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Monitoring and improving the sensitivity of dengue nested RT-PCR used in longitudinal surveillance in Thailand

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ABSTRACT

Background: AFRIMS longitudinal dengue surveillance in Thailand depends on the nested RT-PCR and the dengue IgM/IgG ELISA.
Objective: To examine and improve the sensitivity of the nested RT-PCR using a panel of archived samples collected during dengue surveillance.
Study design: A retrospective analysis of 16,454 dengue IgM/IgG ELISA positive cases collected between 2000 and 2013 was done to investigate the sensitivity of the nested RT-PCR. From these cases, 318 acute serum specimens or extracted RNA, previously found to be negative by the nested RT-PCR, were tested using TaqMan real-time RT-PCR (TaqMan rRT-PCR). To improve the sensitivity of nested RT-PCR, we designed a new primer based on nucleotide sequences from contemporary strains found to be positive by the TaqMan rRT-PCR. Sensitivity of the new nested PCR was calculated using a panel of 87 samples collected during 2011–2013.
Results and conclusion: The percentage of dengue IgM/IgG ELISA positive cases that were negative by the nested RT-PCR varied from 17% to 42% for all serotypes depending on the year. Using TaqMan rRT-PCR, dengue RNA was detected in 194 (61%) of the 318 acute sera or extracted RNA previously found to be negative by the nested RT-PCR. The newly designed DENV-1 specific primer increased the sensitivity of DENV-1 detection by the nested RT-PCR from 48% to 88%, and of all 4 serotypes from 73% to 87%. These findings demonstrate the impact of genetic diversity and signal erosion on the sensitivity of PCR-based methods.

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1. Background

Dengue virus (DENV), a mosquito-borne flavivirus, is transmitted to humans primarily by *Aedes aegypti* and *Aedes albopictus*. DENV infection is estimated to occur in 390 million people per year worldwide [1]. This is likely to increase due to the expanding geographic distribution of both viruses and vectors. There are four antigenically distinct serotypes (DENV-1–DENV-4) that can cause infections in humans with clinical manifestations ranging from asymptomatic infection to undifferentiated fever, self-limited dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2–4]. The 11 kb single-stranded positive-sense viral RNA genome contains a single open reading frame (ORF) encoding the structural capsid (C), pre-membrane/membrane (prM/M) and envelope (E) proteins, and the nonstructural (NS) proteins; NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [5].

DENV surveillance provides important information used for forecasting, outbreak control, and epidemiological studies [6–8]. Routine DENV laboratory testing at the Department of Virology, Armed Forces Research Institute of Medical Sciences (AFRIMS)

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supports DENV longitudinal surveillance. Laboratory detection relies primarily on the dengue nested RT-PCR (nested RT-PCR), dengue IgM/IgG enzyme-linked immunosorbent assay (IgM/IgG ELISA), and DENV isolation using C6/36 culture or intrathoracic inoculation of *Toxorhynchites splendens* mosquitoes [6–8]. Acute serum specimens from patients with suspected DENV infection are tested by nested RT-PCR, while paired acute and convalescent specimens are tested by IgM/IgG ELISA.

Nested RT-PCR method is widely used throughout the world and has been used in our dengue surveillance program since 1994 [6–9]. The results have provided important epidemiological information concerning cyclic serotype predominance and fluctuations in DF/DHF [6–8]. In addition, positive nested RT-PCR samples have been used for molecular epidemiology and evolution studies based on *E* gene and/or complete genome sequences. These studies have provided details of the genotypes or clades circulating in Thailand [10–12]. Although the nested RT-PCR method is more time-consuming and has a higher risk of contamination than real-time RT-PCR (rRT-PCR) [13–20], the cost of the assay is significantly lower making it the preferred method for DENV detection in many laboratories.

2. Objectives

To examine the percentage of false negative nested RT-PCR results found in routine dengue surveillance conducted in Thailand from 2000 to 2013 and to improve the sensitivity of the dengue nested RT-PCR.

3. Study design

3.1. Data analysis

We retrospectively analyzed dengue IgM/IgG ELISA and nested RT-PCR data from previous tests performed on serum specimens collected from patients admitted to Queen Sirikit National Institute of Child Health (QSNICH) in Bangkok and Kamphaeng Phet Provincial Hospital (KPPH) in Kamphaeng Phet province (northern Thailand) from 2000 to 2013. We examined the results from 16,454 acute dengue cases confirmed by IgM/IgG ELISA for which the number of days of illness (DOI) at the time of specimen collection was known. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and 95% confidence interval (CI) of all RT-PCR methods were calculated using MedCalc software [21]. For comparisons between sensitivities obtained from different RT-PCR methods, we used McNemar exact test [22].

3.2. Human sera, extracted RNA and DENV isolates

Acute human serum specimens ($n = 300$) or RNA extracts ($n = 18$) from 318 dengue cases that were previously determined to be positive by IgM/IgG ELISA, but negative by nested RT-PCR were tested by an in-house TaqMan rRT-PCR assay (Table 1). These samples were selected from the available samples collected from 2005 to 2013. The sensitivity and specificity of the nested RT-PCR using the DENV-1 specific primer (TS1) or the newly designed DENV-1 specific primer (TS1.2013TH) were tested using a panel of 87 samples (Table 1) composed of 72 RNA samples extracted from acute serum specimens collected from 2012 to 2013 and 15 DENV isolates (all serotypes at 10^4 pfu/ml or higher) isolated from acute serum specimens collected in 2011 (these specimens were not available). This panel was tested in parallel by the nested RT-PCR using TS1 primer, the nested RT-PCR using TS1.2013TH primer, the TaqMan rRT-PCR and the DENV-1-4 rRT-PCR developed by the U.S. Center for Disease Control and Prevention (CDC) and recently licensed by the U.S. Food and Drug Administration (FDA) for diagnostic use. All these assays are serotyping assays. A flowchart for this study is shown in Fig. 1.

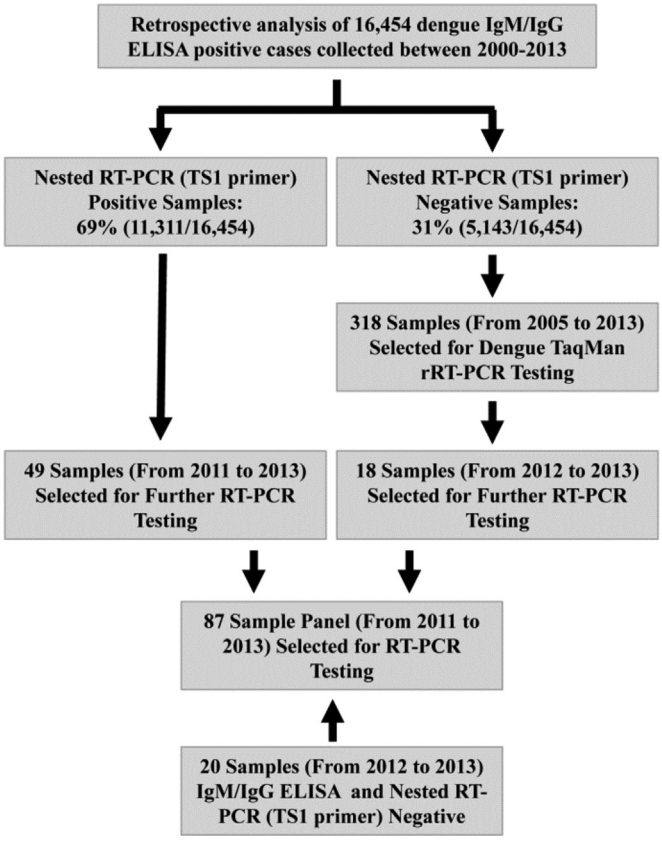

Fig. 1. Flowchart of the study design.

3.3. RNA extraction

Viral RNA was extracted from acute serum specimens using QIAamp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. The extracted RNA was preserved in the elution buffer at −70 °C until use in RT-PCR assays.

3.4. TaqMan rRT-PCR and Nested RT-PCR

TaqMan rRT-PCR was modified from Sadon et al. [13] by using the new DENV-1 primers and probe (TMF 5′-gcattyctaagatttctagccatacc-3′, TMR 5′-tcgctccattcttcttgaatgag-3′, and TMP 5′-aacagcaggaatttt-3′). One-Step RT-PCR kit (Ambion, USA) was used in this assay. Accumulation of PCR products was detected by monitoring the fluorescence emitted by probe hydrolysis using SDS software. The amplification cycle (Ct value) at which fluorescence was detected above the threshold was determined. A sample was defined as positive if the Ct value was <40 based on background cross-reactivity of the primers and probes in non-template control reactions. The nested RT-PCR was performed according to the protocol of Lanciotti et al. with modifications as described by Klungthong et al. [9,23]. A specimen containing DENV-1, 2, 3 or 4 was identified by the detection of a DNA band of 482, 119, 290, or 392 base pairs (bp), respectively, on the gel loaded with nested PCR product corresponding to DNA amplified from the DENV genome in positive controls. The sequences of all primers used in nested RT-PCR are described in Table 2.

Table 1
Number and types of samples tested in this study.

Test	Year of collection	Number of samples	Sample types
TaqMan rRT-PCR	2005	45	Human sera from IgM/IgG ELISA positive and nested RT-PCR negative cases
	2006	44	Human sera from IgM/IgG ELISA positive and nested RT-PCR negative cases
	2007	36	Human sera from IgM/IgG ELISA positive and nested RT-PCR negative cases
	2008	88	Human sera from IgM/IgG ELISA positive and nested RT-PCR negative cases
	2009	43	Human sera from IgM/IgG ELISA positive and nested RT-PCR negative cases
	2010	44	Human sera from IgM/IgG ELISA positive and nested RT-PCR negative cases
	2012	15	RNA from IgM/IgG ELISA positive and nested RT-PCR negative cases
	2013	3	RNA from IgM/IgG ELISA positive and nested RT-PCR negative cases
	Total	318	
Sensitivity test using four RT-PCR methods	2011	15	DENV isolates
	2012	12	RNA from IgM/IgG ELISA and nested RT-PCR positive cases
	2012	15	RNA from IgM/IgG ELISA positive and nested RT-PCR negative cases
	2012	13	RNA from IgM/IgG ELISA and nested RT-PCR negative cases
	2013	22	RNA from IgM/IgG ELISA and nested RT-PCR positive cases
	2013	3	RNA from IgM/IgG ELISA positive and nested RT-PCR negative cases
	2013	7	RNA from IgM/IgG ELISA and nested RT-PCR negative cases
	Total	87	

Table 2
List of primers used in nested RT-PCR.

Primer Name[a]	Sequence (5′-3′)[b]	Serotype specificity	Genome position[c]	Size (bp) of amplified DNA product (primers)[d]
D1	tcaatatgctgaaacgcgcgagaaaccg	DENV-1–4	134–161	NA
D2	ttgcaccaacagtcaatgtcttcaggttc	DENV-1–4	616–644	511 (D1 and D2)
TS1	cgtctcagtgatccggggg	DENV-1	581–599	482 (D1 and TS1)
TS2	cgccacaagggccatgaacag	DENV-2	232–252	119 (D1 and TS2)
TS3	taacatcatcatgagacagagc	DENV-3	400–421	290 (D1 and TS3)
TS4	ctctgttgtcttaaacaagaga	DENV-4	506–527	392 (D1 and TS4)
TS1.2013TH	mgcytcagtgattckagg	DENV-1	582–599	482 (D1 and TS1.2013TH)

[a] D1 is a forward primer and the others are reverse primers. D1 and D2 are used for RT-PCR. D1, TS1, TS2, TS3, TS4 and TS1.2013TH are used for nested PCR.
[b] Sequences of D1, D2, TS1–4 primers are obtained from Lanciotti et al. [9]. Sequence of TS1.2013TH primer is obtained from this study.
[c] The genome positions of D1 and D2 are given according to DENV-2 strain ThNH81/93 (GenBank Accession No. AF169688) and those of TS1.2013TH, TS2, TS3, and TS4 are based on the numbering of DENV-1Cambodia strain (GenBank Accession No.AF309641), DENV-2 ThNH81/93 strain (GenBank Accession No. AF169688), DENV-3 80–2 strain (GenBank Accession No. AF317645), and DENV-4 814669 strain (GenBank Accession No. AF326573), respectively.
[d] The size of the amplified product obtained from each of the type-specific primers (TS1–TS4) is determined from the priming position of primer D1 within each respective genome. The priming position for D1 in each DENV genome is as follows: type 1: 118–145; type 2: 134–161; type 3: 132–159; and type 4: 136–163.

3.5. TS1.2013TH primer design

The *C*/*prM* gene sequences were obtained from 10 contemporary DENV-1 strains tested by IgM/IgG ELISA, nested RT-PCR and TaqMan rRT-PCR, and from 16 other sequences retrieved from GenBank. All sequences were analyzed and compared to the sequence of the nested RT-PCR primers (Fig. 2). The primer TS1.2013TH was designed based on the TS1 primer binding site sequences and was used to replace the TS1 primer (Table 2).

3.6. CDC DENV-1-4 rRT-PCR

The CDC DENV 1-4 rRT-PCR was used for comparison of the sensitivity of DENV detection against the other three methods including the nested RT-PCR using the DENV-1 specific TS1 primers, nested RT-PCR using the DENV-1 specific TS1.2013TH primer, and TaqMan rRT-PCR. The assay was performed using a kit provided by the CDC and following their previously described protocol [15,16].

4. Results

4.1. Analysis of nested RT-PCR results obtained from dengue IgM/IgG ELISA positive cases

We evaluated 16,454 acute serum specimens collected during 2000–2013 and which were positive for DENV by IgM/IgG ELISA. Of these cases, 69% (11,311/16,454) were nested RT-PCR positive and 31% (5143/16,454) were nested RT-PCR negative, suggesting that the nested RT-PCR may have had suboptimal sensitivity. The rate of negative nested RT-PCR results ranged from 17 to 42%, depending on the year (Fig. 3). When we stratified the results into 0–5 and >5 DOI groups (Table 3), the rate of negative nested RT-PCR among specimens collected after 5 DOI was 53% (1823/3,418) compared to 25% (3320/13,036) among specimens collected at 0–5 DOI.

4.2. Detection and serotyping of DENV by TaqMan rRT-PCR

We selected 318 samples that were IgM/IgG ELISA positive (Table 1) but nested RT-PCR negative, and tested them by TaqMan rRT-PCR. These samples included 181 (57%) samples collected within 0–5 DOI and 137 (43%) samples collected >5 DOI. Of these, 194 (61%) were positive for DENV by TaqMan rRT-PCR (Table 4), including 142 DENV-1 (45%), 40 DENV-2 (13%), 4 DENV-3 (1%), 0 DENV-4 (0%) and 8 with both DENV-1 and DENV-2 (2%). Among the positive results, 65% (118/181) and 55% (76/137) were from samples collected within 0–5 and >5 DOI, respectively.

Table 3
Number and percentages of nested RT-PCR positive and negative results of dengue IgM/IgG ELISA positive cases with known days of illness.

Nested RT-PCR results	Number of samples with different days of illness[a] (%)		Total (%)
	0–5 DOI	>5DOI	
Positive	9716 (75)	1595 (47)	11311 (69)
Negative	3320 (25)	1823 (53)	5143 (31)
Total	13036	3418	16454

[a] DOI: days of illness.

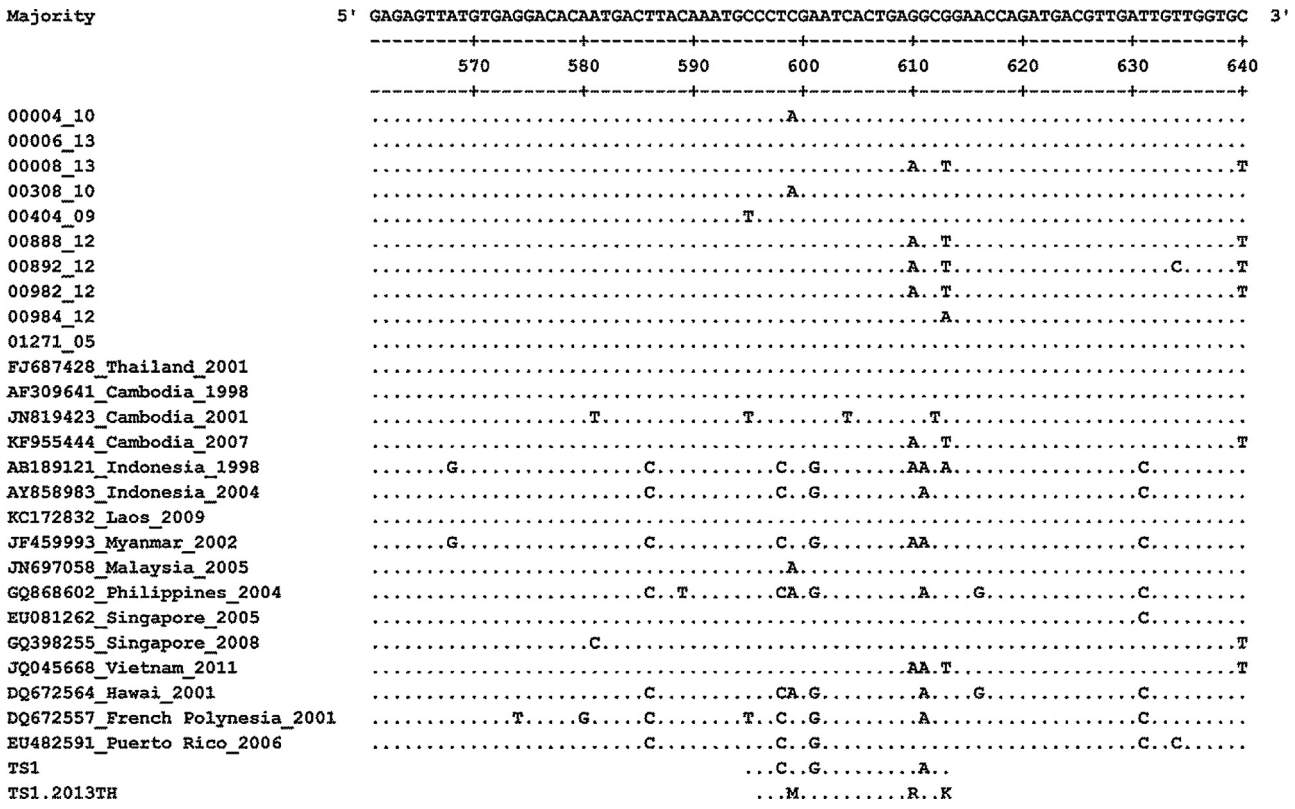

Fig. 2. Nucleotide alignment of TS1 primer binding regions from 26 DENV-1 strains including 10 Thai strains used in this study and 16 other strains from GenBank (GenBank Accession No. AB189121, AF309641, AY858983, FJ687428, JF459993, JN697058, JN819423, KF955444, KC172832, JQ045668, GQ868602, GQ398255, EU081262, DQ672557, DQ672564, and EU482591). TS1 and TS1.2013TH sequences shown in this figure are reverse complement sequences of TS1 and TS1.2013TH primers, respectively. Genome positions are given according to DENV-1 Singapore strain GenBank accession number GQ398255. The dots represent the bases common to most strains.

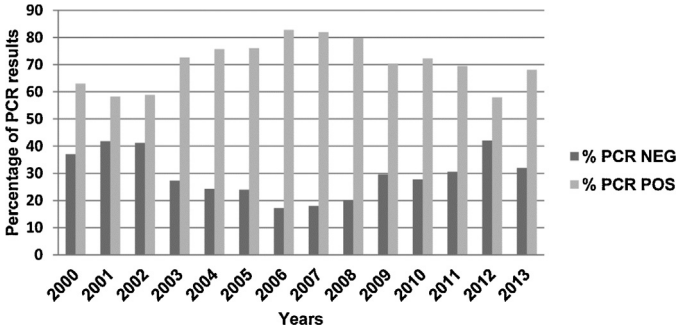

Fig. 3. Percentages of nested RT-PCR positive and negative results from dengue IgM/IgG ELISA positive cases in each year.

4.3. DENV-1 primer binding sites analysis

Given the high percentage (45%) of misdetection by the nested RT-PCR among DENV-1, we analyzed the sequences of the primer binding sites within the *C/prM* gene regions obtained from 10 DENV-1 collected during 2005, 2009–10, and 2012–13 that were positive by IgM/IgG ELISA and TaqMan rRT-PCR, but negative by nested RT-PCR. Table 5 shows the number of mismatched nucleotides of the templates against the sequences of the nested RT-PCR primers. There were no mismatches in the forward primer (D1) binding site in any of the 10 samples. However, the sequences of all 10 samples displayed 4–6 mismatches in the first round reverse primer (D2) binding site and 2–4 mismatches in the second round primer binding site (TS1). Based on these sequences, we designed a new DENV-1 specific primer, TS1.2013TH, to replace TS1. TS1.2013TH is one base shorter than TS1 and is different by 6 bases (Table 2).

4.4. Sensitivity and specificity of DENV detection by nested RT-PCR using the TS1.2013TH primer.

Using the TS1.2013TH primer, we tested the sensitivity and specificity of the nested RT-PCR using a panel of 87 samples collected during 2011–2013 (Table 1). The same samples were tested in parallel with the nested RT-PCR using the TS-1 primer, the TaqMan rRT-PCR and the CDC rRT-PCR. The sensitivity, specificity, PPV, and NPV of DENV detection of all 4 DENV serotypes are summarized in Table 6. The nested RT-PCR using TS-1 primer showed the lowest overall sensitivity (73%) and the lowest NPV

Table 4
TaqMan rRT-PCR results of 318 samples from dengue IgM/IgG ELISA positive cases with nested RT-PCR negative result.

TaqMan rRT-PCR results	Number of samples by DOI[a] (%)		Total (%)
	0–5 DOI	>5 DOI	
Positive	118 (65)	76 (55)	194 (61)
DENV-1	77 (43)	65 (47)	142 (45)
DENV-2	31 (17)	9 (7)	40 (13)
DENV-3	4 (2)	0 (0)	4 (1)
DENV-1 and DENV-2	6 (3)	2 (1)	8 (2)
Negative	63 (35)	61 (45)	124 (39)
Total	181	137	318

[a] DOI: days of illness.

Table 5
Sequences of primer-binding sites of 10 DENV-1.

Primer region on DENV-1 template	Sequences of primer attached regions of 10 samples[a]	No. of mismatched nucleotides	No. of viruses containing the examined sequence/total tested No.
Forward primer D1 region (5′-3′)	tcaatatgctgaaacgcgcgagaaaccg	0	10/10
Reverse primer D2 region (5′-3′)	ttgcaccaacaAtcaaCgtcAtcTggttc	4	6/10
	ttAcaccaacaAtcaaCgtcAtcTggttc	5	3/10
	ttAcaccaGcaAtcaaCgtcAtcTggttc	6	1/10
Reverse primer TS1 region (5′-3′)	cgCctcagtgatccgAggg	2	3/10
	TgCctcagtgatccgAggg	3	1/10
	cgCctcagtgatccTAggg	3	2/10
	AgCTtcagtgatccgAggg	4	4/10

[a] The mismatched nucleotides against the sequences of primers are shown with bold capital letter.

Table 6
Sensitivity and specificity of DENV detection using the four methods.

PCR results	Dengue positive by IgM/IgG ELISA or Isolation	Dengue negative by IgM/IgG ELISA or Isolation	Total	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Nested RT-PCR, TS1primer, positive	49	0	49	73 (61–83)	100 (76–100)	100 (89–100)	53 (36–69)
Nested RT-PCR, TS1 primer, negative	18	20	38				
Total	67	20	87				
Nested RT-PCR,TS1.2013TH, positive	58	0	58	87 (76–94)	100 (76–100)	100 (91–100)	69 (49–85)
Nested RT-PCR,TS1.2013TH, negative	9	20	29				
Total	67	20	87				
TaqMan rRT-PCR, positive	58	0	58	87 (76–94)	100 (76–100)	100 (91–100)	69 (49–85)
TaqMan rRT-PCR, negative	9	20	29				
Total	67	20	87				
CDC rRT-PCR, positive	57	0	57	85 (74–93)	100 (76–100)	100 (91–100)	67 (47–83)
CDC rRT-PCR, negative	10	20	30				
Total	67	20	87				

(53%). The sensitivity improved to 87% when using the TS1.2013TH primer ($P = 0.022$), while the NPV increased to 69%. The sensitivity of the nested RT-PCR using the TS1.2013TH primer was comparable to that of the TaqMan and the CDC rRT-PCR (87% and 85%;respectively, $P = 1.000$). The sensitivity varied for each serotype and is summarized in Table 7. The sensitivity of the nested RT-PCR using the TS-1 primer was only 48% for DENV-1, while that for the other 3 serotypes were 92% or better. Using the TS1.2013TH primer significantly increased the sensitivity for DENV-1 to 88% ($P = 0.006$), comparable to that of the TaqMan (100%) and the CDC rRT-PCR (88%) ($P = 0.250$ and 1.000, respectively). There were no significant differences in the sensitivity of detection for the other serotypes by the nested RT-PCR using the TS1.2013TH primer when compared with the other three methods ($P > 0.05$). We also attempted modifications to the first round reverse primer, D2 primer, but the results showed lower sensitivity and specificity (data not shown). All four methods showed 100% specificity. The nested RT-PCR using the TS1.2013TH primer was also tested using purified RNA obtained from chikungunya, Japanese encephalitis, West Nile, and Zika viruses. There was no PCR product with dengue-specific size detected from these samples (data not shown).

4.5. DENV detection sensitivity by multiple RT-PCR methods and stratified by DOI.

Among the 87 samples tested by all four RT-PCR methods, 72 were RNA samples previously extracted from acute serum. These samples had a recorded DOI. The sensitivities of the four methods tested with these 72 samples were analyzed based on DOI and are shown in Table 8. The nested RT-PCR using TS1 primer showed the lowest DENV detection sensitivity in samples collected at 0–5 and >5 DOI (89% and 38%, respectively). However, replacement of the DENV-1 specific primer with the TS1.2013TH primer significantly improved the sensitivity (96%) of the nested RT-PCR and made it comparable to the sensitivities of all the methods ($P > 0.05$) among samples collected at 0–5 DOI. For samples collected at >5 DOI, the sensitivity of the nested RT-PCR increased to 67% when using the TS1.2013TH primer ($P = 0.07$). This sensitivity was comparable to the TaqMan (71%) and the CDC rRT-PCR (67%) ($P = 1$).

5. Discussion

Nested RT-PCR is a cost-effective, rapid and sensitive diagnostic and surveillance tool used for the detection and identification of

Table 7
DENV serotype detection sensitivity by all RT-PCR methods.

Methods	Sensitivity of DENV detection (95% CI)				
	All serotypes	DENV-1[a] ($n = 25$)	DENV-2[a] ($n = 12$)	DENV-3[a] ($n = 13$)	DENV-4[a] ($n = 13$)
Nested RT-PCR, TS-1 primer	73 (61–83)	48 (28–69)	92 (61–99)	100 (75–100)	100 (75–100)
Nested RT-PCR, TS1.2013TH primer	87 (76–94)	88 (69–97)	92 (61–99)	92 (64–99)	100 (75–100)
TaqMan rRT-PCR	87 (76–94)	100 (80–100)	100 (73–100)	69 (39–91)	92 (64–99)
CDC rRT-PCR	85 (74–93)	88 (69–97)	92 (61–99)	100 (75–100)	85 (55–98)

[a] The DENV serotypes among the ELISA positive samples ($n = 48$) and virus isolates ($n = 15$) were determined by one or more RT-PCR methods tested in this study.

Table 8
Sensitivity of DENV detection of 72 samples based on days of illness.

RT-PCR results	Number of samples from 0 to 5 DOI[a]				Number of samples from >5 DOI[a]			
	Dengue positive by IgM/IgG ELISA	Dengue negative by IgM/IgG ELISA	Total	Sensitivity (95% CI)	Dengue positive by IgM/IgG ELISA	Dengue negative by IgM/IgG ELISA	Total	Sensitivity (95% CI)
Nested RT-PCR, TS-1 primer								
Positive	25	0	25	89 (72–98)	9	0	9	38 (19–59)
Negative	3	10	13		15	10	25	
Total	28	10	38		24	10	34	
Nested RT-PCR, TS1.2013TH								
Positive	27	0	27	96 (82–100)	16	0	16	67 (45–84)
Negative	1	10	11		8	10	18	
Total	28	10	38		24	10	34	
TaqMan rRT-PCR								
Positive	26	0	26	93 (76–99)	17	0	17	71 (49–87)
Negative	2	10	12		7	10	17	
Total	28	10	38		24	10	34	
CDC rRT-PCR								
Positive	26	0	26	93 (76–99)	16	0	16	67 (45–84)
Negative	2	10	12		8	10	18	
Total	28	10	38		24	10	34	

[a] DOI: days of illness.

DENV in acute specimens. It continues to be widely used in clinical, epidemiological and entomological studies [6–8,24–29]. Nonetheless, due to the accumulated spatiotemporally-dependent changes in the virus genome, continuous monitoring of assay sensitivity is required to ensure the validity and accuracy of the assay.

A modified Lanciotti nested RT-PCR method has been used to support surveillance and epidemiological studies [6–8,10–12]. This method was deemed optimal for DENV diagnosis and surveillance by an international external quality control assessment (EQA) [30]. However, this evaluation was based on blind panels of diluted DENV control strains that no longer represent currently circulating, DENV strains: DENV-1 VR344 (Thailand 1958), DENV-2 VR345 (TH-36 strain, Thailand 1958), DENV-3 VR216 (H87 strain, Philippines 1956) and DENV-4 VR217 (H241 strain, Philippine 1956). Routine monitoring of the assay using circulating strains may be a more suitable method for quality control and assay validation. Our analysis of nested RT-PCR results of samples containing DENV collected during the past 14 years (2000–2013) showed a 31% false negative rate, of which 25% were samples collected when viremia is common (5 days or less after the onset of symptoms) [31]. Genetic variations at the sites of primer binding are likely to have contributed to this high rate of false negatives.

DENV has no proof-reading activity during replication and has almost 8% nucleotide sequence diversity among DENVs within each serotype [32]. Nucleotide changes may limit the sensitivity of DENV detection by genomic amplification methods like RT-PCR. The TaqMan rRT-PCR used in this study could detect up to 61% of DENV from samples that could not be detected previously by the nested RT-PCR using the TS1 primer. Among the TaqMan rRT-PCR positive samples, 45% were DENV-1. This was consistent with a previous report that TaqMan rRT-PCR sensitivity for DENV-1 was higher than nested RT-PCR [13]. This was also consistent with our surveillance data showing that DENV-1 circulated at the highest frequency during 2005–2013 (data not published). Using TaqMan rRT-PCR, we detected DENV in acute serum specimens collected at 0–5 and >5 DOI (65% and 55%, respectively), indicating that despite the presence of DENV genomic RNA in samples collected early and late during illness, we could not detect it by nested RT-PCR. This suggested that the TS-1 primer designed based on DENV sequences circulating during 1986–1990 had lower sensitivity for contemporary viruses.

Since DENV-1 detection showed the lowest sensitivity and a high number of mismatches at the TS-1 primer binding site, we designed the DENV-1 specific TS1.2013TH primer based on sequences obtained from contemporary DENV-1 strains. Using TS1.2013TH, the sensitivity of DENV detection improved from 73% to 87% for all DENV serotypes, and for DENV-1 detection from 48% to 88%. In addition, the sensitivity of DENV detection by nested RT-PCR increased when using the new primer not only in samples collected 0–5 days after illness onset but also in samples collected >5 days after illness onset. The sensitivity and specificity for the other serotypes were not affected when using the TS1.2013TH primer. The improved sensitivity obtained by using the new primer was comparable to that of the TaqMan and the CDC rRT-PCR.

In conclusion, the sensitivity of the nested RT-PCR was substantially improved by the development of a new primer. These findings highlight the impact of genetic diversity on the sensitivity of RT-PCR methods, a critical concern for assay development, quality control, and surveillance.

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Competing interests

None declared.

Ethical statement

The research was done after permission granted by Walter Read Armed Institute of Research (WRAIR), USA. WRAIR is the parent organization of AFRIMS.

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