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Dengue virus (DENV) infection is considered a major public health problem in developing tropical countries where the virus is endemic and continues to cause major disease outbreaks every year. Here, we describe the development of a novel, inexpensive, and user-friendly diagnostic assay based on a reverse transcription-insulated isothermal PCR (RT-iiPCR) method for the detection of all four serotypes of DENV in clinical samples. The diagnostic performance of the newly established pan-DENV RT-iiPCR assay targeting a conserved 3′ untranslated region of the viral genome was evaluated. The limit of detection with a 95% confidence was estimated to be 10 copies of in vitro-transcribed (IVT) RNA. Sensitivity analysis using RNA prepared from 10-fold serial dilutions of tissue culture fluid containing DENVs suggested that the RT-iiPCR assay was comparable to the multiplex real-time quantitative RT-PCR (qRT-PCR) assay for DENV-1, -3, and -4 detection but 10-fold less sensitive for DENV-2 detection. Subsequently, plasma collected from patients suspected of dengue virus infection (n = 220) and individuals not suspected of dengue virus infection (n = 45) were tested by the RT-iiPCR and compared to original test results using a DENV NS1 antigen rapid test and the qRT-PCR. The diagnostic agreement of the pan-DENV RT-iiPCR, NS1 antigen rapid test, and qRT-PCR tests was 93.9%, 84.5%, and 97.4%, respectively, compared to the composite reference results. This new RT-iiPCR assay along with the portable POCKIT nucleic acid analyzer could provide a highly reliable, sensitive, and specific point-of-need diagnostic assay for the diagnosis of DENV in clinics and hospitals in developing countries.

Dengue virus (DENV) is a mosquito-borne human pathogen that belongs to the genus *Flavivirus* in the family *Flaviviridae* (1). DENV is an enveloped virus with a single-stranded, positive-sense RNA genome approximately 10.7 kb in length (2). The genomic RNA includes 5′ and 3′ untranslated regions (UTRs) and a single open reading frame that encodes a single polyprotein that is cleaved into three structural proteins (capsid [C], premembrane/membrane [prM/M], and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (3). DENV is comprised of four distinct serotypes (DENV-1, -2, -3, and -4), and all four serotypes are currently circulating in the Pacific Islands, Americas, Asia, and Africa. In addition to this, distinct genotypes have been identified within each serotype (4, 5), highlighting the extensive genetic variability of the DENV serotypes.

DENV infection is considered a major public health problem in developing tropical countries where the virus is endemic, and it is continuously spreading to new geographical areas around the world (6, 7). Moreover, frequent international travel to regions where dengue is endemic or epidemic increases the risk of DENV infection for travelers (8, 9), and travel contributes to the escalating numbers of imported dengue cases in temperate regions. More than 2.5 billion people are at risk of DENV infection each year, and an estimated 50 million dengue virus infections occur annually around the world (10, 11). DENV is primarily transmitted by the mosquito species *Aedes aegypti*, present in tropical and subtropical regions, and less efficiently by *Aedes albopictus* (12). The DENV infection causes a wide range of clinical signs in humans, from acute febrile illness (dengue fever [DF]) to hemorrhagic fever/ dengue shock syndrome (DHF/DSS), mostly characterized by plasma leakage with or without hemorrhage (13).

The diagnosis of dengue virus infections cannot rely solely on clinical manifestations since many patients are either asymptomatic or present with a nondescript fever requiring differential diagnosis to distinguish exposure to DENVs from other febrile-episode-inducing diseases. Therefore, rapid, accurate, relatively low-cost diagnostic tools for DENV are critical for the confirmation of suspected clinical cases, which in turn is the key to effective disease management and control. Currently, laboratory confirmation of DENV infection relies on virus isolation in cell

culture, detection of viral nucleic acid by real-time quantitative reverse transcriptase PCR (qRT-PCR), NS1 antigen detection, and/or detection of virus-specific antibodies in paired sera (e.g., neutralization test, immunoglobulin M [IgM] capture enzyme linked immunosorbent assay [MAC-ELISA], and indirect immunoglobulin G ELISA) (14–16). Although virus isolation is considered the gold standard for laboratory diagnosis of dengue virus infection, it is time-consuming, expensive, and laborious and requires biosafety level 2 (BSL-2)/BSL-3 cell culture facilities with trained personnel. Thus, ELISA is still the most widely used technique for serological diagnosis, providing indirect evidence of infection after seroconversion on the second week of fever; however, it cannot distinguish different DENV serotypes. Furthermore, a number of rapid diagnostic tests targeting NS1 antigen are commercially available; however, false-negative results are still a concern with these assays (17, 18). Molecular diagnostic assays, such as high-throughput quantitative PCR (qPCR) assays or gel-based conventional PCR assays, offer a high degree of detection sensitivity and specificity, but they require laboratories staffed with skilled technicians with relatively expensive equipment that are not available in remote areas or developing countries.

As all potentially exposed individuals require access to rapid molecular diagnostic assays for the detection of DENV RNA in clinical specimens such as blood, plasma, or serum, the development of DENV-specific point-of-need (PON) diagnostics will have a major public health impact by improving clinical outcome and/or by facilitating a significantly more comprehensive dengue surveillance program. Implementation of a sensitive and specific PON test for diagnosis of all four serotypes of DENV in primary care facilities (e.g., clinics and hospitals) is likely to improve patient care in developing countries. As a result, a rapid, affordable, and highly sensitive molecular detection system is still needed for early PON test detection of DENV infection.

Recently, a fluorescent probe hydrolysis-based insulated isothermal PCR (iiPCR) for amplification and detection of nucleic acid has been described (21). The iiPCR is highly sensitive and specific for the detection of both DNA and RNA and could be performed with a single heating source; thus, it does not require an expensive thermocycler (20, 21). The PCR mix in a capillary tube (R-tube; GeneReach USA, Lexington, MA, USA) is heated at the bottom. Rayleigh-Bénard convection drives fluid cycling through temperature gradients, and the three PCR steps, namely, denaturation, annealing, and extension, can be completed at different zones within the capillary tube. Subsequent integration of hydrolysis probe technology and an optical detection module into the device allows automatic detection and interpretation of iiPCR results (21). Taking advantage of the fluorescent probe-based iiPCR methodology and a now commercially available reverse transcription-iiPCR (RT-iiPCR) instrument, the POCKIT nucleic acid analyzer (GeneReach USA, Lexington, MA, USA), we developed an RT-iiPCR assay for the detection of DENV RNA of all serotypes in clinical specimens. The analytical sensitivity, analytical specificity, and reproducibility of the newly established pan-DENV-specific RT-iiPCR assay were assessed to determine effectiveness of the assay for DENV detection. Subsequently, the assay was further evaluated for clinical sensitivity and specificity using RNA extracted from human clinical samples (e.g., plasma) in Sri Lanka and compared to results with a multiplex qRT-PCR assay previously described by the Centers for Disease Control and Prevention (CDC qRT-PCR; Atlanta, GA, USA) (22).

MATERIALS AND METHODS

Cells and viruses. Madin-Darby canine kidney (MDCK; ATCC CCL-22) cells and Vero E6 (ATCC CRL-1587) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained at 37°C in minimum essential medium (MEM; Invitrogen, Carlsbad, CA, USA) and Dulbecco's modified Eagle's medium (Invitrogen), respectively, supplemented with 10% fetal bovine serum (FBS; Invitrogen). The DENV-1 clinical isolate DenKor-02 was kindly provided by the Korea National Institute of Health, Osong, Republic of Korea (23, 24). The laboratory DENV strains, DENV-2/KBPV-VR-29, DENV-3/KBPV-VR-30, and DENV-4/KBPV-VR-31 (serotypes 2, 3, and 4, respectively), were obtained from the Korea Bank for Pathogenic Viruses (KBPV; Republic of Korea [http://kbpv.knrrc.or.kr]) (25). The prototype DENV-1 (Hawaii A), DENV-2 (New Guinea C), DENV-3 (H87), and DENV-4 (H241) strains were purchased from the National Collection of Pathogenic Viruses, Culture Collections of Public Health England (Salisbury, Great Britain). Other human viral pathogens included in this study were West Nile virus (synthetic viral RNA; ATCC VR-3198SD), Japanese encephalitis virus (a gift from Wu-Chun Tu, National Chung Hsin University, Taiwan), hepatitis C virus (synthetic hepatitis C virus RNA, JFH-1, genotype 2a; derived from pJFH1 full-length cDNA clone obtained from T. Wakita, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan), influenza virus type A (H1N1, A/Puerto Rico/8/34 [ATCC VR-1469]), and influenza virus type B (B/Panama/45/1990 [Korea Center for Disease Control and Prevention]). All DENVs were propagated in Vero E6 cells (ATCC CRL-1587), and tissue culture fluid (TCF) supernatants were harvested at 6 to 7 days postinfection. Influenza viruses (type A and B) were propagated by infection of MDCK cells at 33°C for 3 days. TCF was clarified by centrifugation (2,000 × g, 4°C, 15 min), and 1-ml aliquots were stored at −80°C until further use. Individual virus stocks were titrated by plaque assays before use.

Clinical specimens. A total of 220 archived plasma specimens from clinically suspected dengue patients collected over a period of 3 years (2011 to 2013) were obtained from the Professorial Medical Unit, Teaching Hospital, University of Peradeniya, Peradeniya, Sri Lanka. The samples were used for routine diagnosis using the SD Bioline dengue NS1 antigen (Ag) rapid test (Standard Diagnostics, Kyonggi-do, South Korea) assay at the Department of Veterinary Pathobiology, University of Peradeniya. Plasma samples collected from subjects not suspected of DENV infection ($n = 45$) were also included in the study. All the plasma samples used in this study were stored at −20°C until nucleic acid extraction.

Ethics statement. Samples were collected by the Professorial Medical Unit, Teaching Hospital, University of Peradeniya, Peradeniya, Sri Lanka, as a part of routine practice from clinically suspected dengue patients on admission for laboratory diagnosis at the Department of Veterinary Pathobiology, University of Peradeniya, where diagnostic services were available free of charge. Informed consent was obtained from patients at the time of sample collection. Approval of the study was provided by the Ethical Review Committee of the Faculty of Medicine, University of Peradeniya, Peradeniya, Sri Lanka (2015/EC/65).

Nucleic acid extraction. For analytical sensitivity analysis, TCF samples containing each of the four DENV serotypes (1, 2, 3, and 4) were subjected to 10-fold serial dilutions (10^0 to 10^7) in cell culture medium. Subsequently, viral RNA was extracted from 10-fold serial dilutions of each of the four DENV serotypes. The archived human plasma samples were subjected to nucleic acid extraction without further dilution. All samples (TCF and plasma samples) were extracted by using a taco DNA/RNA extraction kit (GeneReach USA) on a taco 24 device (GeneReach USA), according to the manufacturer's instructions. Briefly, 100 μl of TCF or the plasma samples was added into the first well of the extraction plate before the automatic extraction steps. After the extraction steps were completed, nucleic acids were eluted with 200 μl of elution buffer. All nucleic acids were placed at −80°C until further use.

IVT RNA preparation. The analytical sensitivity of the pan-DENV RT-iiPCR assay was determined by using *in vitro*-transcribed (IVT)
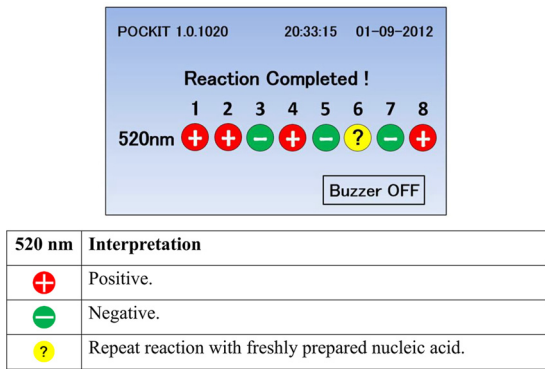
520 nm	Interpretation
⊕	Positive.
⊖	Negative.
❓	Repeat reaction with freshly prepared nucleic acid.

FIG 1 Data interpretation. An example of results displayed on the monitor is shown.

RNA. Briefly, the sequence of the 3′ UTR region of the DENV-2 MD944 isolate (nucleotides [nt] 10162 to 10682; GenBank accession number FM210230.1) was RT-PCR amplified, cloned into the pGEM-T vector downstream of the T7 promoter sequence, and subsequently used as the template in *in vitro* transcription using a MEGAscript T7 kit (Life Technologies, USA). Residual DNA was removed using an Ambion Turbo DNA-free kit (Applied Biosystems, Grand Island, NY, USA).

Concentration of RNA was measured by a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Houston, TX, USA). Single-use IVT RNA aliquots were stored at −80°C. The analytical sensitivity of the RT-iiPCR was determined using a dilution series (10^0 to 10^8 molecules/reaction) of the IVT RNA. The concentration of the IVT RNA molecules per microliter was calculated according to the following formula, as described before (26): number of IVT RNA molecules/microliter = (Avogadro's number × IVT RNA concentration)/IVT RNA molecular mass, where Avogadro's number is 6.022×10^{23}, IVT RNA concentration is measured in grams/microliter, and IVT RNA molecular mass is measured in grams.

Establishment of pan-DENV RT-iiPCR assay. The pan-DENV RT-iiPCR was designed on the basis of the previously described probe hydrolysis-based POCKIT method (21). The primers and probe targeted a highly conserved region in the 3′ UTR of the DENV genome (see Table S1 in the supplemental material). This conserved region was identified by aligning 3,530 DENV sequences available in GenBank. The RT-iiPCR reaction conditions, such as concentrations of primers and probe, *Taq* DNA polymerase, and reverse transcriptase, were tested systematically to obtain the highest sensitivity and specificity. Following optimization of the RT-iiPCR assay conditions, the reagents, including primers and probe, were lyophilized and used in this study. Briefly, R-tubes were labeled with the sample identification numbers (one tube per test sample). After the lyophilized premix was reconstituted in 50 μl of premix buffer B (GeneReach USA), 5 μl of the test nucleic acid extract was added. A 50-μl volume of the premix/sample mixture was transferred into a labeled R-tube, which was sealed subsequently with a cap, spun briefly in a microcentrifuge (cubee; GeneReach USA), and placed into the POCKIT nucleic acid analyzer (GeneReach USA). The default program, including an RT step at 50°C for 10 min and an iiPCR step at 95°C for about 30 min, was completed in less than 1 h. The signal-to-noise (S/N) ratio, i.e., light signal collected after iiPCR/fluorescent signal collected before iiPCR (21), was converted automatically to a plus sign, minus sign, or question mark, according to the default S/N thresholds by the built-in algorithm. The results were shown on the display screen at the end of the program (Fig. 1). A question mark indicated that the results were ambiguous and that the sample should be tested again.

DENV serotype-specific multiplex real-time RT-PCR assays. The CDC multiplex DENV-1-4 qRT-PCR assay described by Santiago et al.(22) (here, the CDC qRT-PCR [http://www.cdc.gov/dengue/clinical Lab/realTime.html]) was performed using a Superscript III Platinum

One-Step RT-PCR System without 6-carboxy-X-rhodamine (ROX) (Life Technologies) in a QuantStudio 6K Flex real-time PCR system (Life Technologies). The CDC qRT-PCR assay was run in the multiplex format as described previously (22). The primers, probes, target genes, and their specific locations in the DENV genome are listed in Table S1 in the supplemental material. Signals were collected using the 6-carboxyfluorescein (FAM), hexachlorofluorescein (HEX), Texas Red, and Cy5 channels for the detection of fluorescence generated from the DENV-1, -2, -3, and -4 probes, which were labeled at the 5′ end with FAM, HEX, Texas Red, and Cy5 fluorescent dyes, respectively. Briefly, each 25-μl reaction mixture included 5 μl of nucleic acid sample, 12.5 μl of 2× Premix, 1 μM forward and reverse primers for DENV-1 and DENV-3, 0.5 μM forward and reverse primers for DENV-2 and DENV-4, 180 nM each TaqMan probe, and 0.5 μl of SuperScript III RT/Platinum *Taq* mix. The thermocycling program was set up as follows: an RT step at 50°C for 30 min, followed by 95°C for 2 min and 45 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. Samples generating a threshold cycle (C_T) of >37 in duplicates were considered negative. Each run included negative controls spiked with water and positive controls with the IVT RNA-containing target sequences.

Dengue virus NS1 antigen detection. All plasma specimens were tested for DENV infection by the NS1 Ag rapid test (Standard Diagnostics). Tests were performed according to the manufacturer's instructions.

Focus-forming assay. Vero cells were seeded in 24-well plates. Tenfold dilutions were prepared in Opti-Pro serum-free medium (SFM) (Invitrogen) supplemented with 2% FBS and 4 mM L-glutamine in duplicate, and 150 μl of each dilution was added to the cells. The plates were incubated for 1 h at 37°C and rocked every 15 min. Unabsorbed virus was removed by a phosphate-buffered saline (PBS) wash, and after which 1 ml of Opti-MEM (Invitrogen) supplemented with 0.8% methylcellulose (MC; Fisher Scientific, Pittsburgh, PA, USA), 4 mM L-glutamine, and 50 μg/ml penicillin-streptomycin (Invitrogen) was added to each well, followed by incubation at 37°C for 3 days. The MC overlay was aspirated, and the cells were washed with PBS and fixed with methanol at 4°C overnight. After fixation, the cells were washed with PBS and incubated at 37°C for 1 h with mouse anti-dengue virus NS1 glycoprotein (catalog no. 41616; Abcam, Cambridge, MA, USA) and mouse anti-dengue virus complex (catalog no. MAb8705; EMD Millipore, Billerica, MA, USA) antibodies, followed by incubation with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 antibody (catalog no. 1070-05; Southern Biotech, Birmingham, AL, USA). The plates were developed with a SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Images were taken using an ImageQuant LAS 4000 (GE Healthcare, Pittsburgh, PA, USA).

Statistical analysis. Limit of detection with 95% confidence ($LoD_{95\%}$) was determined by statistical probit analysis (a nonlinear regression model) using the commercial software SPSS, version 14.0 (SPSS, Inc., Chicago, IL, USA). The clinical sensitivity and specificity of the assays described in the study were calculated based on the analysis of the plasma samples from 220 suspected dengue patients and 45 donors not suspected of DENV infection. A composite diagnosis of each sample (2 out of 3 tests based on the CDC qRT-PCR, the NS1 Ag rapid test, and the pan-DENV RT-iiPCR giving the same result) was used as a reference (27). The degree of agreement between two assays was assessed by calculating Cohen's kappa (κ) values. Sensitivity was calculated as follows: number of true positives/(number of true positives + number of false negatives). Specificity was calculated as follows: number of true negatives/(number of true negatives + number of false positives).

RESULTS

Development and optimization of pan-DENV RT-iiPCR. The pan-DENV RT-iiPCR method, targeting a highly conserved region in the 3′ UTR of the DENV genome was designed to generate

a 110-bp amplicon to be detected by the fluorescent dye-labeled probe. The reaction conditions, such as concentrations of primers and probe, *Taq* DNA polymerase, and reverse transcriptase, were tested systematically to obtain the highest sensitivity and specificity (data not shown). Following optimization of the assay conditions, the reagents, including primers and probe, were lyophilized and used in this study as described in Materials and Methods. The sensitivity of the established assay was determined by using 10-fold serial dilutions of the IVT RNA containing the target DENV sequence. Analysis of replicates showed 20/20 (100%), 19/20 (95%), 5/20 (25%), and 0/20 (0%) positive signals for reaction mixtures containing 100, 10, 1, and 0 copies of the IVT RNA, respectively. The $LoD_{95\%}$ of the pan-DENV RT-iiPCR estimated by probit analysis was 10 genome equivalents/reaction.

Comparison of analytical sensitivity, specificity, and performance of pan-DENV RT-iiPCR. (i) Analytical sensitivity. The performance of the pan-DENV RT-iiPCR was compared to that of the CDC qRT-PCR assay [22] that had been approved by the FDA for detection and typing of DENV in suspected, symptomatic cases in the United States. The analytical sensitivity of the pan-DENV RT-iiPCR was evaluated by using 10-fold serial (10^{-1} to 10^{-9}) dilutions of the TCF samples containing a DENV field isolate (DENV-1/DenKor-02) or three laboratory strains (DENV-2/KBPV-VR-29, DENV-3/KBPV-VR-30, and DENV-4/KBPV-VR-31) of known titers (see Table S2a in the supplemental material). Viral RNAs extracted from 10-fold serial dilutions were analyzed by both assays in parallel. The 100% detection endpoints of the pan-DENV RT-iiPCR and the multiplex CDC qRT-PCR were found both at 2.7×10^{-1} focus-forming units (FFU)/ml for the DENV-1 virus and 4.7×10^{-1} FFU/ml for the DENV-2 virus. Similarly, endpoints of the pan-DENV RT-iiPCR were observed at 2.0×10^{-2} and 1.0×10^{-1} FFU/ml for the DENV-3 and -4 viruses, respectively, while those of the multiplex CDC qRT-PCR were at 2.0×10^{-1} FFU/ml and 1.0×10^{-2} FFU/ml, respectively (see Table S2a in the supplemental material). Therefore, the overall analytical sensitivity of the pan-DENV RT-iiPCR was comparable to that of the multiplex CDC qRT-PCR in detecting viral RNA of the field and laboratory strains.

In addition, the detection endpoints of both assays were compared using the RNA extracted from 10-fold serial dilutions of the TCF samples containing one of the prototype strains of DENV-1 to -4 (DENV-1/Hawaii A, DENV-2/New Guinea C, DENV-3/H87, or DENV-4/H241). The pan-DENV RT-iiPCR and multiplex CDC qRT-PCR had the same detection endpoints for the DENV-1, DENV-3 (4.9 FFU/ml), and DENV-4 (1.4 FFU/ml) viruses (see Table S2b in the supplemental material). For DENV-2/New Guinea C, the detection endpoint of the pan-DENV RT-iiPCR was at 9.7×10^{-1} FFU/ml, and that of the multiplex CDC qRT-PCR was at 9.7×10^{-2} FFU/ml (Table S2b in the supplemental material). The results suggested that the detection limit of the pan-DENV RT-iiPCR was comparable to that of the multiplex CDC qRT-PCR for the prototype DENV-1, -3, and -4 strains while for the DENV-2 prototype strain it was about 10-fold lower than that of the CDC qRT-PCR.

(ii) Analytical specificity. The specificity of the pan-DENV RT-iiPCR was evaluated with viral nucleic acids extracted from TCF samples containing hepatitis C virus, West Nile virus, Japanese encephalitis virus, or influenza virus type A or B strains. The newly established pan-DENV RT-iiPCR assay generated negative

TABLE 1 Diagnostic performance comparison between pan-DENV RT-iiPCR and NS1 Ag rapid test methods[a]

Pan-DENV RT-iiPCR result	No. of results with NS1 Ag rapid test that were:		Total
	Positive	Negative	
Positive	110	25	135
Negative	32	98	130
Total	142	123	265

[a] Agreement (95% CI), 78.49% (73.51 to 83.47%).

signals with all of these viral pathogens (data not shown), implying a high analytical specificity.

(iii) Reproducibility. The reproducibility of the pan-DENV RT-iiPCR was evaluated using the RNA of the 2.7-FFU/ml DENV-1/DenKor-02 sample. Target concentration in this sample was approximately 10-fold above the detection limit of the pan-DENV RT-iiPCR (the assay reached its detection limit for the virus at 2.7×10^{-1} FFU/ml). The test was done in six replicates per device and repeated in three different POCKIT nucleic acid analyzers. All reaction mixtures containing the target template showed positive results (data not shown), indicating that the pan-DENV RT-iiPCR could detect dengue viral RNA at concentrations close to its detection limit with excellent reproducibility.

Evaluation of the pan-DENV RT-iiPCR using clinical samples. The clinical evaluation of the pan-DENV RT-iiPCR was conducted by testing 220 archived plasma samples collected from patients suspected of DENV infection over a period of 3 years (2011 to 2013) and 45 plasma specimens from subjects not suspected of DENV infection in the study. First, these samples were tested by the commercial NS1 antigen detection strip assay (dengue NS1 Ag rapid test; Standard Diagnostics) at the time of sample submission to the laboratory in Sri Lanka. Of the 265 samples, 142 (142/265) were positive whereas 123 (123/265) samples were negative for NS1 antigen. Subsequently, nucleic acids were prepared from these archived samples and subjected to side-by-side testing with the pan-DENV RT-iiPCR and CDC qRT-PCR. When the archived samples were tested with the pan-DENV RT-iiPCR, 32 of the 142 NS1 antigen rapid test-positive samples tested negative for DENV RNA (only 110/142 were positive). Furthermore, 25 of the 123 NS1 antigen-negative samples tested positive by the pan-DENV RT-iiPCR (Table 1). Thus, 135/265 of the plasma samples were positive by the pan-DENV RT-iiPCR. In summary, the agreement between the RT-iiPCR and the NS1 antigen rapid test was about 78.5% (95% confidence interval [CI], 73.5 to 83.5%) with a kappa value of 0.57 when the RNA extracted from plasma samples was tested.

Although virus isolation (VI) is the gold standard for diagnosis of DENV infection, only a few laboratories perform this assay routinely [28]. In most situations accurate diagnosis of dengue virus infection can be best achieved by combining the detection of virus (VI), viral nucleic acid (qRT-PCR), viral protein NS1 (NS1 antigen rapid test), and/or antibody response to the virus (ELISA) [29, 30]. Thus, a composite diagnosis of each sample based on two out of the three methods (CDC qRT-PCR, NS1 antigen rapid test, and pan-DENV RT-iiPCR) giving the same result was used as the reference. A total of 135 out of the 265 samples were detected as positive using the pan-DENV RT-iiPCR. The NS1 antigen rapid

TABLE 2 Diagnostic sensitivity and specificity of pan-DENV RT-iiPCR, CDC qRT-PCR, and NS1 Ag rapid test for detection of dengue virus infection

Assay and result	No. of results with the composite set that were:[a]		Total	% sensitivity (95% CI)	% specificity (95% CI)	% agreement (95% CI)
	Positive	Negative				
Pan-DENV RT-iiPCR				90.5 (85.5–95.4)	98.3 (95.1–100)	93.9 (90.9–96.9)
Positive	133	2	135			
Negative	14	116	130			
Total	147	118	265			
NS1 Ag rapid test				84.3 (78.3–90.4)	84.7 (78.1–91.4)	84.5 (80.1–88.9)
Positive	124	18	142			
Negative	23	100	123			
Total	147	118	265			
CDC qRT-PCR				98.6 (96.1–100)	95.8 (91.6–99.9)	97.4 (95.2–99.5)
Positive	145	5	150			
Negative	2	113	115			
Total	147	118	265			

[a] A composite diagnosis for each sample based on two out of three methods giving the same result was used as the reference.

test and the CDC qRT-PCR detected 142 and 150 positive samples, respectively (Table 2). Of the 150 RT-qPCR positive samples, 145 were positive for DENV-1, and 5 samples were positive for DENV-4 infection. Compared to the composite reference results, the sensitivities of the pan-DENV RT-iiPCR, the NS1 antigen rapid test, and the CDC qRT-PCR tests were 90.5% (95% CI, 85.5 to 95.4%), 84.3% (95% CI, 78.3 to 90.4%), and 98.6% (95% CI, 96.1 to 100%), respectively. The specificities of the pan-DENV RT-iiPCR, the NS1 antigen rapid test, and the CDC qRT-PCR tests were 98.3% (95% CI, 95.1 to 100%), 84.7% (95% CI, 78.1 to 91.4%), and 95.8% (95% CI, 91.6 to 99.9%), respectively (Table 2). Based on the analysis of the 265 clinical plasma specimens, the pan-DENV RT-iiPCR, the NS1 antigen rapid test, and the CDC qRT-PCR tests had an agreement of 93.9% (95% CI, 90.9 to 96.9%; κ = 0.88), 84.5% (95% CI, 80.1 to 88.9%; κ = 0.69), and 97.4% (95% CI, 95.2 to 99.5%; κ = 0.95), respectively, with the composite reference results for DENV detection in patient samples. In summary, compared to the composite reference results, the pan-DENV RT-iiPCR test had 90.5% sensitivity (95% CI, 85.5 to 95.4%), 98.3% specificity (95% CI, 95.1 to 100%), and 93.9% agreement (95% CI, 90.9 to 96.9%; κ = 0.88).

DISCUSSION

DENV infections can result in a range of clinical manifestations from asymptomatic infection to DF and DHF/DSS. The clinical presentation of acute dengue virus infection is nonspecific, and 5 to 10% of patients progress to severe DHF/DSS, which can result in death if not managed appropriately. Diagnosis of dengue virus infection solely based on clinical symptoms is difficult since some infections can resemble other diseases with similar clinical presentations (such as malaria, leptospirosis, typhoid, typhus, chikungunya, and Zika). Sri Lanka is considered a hyperendemic region (a category A country according to the World Health Organization) for DENV infection. The disease is considered a major public health concern locally. Since the early 2000s, large epidemics have occurred at regular intervals in urban areas, involving cocircula-

tion of all four DENV serotypes (31). Despite the high dengue fever burden occurring in Sri Lanka and other developing countries, access to good-quality diagnostic testing is lacking due to limited laboratory infrastructure, technical expertise, and research capacity. PON diagnostics could help shift health services more to local levels, reduce costs and turnaround time, and achieve disease diagnosis at early stages, improving health management quality in underreached communities. Thus, there is a need for specific, inexpensive dengue virus diagnostic tests that can be used for clinical management, surveillance, and outbreak investigations to allow early intervention to treat patients and prevent or control epidemics. Furthermore, a recombinant live attenuated tetravalent dengue vaccine based on the attenuated yellow fever (YF) vaccine virus YF-17D was developed, and this vaccine has now been introduced into the market in some countries around the world (32). The new vaccine consists of four recombinant viruses, expressing the premembrane (prM) and envelope (E) proteins of DENV serotypes 1 to 4 (33, 34). Therefore, the administration of the new recombinant live virus vaccine will complicate the serological diagnosis of dengue virus infection using current IgM/IgG immunoassays which mostly detect antibodies to structural proteins (prM/M and E). Current serological assays use an IgM/IgG ratio of 0.5 as indicative of a primary DENV infection. High levels of IgG in acute-phase samples define secondary infections (35). Thus, having a PON assay like the RT-iiPCR described in the manuscript would be useful to diagnose DENV infection in populations that are vaccinated against dengue virus.

As described, a pan-DENV RT-iiPCR assay which utilized the iiPCR technology was developed. The assay was carried out in the R-tube within a field-deployable device, the POCKIT nucleic acid analyzer (GeneReach USA, Lexington, USA) (21). In addition, iiPCR assays prepared in lyophilized format could be shipped at ambient temperatures and stored for at least 2 years at room temperature, greatly reducing shipping and storage costs for diagnostic laboratories. Thus, the simplicity and efficiency of the RT-

iiPCR coupled with the POCKIT nucleic acid analyzer (RT-iiPCR/POCKIT) to rapidly amplify nucleic acids under isothermal conditions suggested that this assay could be a potential alternative for detection of DENV especially in remote field settings. This system has been shown to offer sensitivity, specificity, and clinical performance comparable to those of reference real-time PCR or nested PCR methods for various microbial pathogens in various clinical sample types (36–45). One of the major advantages of RT-iiPCR is its simple protocol. Assembly of the reaction mixture involves only three simple steps: (i) rehydration of the lyophilized reagents, (ii) addition of sample nucleic acids, and (iii) transfer of the reaction mixture into reaction tubes and placement on the POCKIT nucleic acid analyzer. This simple automatic detection module (POCKIT) made the method rather user-friendly. The reaction could be completed in 1 h. In combination with the easy, field-deployable manual and/or automatic nucleic acid extraction methods available (PetNAD nucleic acid co-prep kit and taco mini-nucleic acid automatic extraction system, respectively; GeneReach), the iiPCR/POCKIT system is ready to serve as a point-of-need pathogen detection tool to facilitate disease management, surveillance, and control.

In this study, the analytical sensitivity, specificity, and repeatability of the pan-DENV RT-iiPCR/POCKIT system were evaluated. The test was shown to be analytically specific and did not cross-react with other important flavivirus pathogens such as Japanese encephalitis virus, West Nile virus, and hepatitis C virus. The emerging Zika virus is also a member of the genus *Flavivirus* and is very closely related to Japanese encephalitis virus (2, 5). Bioinformatic analysis of the genome sequences ($n = 13$) of Zika virus strains available in GenBank indicated that although significant homology was found in the 3′ UTR of the Zika virus genome (GenBank accession number KU820897.1 as a representative) for the forward primer (91.3% identity) of the pan-DENV RT-iiPCR assay, relatively low homology was found in the target sequences for the reverse primer (68.2% identity) and probe (36.8% identity). Thus, the risks of the assay to cross-react with Zika virus should be extremely low.

The relative sensitivities of the pan-DENV RT-iiPCR and the multiplex qRT-PCR differed slightly depending on the DENV serotype. With RNA prepared from laboratory or prototype DENV strains, the pan-DENV RT-iiPCR generally rendered sensitivity equal or comparable ($\pm$ 1 log unit) to that of the multiplex qRT-PCR assay (see Table S2 in the supplemental material). These minor differences in sensitivities with different DENV strains could not be explained solely by the design of primers and probes. For instance, although different relative sensitivities between RT-iiPCR and qRT-PCR were observed with the two DENV-2 strains (GenBank accession numbers KP406804 and AF038403) (23, 25, 46), no mismatches were found in the target regions of the primers and probe of the pan-DENV RT-iiPCR in the two strains (see the supplemental data). Similar observations were made with the DENV-3 (GenBank accession number KP406805 and M93130) and DENV-4 (GenBank accession number KP406806 and AY947539) strains (23, 25, 46). One possible explanation is that primer and probe binding efficiencies could be affected by the stem-loop structures in the 3′ UTR of the RNA genome, which could be different due to the significant sequence divergence, including mutations and deletions, found locally in the 3′ UTR and remote regions in the DENV genome (46). Furthermore, the 3′ end and 5′ end of DENV viral genome are known to interact with each other (47), and prominent sequence heterogeneity was also found among the four serotypes in the 5′ end of DENV genome (46).

When both PCR assays were compared to the composite reference results, the pan-DENV RT-iiPCR demonstrated a promising clinical performance, with 90.5% sensitivity and 98.3% specificity, which were comparable to the 98.6% sensitivity and 95.8% specificity of the CDC qRT-PCR. Both methods had higher sensitivities and specificities than the NS1 antigen rapid test kit (84.3% sensitivity and 84.7% specificity), which was consistent with the observation that immunoassays had lower clinical performance than PCR assays in general (28). However, compared to the RT-iiPCR/POCKIT and the CDC qRT-PCR methods, the NS1 Ag rapid test was simple and rapid.

When the clinical samples were tested with the three assays (NS1 Ag rapid test, CDC qRT-PCR, and pan-DENV RT-iiPCR), several disagreements were found between the test results that could be due to multiple reasons. Of the three direct diagnostic methods used in this study, two detected the viral RNA in plasma (the CDC qRT-PCR and the pan-DENV RT-iiPCR), whereas the NS1 Ag rapid test assay detected the viral antigen present in plasma. It has been reported that the viral nucleic acids can be reliably detected during viremia (5 days after the onset of illness) (28; http://www.cdc.gov/dengue/clinicalLab/realTime.html). In addition, qRT-PCRs could also detect viral RNA up to 10 days after the onset of clinical symptoms in some clinical cases (48). Similarly, NS1 antigen could be detected up to 9 days after the onset of clinical symptoms in patients with primary and secondary dengue virus infections (28, 49). However, the plasma samples used in this study were not sequential clinical samples from a single patient but random clinical samples from individuals who visited the clinic. Therefore, it was not clear whether these patients had primary or secondary dengue virus infection or at what stage of infection they were when the samples were collected (i.e., how many days after the onset of clinical signs). A better understanding of DENV replication and individual variation in host response, as well as the collection and handling of specimens, is required to better explain the reasons for the discrepancies between the two assay systems (nucleic acid detection and NS1 antigen detection). Moreover, the discrepancies between the CDC qRT-PCR and the pan-DENV RT-iiPCR results are difficult to explain, but the secondary structures (such as the stem-loop structure in the 3′ UTR) in the target sequences and RT-PCR conditions used in these assays could give rise to discrepancies in results. The results of several studies have suggested that the sensitivities of RT-PCR and qRT-PCR depend on the region of the genome that is chosen to be amplified and on the primers (and probes, in the case of qRT-PCR assays) used to achieve this goal (48). It has been reported that RT-PCR can be type specific in terms of DENV detection, and the detection threshold is usually less than 100 PFU for all the serotypes (48). Regrettably, the clinical samples included in this study were mainly from patients infected with DENV-1 ($n = 145$) and a few infected with DENV-4 ($n = 5$). Studies to improve the sensitivity of the pan-DENV RT-iiPCR by changing the reaction conditions to achieve sensitivity equivalent to or higher than that of the CDC qRT-PCR are in progress. We plan to further validate the new and improved pan-DENV RT-iiPCR with a significantly larger number of clinical samples that are collected in a methodical manner (i.e., sequential clinical samples with detailed clinical histories and significant samples to cover all four DENV serotypes and multiple genotypes within a serotype) in the near future.

The POCKIT Xpress portable PCR platform (consisting of a POCKIT, a microcentrifuge, micropipettes, and consumables) along with the lyophilized pan-DENV RT-iiPCR reagents (Gene-Reach USA) offers much shorter single-reaction setup time and setup-to-result time than those of the conventional RT-PCR. In addition, its capacity to process eight reaction mixtures in one test run makes the POCKIT system suitable in the near-patient settings where the sample number is small and a sophisticated testing procedure is not possible. Although the POCKIT system shows good sensitivity for DENV detection, the samples would still need to be referred to a centralized laboratory for serotype identification in the case of epidemiological monitoring. Although it cannot provide the amount of information available from a serotype-specific multiplex qRT-PCR, the POCKIT system can serve as an alternative for the molecular diagnosis and routine screening of DENV infections, especially in countries where dengue virus infection is endemic. In summary, the pan-DENV RT-iiPCR coupled with the POCKIT Xpress portable PCR platform is a good candidate as a rapid, specific, and sensitive tool for use as a PON test system in routine diagnosis of DENV infection.

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