

# Persistence of anti-Salmonella O9 IgM as measured by Tubex® TF may contribute to the over-diagnosis of typhoid fever in endemic areas



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## ABSTRACT

**Background:** Diagnosing typhoid fever definitively, using bone-marrow, blood or feces culture is either invasive, time-consuming, expensive, or complicated. Hence, clinicians often use clinical presentations and/or rapid diagnostic methods to examine the indirect evidence of *Salmonella typhi* infections. This study describes the proportion of typhoid fever, challenges of diagnosing typhoid fever, and the kinetics of anti-Salmonella O9 IgM.

**Methods:** During a prospective dengue study in Bandung, Indonesia, 1,431 febrile occurrences were observed among 2,978 adult volunteers over a four-year period. As typhoid fever is endemic and represents a major cause of febrile illness in this study area, acute- and convalescent-phase sera from 964 subjects that had been excluded for dengue and chikungunya infections, were tested with Tubex® TF. To observe the kinetics of anti-Salmonella O9 IgM, we tested sequential sera up to 260 days post-illness from 3 subjects.

**Results:** Based on Tubex® TF, 71 of 964 (7.4%) subjects had anti-Salmonella O9 IgM (score ≥ 4) in their convalescent sera. Anti-Salmonella O9 IgM scores in convalescent sera increased compared to the acute sera in 36 subjects, indicating probable acute typhoid fever. The convalescent scores decreased/remained the same as acute sera in the other 35 subjects, which more likely indicating previous *S. typhi* infections. Using serially collected sera, it was determined that anti-Salmonella O9 IgM could be detected for a long period after illness.

**Conclusion:** Tubex® TF test should be conducted using paired acute- and convalescent-phase sera to reduce the chance of false positive result that may be due to long-lasting anti-Salmonella O9 IgM.

**Keywords:** persistence, typhoid fever, Tubex® TF, Indonesia.

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## INTRODUCTION

Typhoid fever is a systemic infection caused by *Salmonella enterica* serovar Typhi.<sup>1-3</sup> The infection can manifest as a mild to severe illness depending on various factors, such as age, bacterial strain, current intake of H<sub>2</sub> blockers, and co-infection with HIV or *Helicobacter pylori*.<sup>4-6</sup> In a mild or acute non-complicated case, the disease is characterized by fever, malaise, constipation (adult) or diarrhea (children), frontal headache, nausea, and anorexia with slight dry cough.<sup>2,6</sup> In approximately 5-30% of patients, rose-colored spots appear on the chest and abdomen and sometimes on the back and limbs.<sup>1-3</sup> Up to 10% of typhoid fever cases develop into a severe or complicated disease

characterized by increased abdominal discomfort, peritonitis, intestinal perforation, and blood in the stool. Since there are no specific clinical signs and symptom, the diagnosis of typhoid fever requires confirmation by a laboratory test.<sup>2</sup>

Although direct blood culture is more commonly used, bone marrow culture remains the gold standard test.<sup>1</sup> The bacteria can also be cultured from stool, urine, and the skin nip of the rose-colored spots.<sup>7</sup> However, because culture is a time-consuming, costly, and not always a successful procedure, and molecular test using blood is insensitive,<sup>8,9</sup> serological tests have become more widely used, especially in resource limited countries.

The Widal test is a classical serological

test for typhoid diagnosis, but this test has several limitations including a lack of standardization and need to maintain the consistency of the antigen being used.<sup>10</sup> To resolve these issues, several point-of-care tests such as the Tubex® TF rapid diagnostic test have been developed. This test is a particle-inhibition immunoassay that detects anti-Salmonella O9 IgM.<sup>11</sup> In two separate prospective trials in Vietnam and Bangladesh, the Tubex® TF test demonstrated better sensitivity (78-91% vs 64-82%) and specificity (82-94% vs 58-74%) compared to the Widal test.<sup>12,13</sup> Based on a Cochrane review, meta-analyses showed an average sensitivity of 78% (95% CI 71 to 85%) and specificity of 87% (95% CI 82% to 91%) for Tubex®

TF test.<sup>14</sup> The Tubex<sup>®</sup> TF specifically detects anti-Salmonella O9 IgM whereas the Widal test detects both IgM and IgG<sup>8</sup> and the O9 is a common O chain antigen found on the lipopolysaccharide layer of *S. typhi* that discerns it from >99% of other Salmonella subtypes.<sup>15,16</sup>

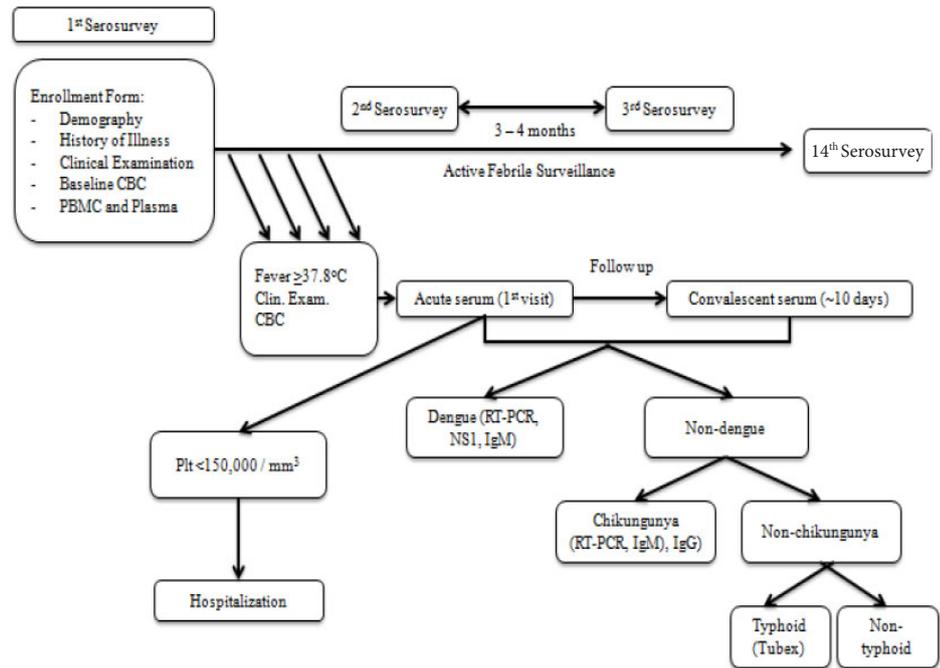
During prospective dengue cohort study conducted from 2000 to 2004, 1,431 febrile episodes occurred among 2,978 adult volunteers enrolled, with 273 of febrile episodes diagnosed as dengue or chikungunya infections.<sup>17</sup> The remaining 1,158 febrile episodes were undiagnosed.<sup>17</sup> The results of a case study in some large hospitals in Indonesia shows an increasing trend in the number of typhoid cases from year to year with an average rate of 500/100,000 population and the deaths are estimated at 0.6–5%.<sup>18</sup> Hence, we would like to determine the incidence of typhoid fever in the cohort retrospectively and the kinetics of anti-Salmonella O9 IgM levels, and subsequently provided further insight into the performance of Tubex<sup>®</sup> TF in diagnosing typhoid fever.

## METHODS

### Sample Collection

The samples used in this study were collected for “An epidemiology study of dengue and dengue hemorrhagic fever in adults”, study approved by the Institutional Review Board of NAMRU#2, Jakarta (IRB#30855) and National Institute of Health Research and Development (NIHRD), Ministry of Health, Indonesia (KS 02.02.2.1.2181, KS 02.01.2.1732 and KS.02.01.2.1.2776) in compliance with all U.S. Federal Regulations governing the protection of human subjects and the Declaration of Helsinki (1975), as revised in 2000.

The study sites, populations, and design of the study have been described in detail previously.<sup>17,19</sup> Briefly, a total of 2,978 employees from two textile factories located in Bandung, participated in this study from September 2000 to June 2004. The age of the participants ranged from 18 to 66 years old, with the majority (90%) was 18–45 years old. Blood samples were collected for sero surveillance at enrollment and then every 3–4 months. Pre-illness sera were serosurvey sera collected prior to acute febrile illness



**Figure 1.** Overall study design

sera. Volunteers who experiencing fever visited the clinic for evaluation and blood sample was collected. The acute-phase specimen was collected during the first visit and a convalescent-phase specimen was collected at least 7 days later. Sera were aliquoted and then stored at -70°C for analysis. The overall study design is shown in [Figure 1](#).

Each participant provided written informed consent prior to participation and agreed to allow their samples to be used in the future for other infectious disease studies. The testing presented in this study was conducted using de-identified specimens and was determined to be non-human subjects research by the NAMRU-2 IRB.

### Tubex<sup>®</sup> TF Rapid Typhoid Test

The Tubex<sup>®</sup> TF (IDL Biotech AB, Bromma, Sweden) assay was performed according to the manufacturer’s instructions. Briefly, reagents were incubated with sera and mixed with indicator antibody bound to particle. The resultant color of the supernatant mixture was scored against a color scale standard ranging from 0 to 10 (provided by manufacturer). A result score  $\leq 2$  was negative for anti-Salmonella O9 IgM, while a result score  $\geq 4$  was positive. Two independent readers read the test

results within 30 minutes after incubation. When they did not agree, the assessments from the third reader decided the final scores.

### Testing Algorithm and Case Definition

During the duration of the study, 1,431 febrile illness episodes were observed, 273 of these episodes were confirmed as either dengue or chikungunya infections, leaving 1,158 undiagnosed episodes. Of those 1,158 cases, 964 had both acute and convalescent samples available for analysis. The convalescent samples from these 964 cases were tested with the Tubex<sup>®</sup> TF and when a positive score ( $\geq 4$ ) was obtained, the corresponding acute sample was also tested. When the acute sample Tubex<sup>®</sup> TF score was lower than the convalescent sample score, the case was categorized as a probable typhoid infection. When the acute sample Tubex<sup>®</sup> TF score was equal to or greater than the convalescent sample score, the case was categorized as a previous typhoid infection. Available pre-illness sera from these cases were also tested to see the score difference with the acute sera. Patients with a negative Tubex<sup>®</sup> TF score in their convalescent sample were categorized as non-typhoid fever illness. The kinetics of anti-Salmonella O9 IgM was followed in 3 subjects whose acute

score was <2 and convalescent sera >6, and had serial sera up to 260 days.

### Statistical Analysis

Data was analyzed using Stata® software version 12.0 (Stata Corp., Tx). The difference in the number of days from the onset of fever to the collection of acute and convalescent samples among probable, previous, and non-typhoid groups was compared using the Kruskal-Wallis test. The difference of age and sex between groups done with the chi-square test.

## RESULTS

### Probable Typhoid, Previous Typhoid, and Non-typhoid Cases

Tubex® TF test from 964 convalescent samples resulted in 892 negative samples and 71 (7.4%) positive samples (Tubex® TF score  $\geq 4$ ). Further testing was performed on the corresponding acute samples of these positive samples which were taken at the mean of 3.26 ( $\pm 1.35$  SD) days post-fever onset (ranging from 2 to 8 days). From these 71 paired samples, 36 (3.7%) were categorized as probable typhoid infections and consisted of 32 seroconversion cases (Tubex® TF score <4 in acute and  $\geq 4$  in convalescent samples) and four cases with positive Tubex® TF scores in both acute and convalescent samples but the convalescent sample score was greater than the acute sample score. The remaining 35 (3.6%) cases were categorized as previous typhoid infections where in 26 cases both acute and convalescent samples had the same score and in 9 cases the convalescent samples scores were lower than the acute samples.

Corresponding pre-illness samples were also tested in 35 subjects and consisted of nine probable typhoid cases with only a slight increase in scores between acute and convalescent samples and 26 previous typhoid cases. All pre-illness samples Tubex® TF score in the probable typhoid cases were lower than its corresponding convalescent samples (Table 1). On the other hand, in the previous typhoid cases, in all but two case, the pre-illness samples scores were higher or the same as its convalescent samples (Table 2). The two cases that had pre-illness scores 2 and 3, but the acute and convalescent scores were the same (4). The Tubex® TF scores of pre-illness sera from probable typhoid

**Table 1.** Tubex® TF scores from pre-illness, acute, and convalescent samples from probable cases.

No	Tubex Score			Duration of (days)	
	Pre-illness	Acute	Convalescent	Pre-illness to acute	Acute to convalescent
1	0	2	4	50	12
2	0	3	4	64	10
3	2	3	5	61	15
4	2	2	4	21	11
5	2	1	4	61	13
6	2	3	4	53	10
7	2	1	4	109	10
8	2	3	4	13	11
9	4	6	10	133	9

**Table 2.** Tubex® TF scores from pre-illness, acute, and convalescent samples from previous cases.

No	Tubex Score			Duration of (days)	
	Pre-illness	Acute	Convalescent	Pre-illness to acute	Acute to convalescent
1	8	6	6	68	11
2	8	4	4	80	19
3	6	4	4	77	12
4	8	7	7	13	10
5	6	6	6	208	11
6	10	10	10	2	11
7	10	10	10	29	10
8	6	4	4	163	4
9	4	4	4	12	10
10	4	4	4	97	10
11	4	4	4	59	11
12	6	4	4	104	17
13	4	4	4	74	10
14	2	4	4	111	10
15	6	4	4	63	10
16	4	4	4	10	10
17	6	6	6	18	12
18	6	4	4	2	12
19	6	4	4	9	10
20	4	4	4	81	18
21	3	4	4	138	15
22	5	6	4	113	13
23	6	5	4	157	17
24	8	6	4	57	13
25	8	8	6	121	15
26	10	8	5	65	10

**Table 3.** The demographic of probable, previous, and non-typhoid cases.

Typhoid Disease Category	N	Age			Sex (Male : Female)	Acute days after onset			Convalescent days after onset		
		Mean [SD]	Median [IR]	Range		Mean [SD]	Median [IR]	Range	Mean [SD]	Median [IR]	Range
Probable	36	34.7[6.8]	34.0[10.3]	25-47	1:1.1	3.4[1.6]	3.0[2.0]	2-8	14.7[2.5]	14.5[3.0]	7-19
Previous	35	28.3[5.2]	28.0[5.0]	19-45	1:3.4	3.2[1.1]	3.0[2.0]	2-6	15.3[3.0]	15.0[5.0]	8-21
Non-typhoid	893	34.4[7.0]	34.0[10.0]	19-57	1:0.7	3.0[1.3]	3.0[1.0]	2-11	14.8[2.6]	14.0[3.0]	7-23

cases ranged from 0-2, while the range for previous typhoid cases was 2-10. As these samples were collected 1-3 months before the acute fever, these samples representing healthy individuals. The median of days from pre-illness to acute in probable vs previous cases was 61(IR 50-64) vs 71(IR 20.75 – 109.25), while the days from acute to convalescent between these two groups was 11(IR 10-12) vs 11(IR 10-13) respectively.

#### Demographics, Clinical Manifestations, and Laboratory Findings

The mean age of patients in the previous typhoid infection group was statistically lower than the probable typhoid and non-typhoid groups ( $P < 0.05$ ). The previous typhoid infection group had significantly more females than males compared to probable typhoid or non-typhoid groups ( $P < 0.05$ ). Female was significantly more common in previous typhoid groups compared to non-typhoid group ( $P < 0.05$ ). There was no significant difference in the time after illness onset to sample collection among these three disease categories (Table 3).

In addition to fever, the most commonly reported symptoms in the probable acute infection cases were headache (92%) and myalgia (83%). Leucopenia was found in only one case. The mean leukocyte and platelet counts upon presentation were 6,933 [SD 2,306] cells/mm<sup>3</sup> and 238,833 [SD 79,408] cells/mm<sup>3</sup>, respectively. The clinical characteristics of the 36 probable typhoid infections are shown in Table 4. Three patients were hospitalized, with one experiencing spontaneous hemorrhage.

#### The Kinetics of Anti-Salmonella O9 IgM

In order to see the kinetics of anti-Salmonella O9 IgM, acute, convalescent, and post-illness serosurvey specimens

**Table 4.** Signs, symptoms, and laboratory findings from probable typhoid cases.

Signs and symptoms	Typhoid cases
Headache	92% (33/36)
Myalgia	83% (30/36)
Nausea	58% (21/36)
Retro orbital pain	44% (16/36)
Cough	44% (16/36)
Abdominal pain	36% (13/36)
Coryza	31% (11/36)
Sore throat	25% (9/36)
Diarrhea	22% (8/36)
Vomitus	11% (4/36)
Tourniquet test	39% (14/36)
Rash/erythema*	4% (1/28)*
Anemia	16.7% (6/36)
Platelet < 150,000/mm <sup>3</sup>	6% (2/36)
Leucocyte < 4,000/mm <sup>3</sup>	3% (1/36)
SGOT > 35	30% (10/33)*
SGPT > 36	27% (9/33)*

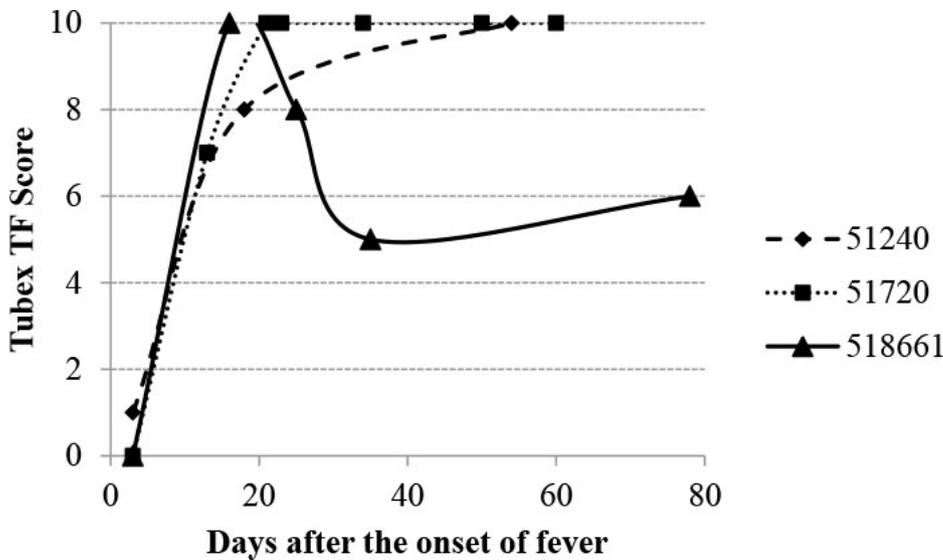
\*The total number of cases collected from available participant/case report form

from three probable cases, whose acute score was <2 and convalescent sera >6, were tested using the Tubex<sup>®</sup> TF, up to 260 days post-infection. Kinetic profiles for all three patients were similar for the first 16-21 days; with a rapid increase in Tubex<sup>®</sup> TF score. In two cases (#51240 and #51720) the levels of anti-Salmonella O9 IgM remained elevated, as reflected by Tubex<sup>®</sup> TF scores of 10, 54-60 days post-fever onset. One (#51720) of these two still had high score until 193 days post-fever onset, while the other had slowly declining, but still positive Tubex<sup>®</sup> TF scores at 260 days post-fever onset. For the third case (#518661), the Tubex<sup>®</sup> TF score declined

at 25 days post-fever onset, but remained positive at 78 days post-fever onset. These results demonstrate that anti-Salmonella O9 IgM can persist for extended periods of time following acute infection (Figure 2).

## DISCUSSION

Using paired sera, our data demonstrated distinct patterns of acute and convalescent Tubex<sup>®</sup> TF scores that could be used to categorize the febrile cases as probable, previous, and non-typhoid cases. The proportion of probable *S. typhi* infections in 964 adults with acute fever was 36 (3.7%) using sero-conversion of Tubex<sup>®</sup>



**Figure 2.** The kinetics of anti-Salmonella O9 IgM from three probable typhoid cases.

TF scores (from  $<4$  to  $\geq 4$ ) or increased scores in those with acute  $\geq 4$  score. This number is not different than previous reports based on the results of the blood culture but less than results based on serological assays.<sup>20,21</sup> As we use the strict criteria for diagnosis, the prevalence was close to blood culture and it was expected. Unfortunately, the Tubex<sup>®</sup> TF has a high cross-reactivity to *Salmonella paratyphoid* A infection.<sup>22</sup> As such, a proportion of the probable cases in our study may have been paratyphoid A infections instead of typhoid fever.

Our study highlighted the importance to carefully interpret the results of Tubex<sup>®</sup> TF as in approximately the same proportion (3.7%) of febrile patients had Tubex<sup>®</sup> TF score  $\geq 4$  both in acute and convalescent samples (previous typhoid infection), and further exploration in a subset of patients showed that pre-illness Tubex<sup>®</sup> TF  $\geq 4$  had been detected approximately 1-3 months before febrile episode. The reasons are described in the kinetics section below. The possibility that anti-Salmonella O9 IgM has not risen in the convalescent was small since the convalescent sera was collected at average 10 days after acute sera (average of 14 days after onset of fever). As anti O9 IgM was negative in convalescent sera of 92.6% subjects with acute fever, we may conclude that the specificity of Tubex<sup>®</sup> TF was considered good. According to our probable cases, clinical symptoms of typhoid infection in the early phase of

illness are similar to other major infectious diseases and consists of non-specific symptoms such as fever, chills, headache, malaise, anorexia, nausea, abdominal pain, a dry cough, or myalgia.<sup>2</sup> Our results showed that the symptoms of probable typhoid infections were in accordance with common symptoms in the early phase. Leucopenia was only found in one case, contrary to the results of hospitalized patients in Turkey and Ghana which showed that 53% and 12% of positive typhoid fever cases had leucopenia, respectively.<sup>23,24</sup> However, as our typhoid cases were outpatients, leucocyte counts were not conducted daily.

To our knowledge, our study was the first to follow the kinetics of anti-Salmonella O9 IgM for extended periods after infection, starting after 3 days of fever while anti O9 IgM was still not detected until almost 10 months later. Our findings show that anti-Salmonella O9 IgM continued to rise up to three weeks after the onset of fever and remained positive for 6-8 months post-illness. The persistence of anti-Salmonella O9 IgM may occur due to the antibody responses to thymus-independent antigen (polymeric antigen such as polysaccharides), in which the B cells mainly produce IgM, but little or no IgG.<sup>25,26</sup> An alternative explanation is the development of a carrier state in those individuals. Previous reports indicate that 1-5% of typhoid patients become carriers as the failure of immune response in

clearing the infection within a year will lead into chronic carrier state where the bacteria remain primarily in hepatobiliary tract and gallbladder.<sup>27,28</sup> This may well explain why the Tubex<sup>®</sup> TF score in some of our patients remained positive for extended periods of time. Interestingly, members of the previous infection group are predominantly female with a male to female ratio of 1:3.4; whereas the male to female ratio in the total volunteers was 1:0.7 and in probable typhoid patients was 1:1.1. This finding indirectly supports a previous study that showed females are more prone to be carriers for *S. typhi*, which might be associated with cholelithiasis which is also more predominant in females.<sup>27,28</sup>

Based on the results presented here, we suggest using paired sera for the Tubex<sup>®</sup> TF test in order to avoid over diagnosis of typhoid infection and to distinguish between acute infection and previous infection with persistent anti-Salmonella O9 IgM that might be associated with carriers. Our study also revealed that using paired sera in the Tubex<sup>®</sup> TF test may avoid false negative for patients when specimens collected too early during the acute phase of illness. If we had only tested acute febrile samples, we would have missed 32 out of 36 probable cases and misdiagnosed the 35 previous cases as a recent typhoid fever. Therefore, it is advisable not to use a Tubex<sup>®</sup> TF score  $\geq 4$  obtained from a single sample as the sole criterion for typhoid infection, especially in female patients and in endemic areas.<sup>27,28</sup>

The limitation of our study was the inability to perform blood and/or bone marrow culture, which is the gold standard for typhoid diagnosis. Due to this limitation, we could not call cases with increasing tubex TF scores as 'confirmed' cases, we were not able to calculate the sensitivity and specificity of the Tubex<sup>®</sup> TF test, and our results should be interpreted carefully.

## CONCLUSION

The Tubex<sup>®</sup> TF is a simple and rapid test but share similar limitations with other serological tests especially when used in endemic settings. Females are predominantly found in previous typhoid cases and are more prone to be carriers for *S. typhi*. It is recommended to use paired

specimens; since using a single specimen may lead to under or over diagnosis, a situation that could lead to inappropriate treatment for patients. To overcome the invasive and insensitive problems of bone marrow and blood culture, a more accurate and reliable test is needed to diagnose typhoid fever at the early stage of infection since serological tests, including the Tubex<sup>®</sup> TF, are not viable options.

## CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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## AUTHOR CONTRIBUTION

Conceptualization and methodology, IWAP, AD, MRH, NL, HK; data collection, IWAP, AD, MRH, NL, HD, DPRB; analysis and writing, IWAP, AD, MRH, NL, HK; review, IWAP, AD, MRH, NL, HK, SW, BA. All authors have read and agreed to publish the manuscript.

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