to nucleotides 969 to 1077) of the S. Type HJ-d flagellin gene differs from those of S. muenchen, which likewise contains the HJ-d gene and nucleotide sequences that are substantially similar to those of *S. typhi* [24, 26]. These results suggested that a PCR-based detection of *S. typhi* flagellin genes from clinical specimens is an essential key in the diagnosis.

PCR-based molecular detection is a fast procedure with high sensitivity and specificity for instant and precise detection and identification of specific Salmonella Spp. However, various PCR methods are available for the Salmonella spp detection with variable sensitivity and specificity.

In this study, we investigated the Salmonella detection using traditional sero-diagnostic method (Widal test), and molecular detection methods (traditional and nested-PCR) [15, 21] in clinical samples collected from Salmonella diagnosed patients in Pune, India. To evaluate these diagnostic tools, we compared the diagnostic accuracy using these methods.

We detected 7 out of 10 samples were positive for Salmonella using PCR approaches published before [15, 21] . The results revealed that targeting the flagellin gene enabled specific detection of *S. Typhi* using conventional PCR. Nested-PCR, in which NST1 and NST2 were targeted interested regions of flagellin gene, the results showed specific detection of *S. Typhi*. *S. muenchen* is a rare cause of gastroenteritis that can be distinguished

from typhoid fever based on clinical signs and symptoms. The PCR technique can distinguish between various acute febrile disorders and *S. typhi* infection. So, this technique demonstrated that it can be utilized to increase the PCR specificity in clinical practice.

PCR-based detection method of *S. typhi* has been the focus of several research [21, 15]. Hatta and Smits [29] showed that PCR is a sensitive approach for diagnosing typhoid fever. As a result, for typhoid fever detection, we employed patient's blood serum. Khan [29] reported the identification of the flagellin gene in *S. Typhi* using PCR approach. The last study comprised 80 patients with a clinical diagnosis of typhoid fever and 40 healthy controls. The result showed that the conducted PCR from blood samples had a sensitivity of almost 100% and a specificity of 76.9%. The PCR's positive predictive value (PPV) was calculated to be 76.9%, with an accuracy of 86%. In the present study, we evaluated the sensitivity of the nested PCR using blood samples from suspected typhoid patients, as well to determine whether it could be utilized to detect *S. typhi* DNA from those samples. The results were compared to blood culture, which is the simplest detection method of salmonella, and the Widal test. Interestingly, the results are in line with Khan et al. (2012) [15] study, which proved high sensitivity and specificity of the used nested-PCR.

Nevertheless, the sensitivity of the nested PCR appears to be higher than the blood culture sensitivity [29], which is similarly indicated in our study. Positive PCR results of blood samples from culture-negative typhoid patients were consistent with prior findings indicating the same nested PCR, as well as other PCRs for typhoid fever, may have higher sensitivity than blood culture [30]. Prakash [30] and others utilized the same nested PCR but a different DNA extraction method, reporting a blood culture sensitivity of 29.8% and a nested PCR sensitivity of 92.8 percent. Subsequently, he PCR can be used to confirm the clinical diagnosis of typhoid fever in patients with suspected clinical symptoms of typhoid fever, such as high fever, leukopenia, and hepatosplenomegaly.

5. Conclusions

PCR can be used as a promising, fast, and precise technique for the early detection of Salmonella infection. So that correct and specific treatment can be implied. The PCR approach and primers evaluated in this study have vast possibility for specific detection of Salmonella typhoid. On further simplification, this protocol can be used for specific detection of typhoidal Salmonella in clinical application due to its high sensitivity and specificity to diagnose clinically suspected, culture negative cases of typhoid fever. The sensitivity of PCR and its potential use in routine diagnosis and epidemiological studies of typhoid fever can be exploited to complement studies by serum samples. Nevertheless, the evaluation of Salmonella detection methods conducted in this study clearly indicated the accuracy and ease of use of the PCR assay compared to the rest of the traditional methods, which are sometimes inaccurate.

Supplementary Materials: No supplementary materials.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, A.O.H.; methodology, A.O.H., P. M., H.A.; validation, A.O.H., T.A.K., P. M.; formal analysis, A.O.H., H.A., T.A.K., S.H.A., A.M.S.A., N.H.I., R.N.T., M.H.G., T. M. S., Y.A.N.A, P. M.; investigation, A.O.H., H.A., T.A.K., S.H.A., R.A., T.M.S., A.M.S.A., N.H.I., P. M., R.N.T., M.H.G., Y.A.N.A.; resources, A.O.H.; data curation, A.O.H.; writing—original draft preparation, A.O.H. and H.A.; writing—review and editing, A.O.H., H.A., T.A.K., S.H.A., A.M.S.A., N.H.I., R.A., R.N.T., M.H.G., Y.A.N.A., T. M. S., P. M.; project administration, A.O.H. All authors have read and agreed to the published version of the manuscript.

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