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ORIGINAL ARTICLE

Evaluation of a rapid diagnostic test for detection of dengue infection using a single-tag hybridization chromatographic-printed array strip format

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Abstract

A dipstick DNA chromatography assay, a single-tag hybridization-printed array strip (STH-PAS), was evaluated for its efficacy to detect dengue virus (DENV). Reverse-transcribed DNA was amplified by PCR, and the amplified DNA was detected using the STH-PAS system. The method was evaluated using stored RNA samples previously identified to carry all 4 serotypes of dengue, chikungunya, and influenza viruses. Clinical performance was also assessed in a prospective study using plasma from 269 febrile cases from the Emergency Department of St. Luke's Medical Center, Quezon City, Philippines, and 30 afebrile normal healthy volunteers. A Taqman real-time PCR (RT-PCR) assay and a rapid Dengue NS1 test, SD Bioline, were used for comparison. The STH-PAS system was more sensitive in detecting dengue infection compared to Taqman RT-PCR. For DENV serotypes 1, 2, and 3, the detection was 1 to 2 dilutions (10-fold) higher, and for DENV serotype 4, the detection was 2–4 dilutions higher. In clinical studies, the STH-PAS system showed 100% sensitivity with 88.9% and 86.6% specificities compared to Taqman RT-PCR and SD Dengue Duo NS1 test, respectively. The STH-PAS system was found to have a superior sensitivity than the Taqman system. Further evaluation of its performance in the field may provide important data to extend its usefulness for surveillance and epidemiological research in outbreak situations.

Keywords Dengue · Rapid diagnostics · Polymerase chain reaction · STH-PAS

Introduction

Dengue is one of the most important re-emerging infectious diseases affecting tropical and subtropical regions of the world. The incidence of infection caused by dengue virus (DENV), which is transmitted to humans through the bite of an infected mosquito, is increasing and expanding in terms of geographical range, with an estimated 390 million infections every year [1] and 3.9 billion people in 128 countries at risk of infection [2]. Dengue is classified as dengue without warning signs or dengue with warning signs or severe dengue based on the WHO Dengue Guidelines for Diagnosis, Treatment, Prevention and Control 2009 [3, 4]. Its manifestations overlap with those of other microbial infections and an accurate clinical diagnosis requires confirmation by laboratory tests. Physicians in countries in which these diseases are not

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endemic are often unfamiliar with the symptoms and unaware of the potential for importation of DENV via the movement of infected travelers. In such cases, dengue is often not considered part of a differential diagnosis.

In many dengue-endemic areas, laboratory diagnosis is hampered by the lack of proper services for the timely collection and transport of requisite blood specimens, as well as the availability of modern facilities with the capability to conduct molecular diagnostics for dengue. Several laboratory diagnostic techniques are used for the confirmation of DENV infection: viral isolation by in vitro culture in mosquito cells, viral antigen detection, viral nucleic acid detection, and anti-dengue virus antibody (Ab) detection. Current laboratory methods target the viremic phase, which occurs upon introduction of the virus, the succeeding acute to critical stages when early IgM antibodies begin to circulate, and the convalescent phase when long-term IgG antibodies start to appear. Nucleic acid–based amplification, enzyme-linked immunosorbent assay (ELISA), and dengue nonstructural protein 1 (NS1) antigen detection techniques are commonly used. The antibody and NS1 immunoassays are limited in terms of sensitivity and specificity [5, 6] compared to nucleic acid–based reverse transcriptase PCR (RT-PCR). The latter's sensitivity has been further improved with the use of various RT-PCR platforms; however, this requires expensive equipment and reagents that are beyond the resources of laboratories at the frontline [7].

In this study, we used and evaluated a novel dengue testing technology in which amplified DNA was detected by a dipstick DNA chromatography assay known as the single-tag hybridization-printed array strip (STH-PAS) system. It runs on a multiplex reaction system that can detect several amplified DNA fragments of target genes in one strip, such as those belonging to food-borne pathogens [8], oral microbiota [9], or shrimp DNA and RNA virus–induced diseases [10, 11]. It allows for the rapid visualization of the signals of the gene product amplified by polymerase chain reaction (PCR) in a single reaction with great sensitivity. Moreover, it does not require the preparation or staining of a gel or the use of other apparatus. In this study, we evaluated whether this approach could also be applied for the detection of dengue viruses using reverse-transcribed DNA template for its sensitivity and specificity in the differential detection of dengue against other viral febrile illnesses, such as chikungunya fever and influenza. We also assessed its use as an aid in the clinical diagnosis of dengue among febrile cases at the Emergency Department for appropriate treatment.

Materials and methods

Retrieval of samples for retrospective study

Stored RNA samples that were previously identified as infected with DENV serotypes 1, 2, 3, and 4, chikungunya (CHIKV), or influenza (INFV) viruses were selected and retrieved from storage at − 80 °C.

The samples were identified as carrying either chikungunya virus (from serum) or influenza virus (from nasal swab) by RT-PCR of the envelope 1 (E1) and nonstructural protein 1 (nsP1) genes for CHIKV and matrix (M) genes for INFV, respectively, in previous studies. Ethics approval for access to these samples for the retrospective study was given as indicated below in the "Patient enrollment and specimen collection for the prospective study" section. These were used to prepare the cDNA template for RT-PCR assays as described below in the "Dengue detection by nucleic acid chromatography and single-tag hybridization-printed array strip assay" and "Taqman real-time PCR" sections. The operators performing the RT-PCR assays were blinded as to the source and identity of the samples.

Patient enrollment and specimen collection for the prospective study

The study was approved by the Institutional Ethics Review Committee, EC No. CT-17010, and conducted in St. Luke's Medical Center, Quezon City, Philippines. Patients from the Emergency Department (Adult and Pediatrics Units) were evaluated by clinicians and research staff based on the following criteria: fever with temperatures ≥ 38 °C of unknown cause within 5 days of fever onset. The samples were collected from February to June 2017. The healthy controls consisted of volunteers who did not present with a fever for the past 8 weeks prior to enrolment into the study. Patient information, which included demographics, clinical presentation, and laboratory test results, were also collected at the same time as blood extraction. Clinical diagnosis of dengue was made using the following criteria: the clinical diagnosis as recorded in the patient's chart, and classification as dengue based on symptoms according to the 2009 WHO criteria as previously described. Thereafter, 5 ml of peripheral blood in EDTA tube were collected, from which plasma was separated and stored at − 80 °C for further analysis.

Viral RNA isolation

Virus RNA was obtained from 140 μl plasma using QIAamp Viral RNA Mini Kit (QIAGEN, GmbH, Germany) according to the manufacturer's instructions, and stored at − 20 °C until use.

Dengue detection by nucleic acid chromatography and single-tag hybridization-printed array strip assay

Two microliters of viral RNA extract were added to 8 μl of OneStep RT-PCR Master Mix (QIAGEN, GmbH, Germany) in combination with the set of primers carrying the tag-spacer sequence and biotin-labeled primer sequence (AVSS Co., Ltd., Japan, Fig. 1). The primers were designed targeting the capsid-pre-membrane (C-prM) gene of the DENV genome, as described previously [12]. The reaction mixture was subjected to programmed amplification using the Veriti™ thermocycler (Applied Biosystems) and was cycled once at 50 °C for 30 min, 95 °C for 15 min, followed by 40 cycles of 94 °C for 20 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension for 10 min at 72 °C.

After RT-PCR, 10 μl of avidin-coated blue beads with developing buffer was added to 10 μl PCR product and allowed to react for 5 min. Then, a single-tag hybridization-printed array strip (STH-PAS, AVSS Co., Ltd., Japan) was dipped into the reaction mixture and the amplified target DNA was allowed to hybridize to the probe region of the strip. A positive result was visualized by the production of a blue color on the reaction line of the strip. Positive controls and no template controls were included in each run. Three replicate runs were provided for each sample, and a consensus score was obtained to signify a positive or negative result (Fig. 1). In Fig. 2, representative images of the STH-PAS results for dengue-negative and dengue-positive samples are shown. Selected STH-PAS reactions were run in parallel with gel electrophoresis, and also compared to the threshold cycle number from the comparator Taqman real-time RT-PCR, to determine the relative sensitivities of the different systems of amplified DNA detection.

Taqman real-time PCR

Amplification by real-time PCR was performed in an ABI 7500 Fast thermocycler using Taqman probe and primers, targeting the 3′-UTR region of the dengue genome, according to Alm et al. [7], with some modifications. First-strand cDNA synthesis was performed using Superscript III reverse transcriptase (Invitrogen) and random primers (Invitrogen), according to the manufacturer's instructions. The PCR mixture was prepared in a 10 μl volume containing 1 μl of cDNA template, 9 μl Taqman GTXpress Master Mix, with primers at 0.9 μM and probe at 0.2 μM final concentrations. Positive controls and no template controls were included in each run. Amplifications with Ct values less than 35 ($Ct < 35$) were considered positive, while values greater than 35 cycles ($Ct > 35$) were considered negative. Each PCR was run in duplicate.

Dengue detection by SD Dengue Duo test

All components of the SD Dengue Duo test and plasma samples were brought to room temperature prior to testing. Dengue NS1 antigen and anti-dengue IgG/IgM were simultaneously detected following the manufacturer's instructions [5]. Briefly, 100 μl and 10 μl of plasma were added to the sample wells of the NS1 and IgM/IgG strips, respectively. Four drops of the antibody assay diluent were added to the assay diluent well of the IgM/IgG strip. The signals in both

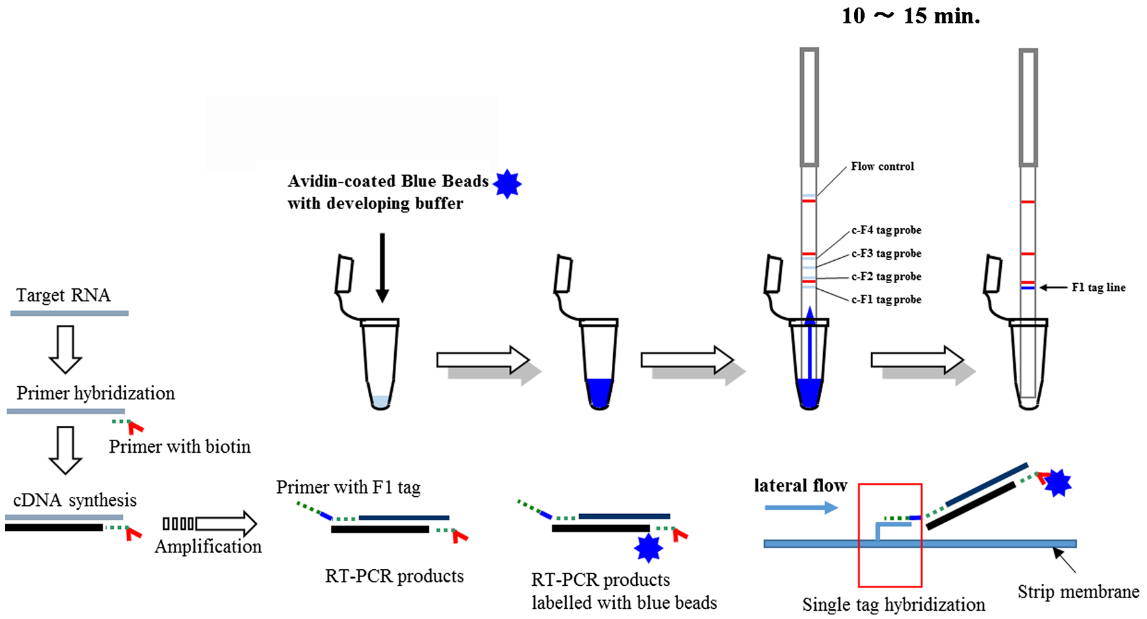

Fig. 1 Schematic diagram of nucleic acid chromatography detection using single-tag hybridization-printed array strip (STH-PAS). cDNA was synthesized from RNA by reverse transcriptase using specific biotin–labeled primer. From the cDNA, PCR amplification was carried out using a primer having a tag-linker sequence. The PCR product was labeled with blue latex. The complementary oligonucleotides of the tag sequence (gray line) were preliminarily printed on the STH-PAS, which develops single-strand tag hybridization between the tag-linker sequence of the PCR product and the STH-PAS

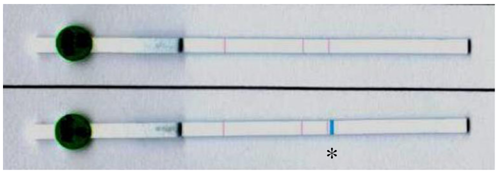

Fig. 2 Images of actual negative (top) and positive (bottom) results of the DENV NAC STH-PAS strips. The asterisk below the blue line shows the positive band

strips of the device were allowed to develop and were read after 15–20 min.

Data analyses and biostatistics

The comparative performances of the diagnostic tests were computed for the dengue nucleic acid chromatography (NAC) and STH-PAS system vs. Taqman real-time RT-PCR, and Dengue NAC and STH-PAS system vs. SD Dengue Duo NS1 rapid test, using the Open Source Statistics for Public Health (www.openepi.com/DiagnosticTest).

Results

Retrospective study

Representative samples, previously identified as carrying DENV serotypes 1, 2, 3, and 4, chikungunya, and influenza viruses, were selected and used in a randomly blinded experiment to determine the analytical sensitivity, specificity, and accuracy of the dengue detection NAC STH-PAS system. All samples of dengue serotypes were collectively identified as dengue, while all dengue-negative and influenza-positive samples were determined to be negative for dengue. Three out of 17 chikungunya-positive sera were mis-identified as dengue by the STH-PAS system, but were dengue-negative by Taqman real-time RT-PCR (Table 1). Based on these results, the Dengue NAC and STH-PAS system exhibited 100% sensitivity, 93.75% specificity, 96.81% positive predictive value, 100.0% negative predictive value, and a diagnostic accuracy of 97.84%.

The limit of detection for the STH-PAS system in comparison with Taqman real-time RT-PCR was also determined using a tenfold dilution series of stored RNA extracts of all four dengue serotypes. The STH-PAS system was more sensitive in detecting dengue infection compared to Taqman RT-PCR: for DENV-1, 2, and 3 by 1 to 2 dilutions higher, and for DENV-4 by 2–4 dilutions higher (results not shown).

Prospective study

The clinical performance of the Dengue NAC and STH-PAS system was determined in a prospective study conducted on 269 individuals who were treated for fever at the Emergency Department of St. Luke's Medical Center in Quezon City, Philippines, and 30 afebrile healthy volunteer controls. The study participants were comprised of 43.9% male and 56.1% female, within an age range of 5–83 years old.

Clinical diagnosis of dengue was made using the following criteria: the clinical diagnosis as recorded in the patient's chart, and classification as dengue based on symptoms according to 2009 WHO criteria, in which dengue is classified according to whether warning signs were present or not, or as severe dengue. Only 41.0% of 217 participants were diagnosed as having dengue, and 59.0% of the febrile cases were diagnosed as having upper respiratory tract infection, systemic viral illness, acute gastroenteritis, or other illnesses.

The dengue virus NAC and STH-PAS system were evaluated for the laboratory diagnosis of dengue infection using plasma samples from the participants. A Taqman-based real-time RT-PCR method modified from the protocol of Alm et al. [7] and a commercial point-of-care rapid test, SD Dengue Duo, were used as comparator assays [5]. The DENV NAC which contains the STH-PAS strip and Taqman real-time RT-

Table 1 Determination of the analytical specificity of the Dengue NAC STH-PAS test using retrospective samples previously identified as carrying dengue virus serotypes 1–4, chikungunya, and influenza viruses

Viral illness previously identified	No. of samples tested	No. of DENV (+) samples by STH-PAS test	No. of DENV (+) samples by Taqman RT-PCR
*DENV-1	26	26	26
DENV-2	21	21	21
DENV-3	22	22	22
DENV-4	22	22	22
DENV (−)	20	0	0
#CHIKV	17	3	0
&INFV	11	0	0
Total	139	94	91

* Dengue virus

Chikungunya virus

& Influenza virus

PCR both detected amplified target regions in the dengue genome by probe hybridization signaling and Taqman probe–mediated notification, respectively. The differential detection of the viral nucleic acid by the STH-PAS system, and the circulating viral nonstructural protein 1 (NS1), anti-dengue IgM, and IgG by the SD Dengue Duo rapid test was also compared. Among the 269 collected samples, 247 samples were processed for all three laboratory methods for dengue detection.

The STH-PAS system gave higher detection rates for both dengue-diagnosed (32.6%) and non-dengue cases (12.5%), compared to Taqman RT-PCR (19.1% and 3.9%, respectively) and the SD Dengue Duo rapid test (13.5% and 3.1%, respectively). In total, 19.0%, 8.9%, and 6.5% were detected by STH-PAS, RT-PCR, and the SD Dengue Duo rapid test, respectively (Table 2). From these data, it was found that 13 (56%) were clinically diagnosed as non-dengue in 23 STH-PAS single-positive cases. Moreover, 16 samples from non-dengue cases and 2 samples from healthy volunteers tested positive for dengue by the STH-PAS system, of which five samples also tested positive by real-time PCR and SD Dengue Duo.

The results of three diagnostic methods among cases that were clinically diagnosed as dengue, as well as for illnesses other than dengue, are listed in Table 3. All three tests were positive in 16 cases, strongly indicating dengue infections. However, 4 cases were diagnosed to be infections other than dengue (25%) (Table 3). For this group of STH-PAS-positive cases, the accompanying heterogeneous clinical diagnoses of viral illness and other illnesses may not clearly rule out dengue infection. Two STH-PAS-positive samples from healthy volunteers were found to be negative by Taqman RT-PCR and SD Dengue Duo. On the other hand, 60 samples from cases diagnosed as dengue were found to be negative for dengue by all three methods (Table 2).

The clinical performance of the dengue STH-PAS test was determined using Taqman RT-PCR as the reference assay. Both methods were positive for dengue in 22 samples and negative in 200 samples. Twenty-five samples were positive for dengue using the STH-PAS system but were negative by the Taqman method. With a detection rate of 19.0%, the DENV NAC and STH-PAS system gave a sensitivity of 100.0%, a specificity of 88.9%, a positive predictive value (PPV) of 46.8%, a negative predictive value (NPV) of 100.0%, and a diagnostic accuracy of 89.9% in this study.

When the SD Dengue Duo NS1 test was used as comparator, the DENV NAC and STH-PAS system exhibited a sensitivity of 100.0%, a specificity of 86.6%, a PPV of 34.0%, a NPV of 100.0%, and a diagnostic accuracy of 87.5%. Both methods were positive for dengue in 16 samples and negative in 200 samples. Thirty-one samples were dengue-positive using the STH-PAS system but tested negative with SD Dengue Duo.

Discussion

The STH-PAS system was developed as a sensitive PCR–based dipstick DNA chromatography assay to provide visual multiplex and semi-quantitative analyses of various DNA and RNA viruses, and bacteria [8–11]. This report describes for the first time the detection of dengue viruses by the NAC and STH-PAS system. The system allows specific reverse transcription and amplification of viral RNA followed by immobilization of parallel oligonucleotide probes to which target regions of the dengue genome can hybridize and trigger signal production. Positive blue lines appear as a result of the formation of biotin-streptavidin complexes upon binding of the probe and target DNA. Accumulation of blue latex microspheres on the test and control lines allows for visualization by the naked eye.

This paper reports on the performance of the Dengue NAC and STH-PAS system in comparison to common laboratory diagnostics tests for dengue, and as assessed on the basis of clinical parameters used to diagnose dengue infection.

Both the analytical and clinical performance of the Dengue NAC and STH-PAS system exhibited 100% sensitivity and comparable specificity compared to Taqman RT-PCR assays. In previous studies, Taqman RT-PCR exhibits a detection limit between 600 and 1100 genome copy equivalents per milliliter

Table 2 Comparison of three detection methods for dengue: DENV NAC Universal Primer kit and STH-PAS system, RT-PCR, and SD Bioline Dengue Duo System

Group	No. of cases	STH-PAS		RT-PCR		SD Dengue Duo NS1	
		+	–	+	–	+	–
Patients diagnosed with dengue	89	29	60	17	72	12	77
Patients with diagnoses other than dengue	128	16	112	5	123	4	124
Afebrile healthy volunteers	30	2	28	0	30	0	30
Total	247	47 (19.0%)	200	22 (8.9%)	225	16 (6.48%)	231

Table 3 Profile of the laboratory-based molecular test results and clinical diagnoses of patients seen at the Emergency Department

Laboratory-based molecular tests			Dengue with warning signs	Dengue without warning signs	Viral illness	[#]Others	No diagnosis
STH-PAS	Taqman RT-PCR	SD Duo NS1					
+	+	+	7	5	0	4	0
+	+	–	2	3	1	0	0
+	–	+	0	0	0	0	0
+	–	–	7	5	2	9	2
–	+	+	0	0	0	0	0
–	+	–	0	0	0	0	0
–	–	+	0	0	0	0	0
–	–	–	32	28	24	88	28
Total			48	41	27	101	30

[#] Include upper respiratory infection, gastrointestinal tract infection, or other infection

[7], while the SD Dengue Duo NS1 test has been reported to exhibit a sensitivity of 59% and a specificity between 76% and 80% [5]. In this study, the concordance between the STH-PAS system and Taqman RT-PCR is 89.9%, and 87.5% between the STH-PAS system and the SD Dengue Duo NS1 test, respectively. The STH-PAS system was found to be more sensitive in detecting dengue infection compared to Taqman RT-PCR using a dilution assay. The lower sensitivity obtained with the comparator Taqman test may be a result of the lower input of RNA template in the two-step RT-PCR used compared to the one-step format of the STH-PAS system.

It is worth noting that a stored CHIKV sample showed a positive reaction to STH-PAS for DENV but was negative with Taqman RT-PCR, highlighting the superior specificity of the latter. Also, this may indicate possible co-infection which we have previously identified in earlier studies (unpublished data), as supported by reports that co-infections between dengue and chikungunya are increasing [13–15]. In addition, chikungunya and dengue infections are spatio-temporally related, because both pathogens are transmitted by the same *Aedes* spp. mosquitoes. Moreover, because symptoms presented by infected patients are similar and diagnosis of both infections is predominantly symptom-based, there will inevitably be ambiguity in disease recognition in inhabitants of endemic/epidemic regions and travelers returning from the regions. The frequency of co-infections suggests that the proper diagnosis, surveillance, and management of cases of co-infection are necessary to avoid poor prognosis of the underlying etiology [13, 14]. One case has been previously reported where Zika infection, chikungunya, and DENV were all detected in one person [15].

Our data showed that a substantial number of cases diagnosed as dengue, based on the symptoms and classification of WHO 2009 criteria, tested negatively for DENV, Dengue NS1 antigen, and anti-dengue IgM and IgG by the three methods used in this study. A possible explanation for this is the different number of days from the onset of illness at which patients sought medical attention at the Emergency Department. The three laboratory tests used here target the circulating virus and NS1 antigen, as well as anti-DENV IgM, which are produced in the early stages of dengue infection. The window for detection may thus have been missed for the identification of dengue at the later stages of illness. Alternatively, in a similar study performed on Cambodia children, a rapid diagnostic test (RDT) for DENV showed low sensitivity for the diagnosis of DENV infection, with a sensitivity of 58% and a specificity of 85%, for NS1 and IgM combined [16]. On the other hand, samples from several non-dengue cases were found to be positive for dengue by all of the three methods employed here. The initial masking of dengue symptom profiles may have been caused by prior intake of medication, the prevailing co-morbid conditions, and the limitations of manual capture of clinical data. This highlights the importance and urgent need for the development of a specific and sensitive point-of-care test (POCT) for DENV infection.

Some of the limitations of this study included the small number of dengue cases and the variable number of days from the onset of illness at which patients visited the ER to seek medical assistance. Further evaluation of the STH-PAS system for the detection of dengue and other pathogens in more carefully profiled clinical cases may be needed.

In conclusion, the Dengue NAC and STH-PAS system has been shown to provide 100% sensitivity in detecting dengue infection in acute febrile cases, superior to Taqman real-time RT-PCR and a Dengue NS1–based rapid diagnostic test.

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Authors' contributions Conceived and designed the experiments: YT, TH, MK, HCY, MLGD, MTAD.

Performed the experiments: VRL, LASP, RSR, MKB.

Analyzed the data: VRL, LASP, RSR, MLGD, MTAD, MPSD, YT, TH, MK, HCY.

Contributed reagents/materials/analysis tools: MK, YT, TH, PASL, AM, GB.

Wrote the paper: MLGD, VRL, LASP, HCY, TH.

Contributed to the logistics of kits: YT, TH.

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Compliance with ethical standards The study was approved by the Institutional Ethics Review Committee, EC No. CT-17010, and conducted in St. Luke's Medical Center, Quezon City, Philippines.

Competing interest The authors declare that they have no conflict of interest.

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