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Rapid detection and differentiation of dengue virus serotypes by NS1 specific reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay in patients presenting to a tertiary care hospital in Hyderabad, India

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A B S T R A C T

Early and rapid detection of dengue virus (DENV) infection during the acute phase of illness is crucial for proper patient management and prevention of the spread of the infection. In the present study, the standardization and validation of a one step, four tube reverse transcription loop-mediated isothermal amplification assay (RT-LAMP) for rapid detection and serotyping of the DENV targeting NS1 gene using the Genie® II flourometer was carried out. The performance of the RT-LAMP was compared to RT-PCR, CDC 1-4 Real time PCR and the NS1 antigen ELISA, IgM and IgG anti DENV antibodies. Acute DENV infection was confirmed in 250/300 patients suspected clinically of DENV infection. RT- LAMP and CDC 1-4 Real time PCR assay was positive in 148/250 patients, while 92/250 patients were positive for anti- Dengue IgM and IgG antibodies. The RT-LAMP assay and the CDC real-time RT-PCR assay showed high concordance (k = 1.0). The detection rate of acute DENV infection improved to 96% (240/250) when the results of RT-LAMP were combined with NS1 Ag, IgM and IgG ELISA. The RT-LAMP had a detection limit of 100 copies for DEN-1 and DEN-2, 10 copies for DEN-3 and DEN-4 compared to 1000 copies for DEN-1 and DEN-2, 100 copies for DEN-3 and DEN-4 by the conventional RT-PCR. The assay showed 100% specificity. The RT-LAMP assay developed in this study has potential use for early clinical diagnosis, serotyping and surveillance of DENV infection in endemic countries such as India.

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1. Introduction

Dengue is a mosquito borne flaviviral infection, affecting the tropical and subtropical regions of the world and is one of the major emerging global public health problems. There are four antigenically distinct dengue virus serotypes DEN-1, DEN-2, DEN-3 and DEN-4 and each serotype contains phylogenetically distinct genotypes (Teoh et al., 2013). The Dengue virus (DENV) infection induces a lifelong protective immunity to the homologous serotype but confers only partial and transient protection against subsequent infection by the other three serotypes. Therefore, multiple and sequential infections with the four DENV serotypes would be expected for people living in a region where the infection is hyper endemic due to the lack of cross-protective neutralizing antibodies. Seroepidmiological studies have shown that the secondary infection is a major risk factor for Dengue hemorrhagic Fever (DHF) and Dengue shock syndrome (DSS) through antibody-dependent enhancement (Halstead et al., 1970; Monath and Heinz, 1996). Diagnosis of DENV infection on the basis of clinical signs and symptoms is not reliable as more than half of the infected individuals either are asymptomatic or have a mild undifferentiated fever (Burke et al., 1988; Endy et al., 2002). Early diagnosis of dengue infection can reduce the number of cases of DHF and DSS. Therefore, there is a great demand for the rapid detection of the infection and differentiation of DENV

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serotypes for timely clinical management and disease control, respectively.

The most common methods for laboratory diagnosis of DENV include serological methods detecting antibodies (IgM and IgG) against DENV and additionally various methods are used in detecting DENV RNA or antigens: non-structural protein 1 (NS1) and envelope protein (E). The serological methods are vulnerable to cross reactions caused by antibodies against related flaviviruses and are therefore not DENV-specific tests like DENV NS1 antigen and RNA detection methods. The detection of dengue specific secretory NS1 (non-structural protein 1), a highly conserved glycoprotein represents a new approach to the diagnosis of acute DENV infection, in recent times. Enzyme-linked immunosorbent assays (ELISA) directed against NS1 antigen (NS1 Ag) have demonstrated its presence at high concentrations in the sera of DV infected patients during the early clinical phase of the disease (Dussart et al., 2006). Assays based on the detection of nonstructural protein 1 (NS1) tend to be specific for DENV infection. NS1 antigen levels correlate well with viremia and it circulates at high levels during the first few days of illness especially in patients with DHF. NS1 antigen remains circulating in patients' blood for longer periods than does viral RNA and is reported to be detectable even up to the 14th day of illness.

The first isothermal amplification methods introduced in 1990s included the transcription mediated amplification (TMA), nucleic acid sequence based amplification (NASBA) and the strand displacement amplification (SDA). The Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method and has the potential to replace PCR because of its simplicity, rapidity, specificity, sensitivity and cost-effectiveness without the need of specialized equipment (Notomi et al., 2000; Parida et al., 2005; Tomita et al., 2008). The RT-LAMP assay is being increasingly used by various investigators for rapid detection and typing of emerging viruses (Chan and Fox, 1999; Mori et al., 2001; Parida et al., 2005). These earlier reports, however, evaluated their RT-LAMP assays for the detection of DENV infection with a small clinical sample size (<100) and using the C-prM gene (Lu et al., 2012) or serotype-specific regions of the 3′ untranslated region (UTR) (Parida et al., 2005; Li et al., 2011; Sahni et al., 2013). The C-prM gene, however, was relatively less conserved among all the four DENV serotypes (inter-serotype) in comparison to the 3′UTR (Teoh et al., 2013). However, we have targeted a highly conserved region of NS1, revealing >90% sequence identity among various genotypes within each serotype. As such genotyping of dengue serotypes can be done employing many gene including NS3 and NS5 (Klungthong et al., 2008).

In the present study, the RT-LAMP assay was developed for the detection and serotyping of DENV infection targeting the serotype specific regions of the NS1 gene using a real-time flourometer (Genie® II from Optigene, U.K.). The detection sensitivity and the specificity of the reported DENV NS1 serotype specific RT-LAMP in freshly obtained blood samples from 300 patients suspected clinically of DENV infection, is compared with available test system for suitable algorithm. To the best of our knowledge this is the first report of the detection and differentiation of dengue using NS1 RT-LAMP with real time fluorometer (Genie® II from Optigene, U.K.) from South India.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Institutional Ethics Committee of Nizam's Institute of Medical Sciences (EC/NIMS/1336/2012). Written informed consent was obtained from each patient.

2.2. Viruses

Reference Strains of the four dengue virus serotypes DEN-1, RR107 (KF289072), DEN-2, GWL18 (AY324614), DEN-3, ND143 (FJ644564), DEN-4, ND 73 (HM237348) were used in this study (Dash et al., 2004, 2011; Neeraja et al., 2013).

2.3. Clinical samples

300 Patients suspected clinically of Dengue/DHF/DSS, who either reported directly or were referred to a tertiary care institute for treatment from the regions in and around Hyderabad, from July 2011 to December 2013, were included in the study. The Dengue/DHF/DSS case proformas prepared as per the WHO protocol (World Health Organization, 2009) for DENV infection was filled by the treating clinicians. 300 acute phase and early convalescent serum and plasma samples based on reporting time were collected from patients with a history of sudden onset of fever, and the presence of two or more of the symptoms viz. headache, eye pain, nausea, vomiting, rash, myalgia, abdominal pain suggestive of DENV infection.

Samples collected within 7 days of fever were categorized as acute phase samples and those collected after 7 days of fever were considered as convalescent phase sample.

In order to check the cross-reactivity, within 4 serotypes and with other closely related members of flavivirus family i.e., 10 JE, 10 WNV archived samples from DRDE Gwalior and 10 HCV positive samples from our tertiary care hospital were included in the study.10 confirmed Chikungunya (CHIKV) RNA positive samples were also included as symptoms of DENV and CHIKV mimic each other. In addition, a panel of 50 samples collected from healthy individuals was included as negative controls. Before performing the RT-LAMP assay, all the 350 samples were also screened for DENV-specific RNA by RT-PCR (Lanciotti et al., 1992; Neeraja et al., 2013) and NS1 antigen by Panbio Dengue Early ELISA assay (Inverness Medical Innovations, Australia), Dengue IgG and IgM capture ELISA (Pan Bio, Queensland, Australia).

2.4. Design of DENV serotype -specific RT-LAMP primers

DENV serotype specific oligonucleotide primers were designed from the NS1 region of DENV genome. The nucleotide sequence of the NS1 gene of DENV, representative of respective genotype and serotype strain was retrieved from Gen Bank (DEN-1, accession no. EU863647; DEN-2, accession no. AF162448; DEN-3, accession no. EU371057; and DEN-4, accession no KC620380) and was aligned with the available NS1 gene sequences from global DENV strains including the circulating strains in India, to identify the conserved regions using DNASIS software (Hitachi, Japan). The primers were selected based on criteria described by Notomi et al. Percent gene homology among each serotype were found to be ≥90%.

The potential target region corresponding to the genome positions was selected from the aligned sequences, and the RT-LAMP primers were designed from conserved region of each serotype using the Primer Explorer version 4 software (Eiken Chemical Co., Tokyo, Japan). A set of six primers comprising two outer (F3 and B3), two inner (FIP and BIP), and two loop primers (FLP and BLP) that recognize eight distinct regions on the target sequence was designed. The primers were selected based on criteria described previously by Notomi et al.

All the primers were assessed for specificity before use in LAMP assays with a BLAST search with sequences in the Gen Bank (Table 1).

Table 1
Details of the NS1 serotype specific RT-LAMP assay primer sets used for rapid detection and differentiation of dengue virus serotypes 1, 2, 3, and 4 targeting the NS1 region of the viral genome.

Virus serotype	Primer	Sequence (5′ → 3′)	Target size
DENV-1	F3	TGTGGGAAAACTGGTACAC	191 bp
	B3	CCTGGACCATGACTCCTAG	
	FIP	ATCCCTATTCCTATTTTCATGGTCCTTTTCAGATCTTTGGAACTGCGT	
	BIP	CTGCTGACATGGCTGGGATTATTTTGTACAGTGTGACCATGCC	
	FLP	CACCGCTGAACAAAACTCCAT	
	BLP	TCCCTTTCAATGACGTGTATCG	
DENV-2	F3	CAAACAGCAGGACCTTGG	199 bp
	B3	ATCCATCCTCACCTCTGT	
	FIP	ACATTCTTCGGTTACCACCACTTTTTGCAAGCTTGAGATGGACT	
	BIP	AGAGGGCCTTCTTTAAGAACAACTTTTTGGTGGTAGTGTGCAAGA	
	FLP	TGGTCCCTTCGCAGAAATCAA	
	BLP	CACTGCCTCAGGAAAACTCATAA	
DENV-3	F3	CCCTCATAGAGGTGAAAACC	186 bp
	B3	TGAAGTCCAGCTCCAATT	
	FIP	GGAATGATCATGTCACTCTCTAGCTTTTGCACATGGCCAAAATCAC	
	BIP	AGAGTCTAGCTGGTCCTATTTCGTTTTTTCCTAAGTGCCAGGGTC	
	FLP	ACACCGTTGCTCCAAAGAGT	
	BLP	CAACACAACTACAGGCCCG	
DENV-4	F3	CGAGCTAAACTATGTTCTCTG	185 bp
	B3	TCAAAAATGTGCTATTTCTTGC	
	FIP	TGCCTTTGGTTAACACCCCTTTTTTGGAAGGAGGACATGACCT	
	BIP	GAGCACTCACACCTCCAGTGTTTTCTGGAGTGAAGATTTTTGC	
	FLP	GGCATAGCCCTGGCGATAA	
	BLP	GAATGCCCCGGAACAACAGTCAC	

F3, forward outer primer; B3, backward outer primer; FIP, forward inner primer; BIP, backward inner primer; FLP, forward loop primer; BLP, backward loop primer.

2.5. RNA extraction

The viral RNA was extracted from 140 µl of the serum/plasma samples by using the QIAamp viral RNA mini kit (Qiagen, Germany). The RNA was eluted from the QIA spin columns in a final volume of 50 µl of the elution buffer and stored at −80 °C until testing.

2.6. Optimization of the RT-LAMP reaction

The RT- LAMP was carried out in a final reaction volume of 25 µl. The reaction mixture contained 15 µl of Isothermal Master Mix ISO-001 (Optigene, U.K.) containing, *Geobacillus* species DNA polymerase, thermostable inorganic pyrophosphatase, optimized buffer including MgCl2, dNTPs and ds-DNA dye (Optigene, U.K.), 1 µl Primer mix consisting of 6 primers each for DENV-1, DENV-2, DENV-3, and DENV-4 (F3 and B3 primers at 0.2 µM, FIP and BIP primers at 0.8 µM, LF and LB primers at 0.4 µM), 0.25 Units AMV reverse transcriptase (Promega, Madison, WI.), 3.75 µl Nuclease free water and 5 µl extracted nucleic acid.

The RT-LAMP assay was run at temperatures between 62 and 67 °C and time between 60 min and 35 min in the real-time fluorometer (Genie® II from Optigene, U.K.) to determine the optimal temperature with the shortest amplification time and the highest fluorescence reading. All the RT-LAMP assays were subsequently run at 63 °C for 35 min followed by a heating and cooling step to 98 °C to 80 °C (0.05 °C/s) to allow re-annealing of amplified DNA and display of the annealing curve. The Genie II displays amplification signals in real time and at the end of the run displays the time to positivity that is expressed in terms of plots of fluorescence signals (real time curves) and T_m for each specimen. The analysis of each sample was done in a set of four tubes, with serotype specific primer mixture. The T_m for DENV-1 was 82.42 °C, DENV-2 was 84.67 °C, DENV-3 was 86.17 °C and DENV-4 was 88.12 °C.

Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed. Amplification of the DNA leads to an increase in fluorescence emitted from a DNA intercalating dye. This increase was monitored in real time using the Genie® II fluorometer.

2.7. Detection methods for RT LAMP results

2.7.1. Agarose gel analysis

Following incubation at 63 °C for 35 min, a 10 µl of aliquot of the RT-LAMP assay products was electrophoresed on 3% NuSieve 3:1 agarose gel (Biowhittaker Molecular Applications, Rockland, Maine) in trisborate buffer, followed by staining with ethidium bromide and visualization on a UV transilluminator at 302 nm.

2.7.2. Visual detection

In order to facilitate the field application of the RT-LAMP assay, monitoring of amplification was done visually with an unaided eye. Following amplification in Genie® II flourometer 1 µl of SYBR Green I intercalating dye was added to the reaction tube. The RT-LAMP amplification was visually monitored for colour change. Positive reaction turned the reaction mix green and fluoresces under the white light and UV irradiation, respectively. The reaction mix remained orange and non-fluorescent in the absence of amplification. This change of color is permanent and thus can be kept for record purposes.

2.8. Comparison of RT-LAMP with RT-PCR and CDC 1-4 Real time PCR

2.8.1. RT-PCR assay with F3, B3 primers

In order to compare the sensitivity and specificity of the RT-LAMP assay, one-step RT-PCR was done by employing the two outer primer pairs (50 pmol of F3 and B3) targeting the NS1 gene of each serotype. Amplification of the RNA was carried out in 50 µl reaction volume with the PCR mix containing PrimeScript™ 1 step Enzyme Mix and its buffer along with respective sense (F3) and anti sense (B3) primer in a thermal cycler (Applied Biosystems, USA). The thermal profile of the RT-PCR reaction was- reverse transcription at

50 °C for 30 min, initial denaturation at 95° C for 2 min, followed by 35 cycles of denaturation at 95° C for 1 min, annealing at 60° C for 1 min, extension at 72° C for 2 min and final extension at 72° C for 10 min (Neeraja et al., 2013).

2.8.2. CDC DENV 1–4 real-time RT-PCR assay

The real-time RT-PCR assay from CDC was used as a standard test for the DENV serotype specific identification in ABI 7500 quantitative PCR system (ABI, USA). The assay is based on Taqman chemistry including a panel of oligonucleotide primers and dual labeled hydrolysis probe sets [D1, D2, D3, D4] employing Invitrogen Super Script TMIII Platinum® one step quantitative kit. The amplification was carried out in a 25 μl reaction volume. Instruction and standard thermal profile for sample screening was as follows, reverse transcription 50 °C for 30 min, initial denaturation and enzyme inactivation 95 °C for 2 min, 45 cycles of extension at 95 °C for 15 sec and 60 °C for 1 min of denaturation and annealing extension respectively (Chien et al., 2006). Briefly, the reagents include 2× buffer (Invitrogen One-step RT-PCR kit, USA) 12.5 μl, enzyme mix 0.5 μl, D1/D3 both forward and reverse primers 0.5 μl (5 nM), D2/D4 both forward and reverse primers 0.25 μl (5 nM) and D1–D4 probe 0.45 μl (1 nM) each and DEPC treated water added up to a total volume of 25 μl. Finally, 5 μl of viral RNA elute extracted from different samples was added for real-time RT-PCR assay.

2.9. Performance parameters of DENV RT-LAMP

2.9.1. Sensitivity of serotype-specific dengue virus-specific RT-LAMP assay

The sensitivity of the NS1 serotype specific RT-LAMP assay was determined through serial dilutions of in vitro transcribed DENV with known copy number.

2.9.2. Specificity of RT-LAMP assay

The specificity of the primers for detecting DENV serotypes was validated by testing 10 samples that were positive for other *Flavivirus* including JE, WNV, HCV and CHIKV. In addition, the authenticities of the amplified products were also established by nucleotide sequencing of amplified products with outer (F3) and inner (B3) primers (Parida et al., 2007).

2.9.3. Nucleotide sequencing and phylogenetic analysis

Nucleotide sequencing of the NS1 gene of randomly selected Dengue viruses from the clinical samples, that included one DEN-2 (VL998) and two DEN-3 (VL524, VL2086), was carried out by employing the Big Dye Terminator Cycle Sequencing Ready Reaction kit with an ABI 3100 sequencer (Applied Biosystems, USA) for identifying the genotype of the DENV serotype by following the standard protocol (Dash et al., 2004) The sequences were initially subjected to BLAST to find the closest sequence identity. Further, phylogenetic analyses based on the NS1 gene junction of DEN-2 and DEN-3 were carried out by including a large number of geographically diverse DENV gene sequences, by using the Neighbour-Joining (NJ) method of the MEGA3 software version 3.1 (Kumar et al., 2004). The sequences of VL998, VL524, and VL2086 were submitted to GenBank under the accession numbers KC571834, KJ584531, KF301600, respectively.

2.9.4. Inter run assay

Inter-assay variability for reproducibility was assessed by testing 1 sample of each serotype in 5 separate LAMP runs and recording time and T_m for each serotype.

2.9.5. Intra run assay

The intra-assay variability for repeatability was assessed by simultaneously testing 4 samples of each serotype that included 2 strong positive and 2 weak positive of each serotype.

2.10. Statistical analysis

The degree of agreement between RT-LAMP and the CDC real time PCR test results was measured by kappa value (k). Fisher's exact test (two tailed) was done to calculate p value, p value <0.0001 was used to suggest significant results. The diagnostic performance of RT-LAMP assay and the CDC real time PCR assay as compared with NS1Ag and NS1 RT-PCR assay was calculated using Med Calc easy to use statistical software (http://www.medcalc.org/calc/diagnostic_test.php).

3. Results

250/300 Patients suspected clinically of DENV infection, were confirmed as acute DENV infection by detection of the NS1 Ag, anti-IgM, conventional RT-PCR, and the real-time RT-PCR either alone or in combinations.

138/250 Patients were positive by NS1 Ag alone. 92 out of the remaining 112 patients were positive only for anti-Dengue IgM and IgG antibodies. NS1 serotype specific RT-PCR assay was positive in 140/250 patients, NS1 serotype specific RT-PCR assay detected 2 patients that were negative for NS1 Ag by ELISA test. RT-LAMP assay detected 8 additional patients that were negative for NS1 Ag by ELISA and NS1 RT-PCR assay. 10/250 patients were identified as past DENV infection as dengue IgG antibodies alone were tested positive among them (Table 2).

3.1. Specificity of DENV RT LAMP primers

All the four DENV serotype-specific primers were highly specific for the detection and differentiation of the appropriate serotypes with no cross reaction. None of the serotype-specific primer sets amplified or cross reacted with any of the JE, WNV, HCV or CHIKV viral RNA template and 50 samples from healthy individuals, there by indicating their specificity. As depicted in Fig. 1, the size of the resultant product by RT-PCR using outer primers F3 and B3 was in good agreement with the predicted size for each serotype, i.e., 191 bp for DENV-1, 199 bp for DENV-2, 186 bp for DENV-3, and 185 bp for DEN-4, (Fig. 1). 100% sequence homology was also observed between the primers and the corresponding nucleotide sequences.

3.2. Sensitivity of serotype-specific dengue virus-specific RT-LAMP assay

The sensitivity of the in house developed DENV NS1 serotype specific RT-LAMP assay was same as that of the CDC 1–4 real time RT-PCR assay. These two methods showed high concordance with kappa value of 1.0.

The diagnostic accuracy improved to 96% (240/250, 95% confidence interval = 92.8–98.0) when the results of the DENV RT-LAMP or the CDC Real time assay were combined with the results of the NS1 antigen and anti -dengue IgG and IgM ELISA (Table 2)

The diagnostic performance of RT-LAMP compared to NS1 Ag by ELISA and the NS1 RT-PCR is summarized in Table 3.

The sensitivity of the NS1serotype specific RT-LAMP assay as a function of the timing of the test (days after onset of fever) was studied and it was found that the sensitivity was optimal, at 82.2% (95% CI, 75.9% to 87.7%), between days 0 and 5 for NS1 serotype specific RT-LAMP assay (Table 4).

Table 2
Sensitivity of the NS1serotype-specific dengue virus RT-LAMP assay compared to NS1Ag, IgG + IgM antibody, NS1 RT-PCR and the CDC real time RT-PCR assay.

Test	Positive	Negative	Sensitivity (%)	95% Confidence interval
NS1 Ag only	138	112	93.2	87.9–96.4
IgG + IgM only	92	158	62.2	54.1–69.6
[a]NS1RT-PCR + NS1Ag	140	110	94.6	89.5–97.4
[b]CDC real time PCR + NS1Ag	148	102	100	96.9–100
[b]RT-LAMP + NS1Ag	148	102	100	96.9–100

The CDC-Real time PCR assay was considered as gold standard control.
[a] NS1 RT-PCR detected 2 samples that were negative for NS1 Ag by ELISA.
[b] RT-LAMP and CDC real time RT-PCR assay detected 8 samples which were negative for NS1 Ag by ELISA and NS1 RT PCR assay.

Table 3
The diagnostic performance of the RT-LAMP assay against NS1Ag and the RT-PCR assay in dengue patients in tertiary care hospital in Hyderabad.

Assay	Results	RT-LAMP		Sensitivity% (95%CI)	Specificity% (95%CI)	PPV% (95%CI)	NPV (95%CI)
		Pos	Neg				
NS1Ag	Pos	138	0	93.2 (87.9–96.7)	100 (97.6–100)	100 (97.3–100)	93.8 (88.9–97)
	Neg	10	152				
RT PCR	Pos	140	0	94.6 (89.6–97.6)	100 (97. 6–100)	100 (97.3–100)	95 (90.4–97.8)
	Neg	8	152				

Table 4
The sensitivity of the NS1serotype specific RT-LAMP assay related to number of days after onset of fever (n = 148).

Days of onset of fever	RT-LAMP positive
1	38
2	29
3	26
4	20
5	20
6	10
≥7	5

The RT-LAMP assay was positive more in the patients with primary infections (110/148) compared to patients with secondary infections (38/148). p Value for the detection of primary infections by RT-LAMP was statistically significant when compared with secondary infections ($p < 0.0001$, by Fishers exact test). RT-LAMP was positive among 133 patients with DF, 10 patients with DHF and 5 patients with DSS.

The RT-LAMP assay detected 100 copies of DEN-1 and DEN-2 and 10 copies of DEN-3 and DEN-4 RNA, respectively, as shown in Fig. 2a and b and the sensitivity of RT-PCR was 1000 copies of DEN-1 and DEN-2 and 100 copies of DEN-3 and DEN-4 as shown in Fig. 2c and d.

Table 5
Amplification time and annealing temperatures for 4 DEN-1, 4 DEN-2, 4 DEN-3and 4 DEN-4 positive samples in Genie II instrument.

Specimen number	DENV-serotype	Amplification time (min)	T_m (°C)
VL1482	DENV-1	12:00	82.43
VL1555	DENV-1	11:45	82.48
VL1483	DENV-1	12:15	82.42
G1	DENV-1	12:00	82.45
VL1595	DENV-2	27:00	84.65
VL998	DENV-2	26:45	84.63
VL1465	DENV-2	27:00	84.68
VL2007	DENV-2	27:15	84.67
VL2086	DENV-3	18:00	86.17
VL514	DENV-3	18:15	86.18
VL1695	DENV-3	17:45	86.15
VL240	DENV-3	18:00	86.19
VL1893	DENV-4	11:30	88.12
VL1659	DENV-4	11:45	88.10
VL1866	DENV-4	11:15	88.15
VL1829	DENV-4	11:00	88.14

3.3. Optimization of DENV RT LAMP on the Genie® II fluorometer for DENV serotypes

The optimized amplification time of samples from 20 patients (5 of each serotype) by DENV RT-LAMP was 35 min. as shown in Table 5. The mean time to positivity for all 148 positives was 17 min

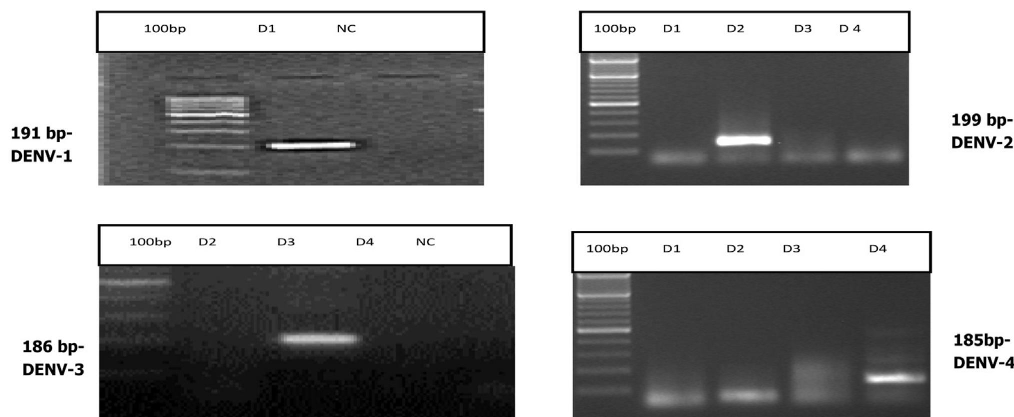

Fig. 1. Agarose gel electrophoresis of DENV serotype-specific RT-PCR assay products on a 3% agarose gel employing F3 and B3 primers of respective serotypes. D1—DENV-1 RT-PCR assay product, 191 bp; D2—DENV-2 RT-PCR assay product, 199 bp; D3—DENV-3 RT-PCR assay product, 186 bp; D4—DENV-4 RT-PCR assay product 185 bp.

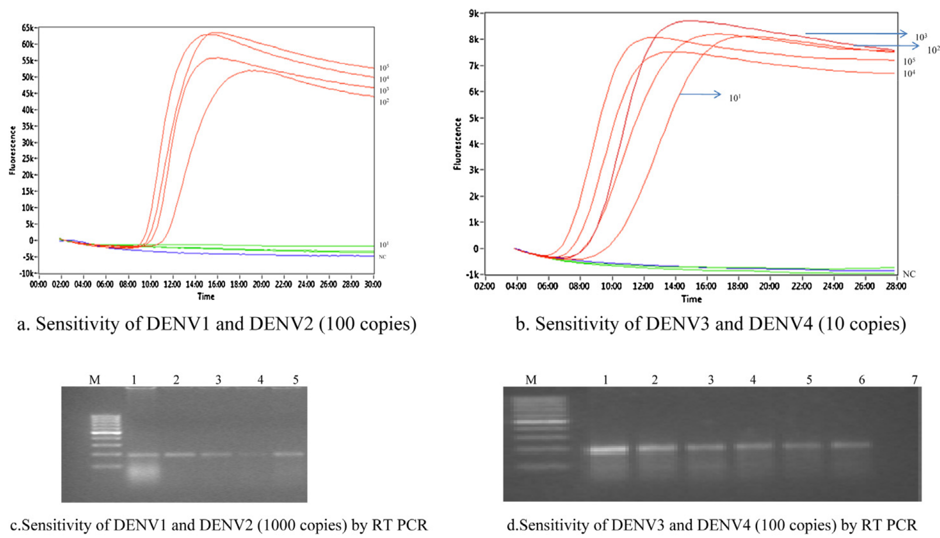

Fig. 2. Comparative sensitivity of RT-LAMP versus RT-PCR for detection of the NS1 gene of DENV. Sensitivity of the RT-LAMP assay as monitored by real-time measurement of fluorescence. Shown from left to right are the curves of decreasing concentrations of virus from 1×10^5 to 1×10^1 copy numbers of the template in a serial 10-fold dilution. The detection limit for the assay was 100 copy numbers for DENV1 and DENV2 (a) and 10 copy numbers for DENV3 and DENV4 (b). (c and d) Sensitivity of RT-PCR for the detection of the DENV NS1 gene as observed by agarose gel analysis with a detection limit of 1000 copy numbers for DENV1 and DENV2 and 100 copy number for DENV3 and DENV4. Lane M, 100-bp DNA ladder (Sigma); lanes 1 to 7, different concentrations of virus ranging from 1×10^7 to 1×10^{-1} copy numbers in a serial 10-fold dilution pattern.

(range 9–30 min). The mean amplification time for 4 DEN-1 positives was 12.5 min (range 10–15.5 min), for 60 DEN-2 positives was 27 min (range 22–30 min), 80 DEN-3 positives was 18 min (range 15–20.5 min) and 4 DEN-4 positives was 11 min (range 9–15 min). The primers designed for RT- LAMP displayed a T_m for DEN-1 as 82.42 °C, DEN-2 as 84.67 °C, DEN-3 as 86.17 °C and DEN-4 as 88.12 °C. The lack of overlap of T_m allowed for the easy identification of all 4 serotypes of dengue.

The real-time amplification of each dengue virus serotype in Genie® II fluorometer is shown in Fig. 3a and b, that shows different amplification times and annealing temperatures (c and d). On 3%agarose gel electrophoresis the amplification product was

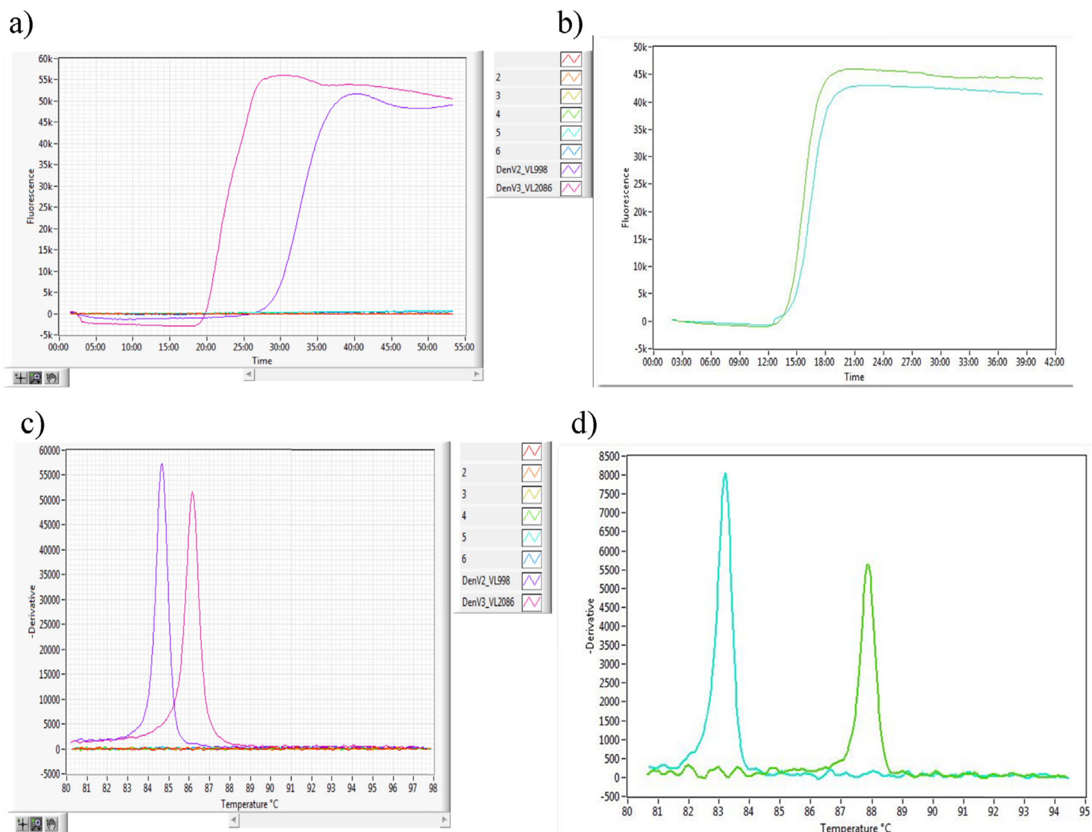

Fig. 3. (a) Amplification Curve of Dengue serotype 2 and 3 in Genie® II flourometer, (b) Amplification Curve of Dengue serotype 1 and 4 in Genie® II flourometer. (c) Anneal Curve of Dengue serotype 2 and 3 in Genie® II fluorometer, (d) Anneal Curve of Dengue serotype 1 and 4 in Genie® II flourometer.

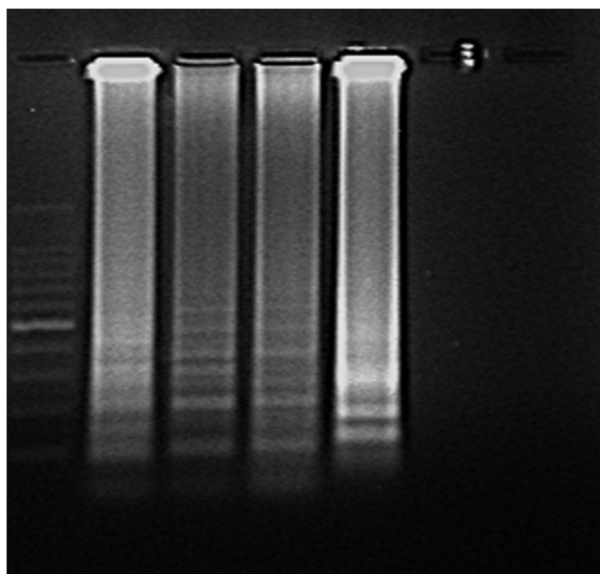

Fig. 4. Agarose gel electrophoresis of dengue virus serotype-specific RT-LAMP assay products on a 3% agarose gel. Lane 1—100-bp DNA ladder (Sigma Genosys, Japan); lane 2, DENV-1 RT-LAMP assay amplification; lane 3, DENV-2 RT-LAMP assay amplification; lane 4, DENV-3 RT-LAMP assay amplification; lane 5, DENV-4 RT-LAMP assay product, lane 6, negative control without target RNA.

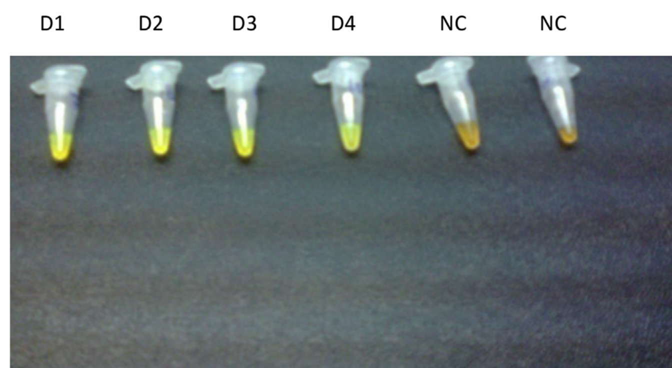

Fig. 5. Visual detection of amplified LAMP products using SYBR green I. The color changes from orange (negative reaction) to green (positive reaction). D1—DENV-1, D2—DENV-2, D3—DENV-3, D4—DENV-4, NC—negative control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

detected as a ladder-like pattern due to the formation of a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Fig. 4). The visual detection of the RT-LAMP results is shown in Fig. 5.

The most predominant serotype documented in our study was DEN-3 (80 patients), followed by DEN-2 (60 patients), DEN-1 (4 patients) and DEN-4 (4 patients). DEN-2 and DEN-3 serotypes were confirmed by sequencing, with accession numbers KJ584531 and KF301600 for DEN-3 and KC571834 for DEN-2. Phylogenetic analysis of DEN-2 showed that the strain belonged to genotype IV and DEN-3 belonged to genotype III (Fig. 6).

3.4. Repeatability and reproducibility of RT-LAMP

Precision of the RT-LAMP for identification and serotyping of DENV was determined by testing 4 samples of each serotype that included 2 strong positive and 2 weak positive of each serotype.

The mean amplification times of the replicates for strong and weak positives for DEN-1 was 10.00 min (standard deviation [SD], 0.14) and 16.00 min (SD, 0.193), DEN-2 was 26.00 min (SD, 0.15) and 30.15 min (SD, 0.30), DEN-3 was 16.0 min (SD, 0.196) and 23.15 min (SD, 0.2) and DEN-4 was 10.0 min (SD, 0.15) and 16.15 min (SD, 0.193), respectively.

To further assess the reproducibility of RT-LAMP, we tested 1 sample of each serotype in 5 separate LAMP runs and recorded the time to positivity and T_m for each serotype. The difference in the amplification times for each serotype across 5 separate runs were within 1.0 min for each serotype and SDs ranging from 0.13 to 0.62 indicating that the RT-LAMP for DENV identification and serotyping is highly reproducible (Table 6).

4. Discussion

The nonstructural protein 1 (NS1), of dengue viral genome has been shown to be a useful tool for the early diagnosis of acute dengue infections (CDC-Laboratory Guidance Dengue) and was found to be highly conserved for all dengue serotypes. The DENV NS1 antigen ELISA, a widely used test in recent times, is highly sensitive and specific (Young et al., 2000; Alcon et al., 2002) but it can be compromised by pre-existing NS1-IgG immunocomplexes in the acute stage of secondary DENV infection, a common feature in dengue endemic regions (Lapphra et al., 2008; Hang et al., 2009). Although, WHO (World Health Organization, 2009) recommends the detection of DENV RNA, as the most effective diagnostic method in the acute phase of the illness, its use is limited due to lack of infrastructure and technical expertise. More recently, molecular techniques to detect virus genomic RNA sequence by the reverse transcription-polymerase chain reaction (RT-PCR) and the real-time quantitative RT-PCR (qRT-PCR) are gradually being accepted as new standards over virus isolation for the detection of DENV in the acute sera (Lanciotti et al., 1992; Shu et al., 2003). These PCR-based methods require either high-precision instruments for the amplification or elaborate methods for detection of the amplified products. In addition, these methods are often cumbersome to adapt to routine clinical use, especially in the peripheral health care settings and the private clinics (Parida et al., 2005).

The RT-LAMP assay has emerged as a powerful gene amplification tool for rapid identification of microbial infections and is being increasingly used by various investigators for rapid detection and typing of emerging viruses, such as the West Nile, severe acute respiratory syndrome, dengue, and Japanese encephalitis viruses (Hong et al., 2004; Parida et al., 2004, 2005, 2006, 2007).

A four tube DENV NS1 serotype specific RT-LAMP assay was developed in this study for the rapid detection and differentiation of dengue serotypes in this study with high sensitivity and specificity. Using the primer concentrations (F3 and B3 primers at 0.2 μM, FIP and BIP primers at 0.8 μM, LF and LB primers at 0.4 μM), together with the use of commercially available isothermal master mix from OptiGene, UK, containing an engineered large fragment DNA polymerase (*Gsp*SSD), amplification time of 35 min and temperature of 63^0C was optimized for rapid detection and serotyping of the DENV. *Geobacillus* DNA Pol enzyme demonstrated superior LAMP amplification speed compared to Bst DNA Pol I (Parida et al., 2005; Sahni et al., 2013; Boon-Teong et al., 2013). This isothermal amplification mix allows fluorescence detection of the product on the Genie® II platform but may also be used on generic qPCR instrumentation (www.optigene.co.uk, 2014).

Very few RT-LAMP assays have been described for the detection and serotyping of DENV. Recently Teoh et al. (2013) developed single tube RT-LAMP assay by targeting 3′UTR of DENV using nine sets of primers for serotyping of DENV. However no information on the serotypes detected from the clinical samples was reported in

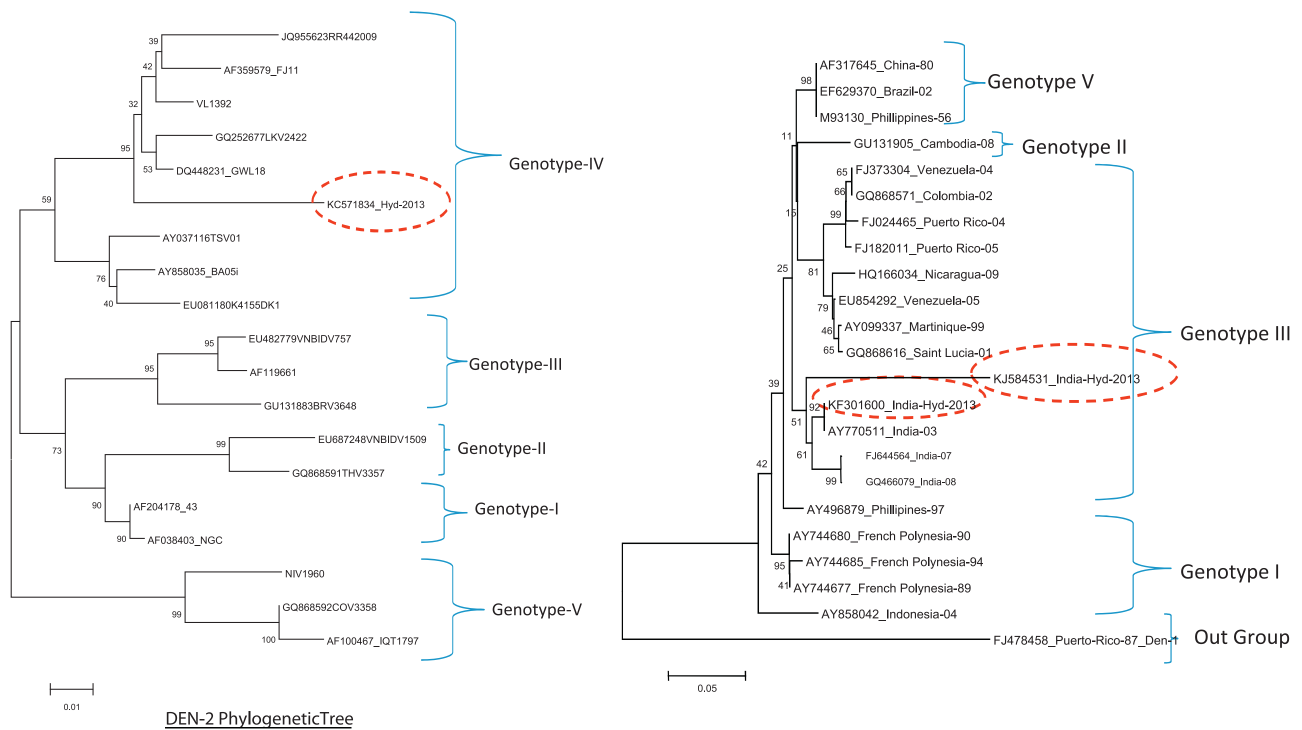

Fig. 6. Phylogenetic tree of studied sequences with geographical strains of serotype 2 and 3 generated by neighbor-joining method. The tree is based on NS1 regions (nt 86–398 bp for DENV-2 and nt 128–397 bp for DENV-3) of the selected strains.

this study. Our experience revealed that because of emergence of many genotypes across multiple DENV serotypes, the designing of pan-Dengue primers is an impossible task and is most likely to miss some genotypes. The reproducibility of RT-LAMP assay in this study was low and sensitivity was slightly lower than q RT-PCR. The specificity of the RT-LAMP assay developed by Teoh et al. was tested by single site restriction enzyme digestion that is not cost effective and also laborious. These reported methods for detection and differentiation of LAMP results were done by real time monitoring of turbidity (at 400 nm) with a Loopamp real-time turbidimeter, "ladder-like feature" on agarose gel electrophoresis and color change from orange to green caused by fluorescent detection reagent. However real time monitoring of amplification for the detection and differentiation of DENV serotypes in a fluorometer has rarely been reported. A few studies have reported the LAMP assay for rapid detection of viruses by real-time fluorometer (Genie II from Optigene, U.K.) (Mahony et al., 2013a,b). In this study we established real-time fluorescence monitoring of isothermal method using simpler and less costly Genie® II instrument. The most common method for real-time fluorescence monitoring of LAMP reactions uses intercalating dyes such as SYBR Green (Maeda et al., 2005; Ohtsuka et al., 2005). Fluorescence detection using intercalating dyes has the advantage of allowing further analysis in terms of the temperature at which amplification products melt or anneal. LAMP products contain structures of differing lengths containing catenated repeats of the target sequence which melt/anneal at a specific temperature determined by the length and G/C content of the target. After amplification, the reactions can be subjected to a gradual melting or annealing step with fluorescence monitoring to discriminate the specific amplification products from the non-specific artefacts in Genie® II instrument. This eliminates the need for gel electrophoresis or turbidity detection and allows for a closed-tube system thus reducing the cost of the assay. In our study the lack of overlap of T_m allowed for the easy identification of all 4 serotypes of dengue.

The color change from orange to green in the positive control and the samples was evident in first 20–25 min in this study. The DENV NS1serotype specific RT-LAMP assay developed in this study was significantly faster with a mean amplification time of 17 min compared with earlier studies (Parida et al., 2005; Sahni et al., 2013; Teoh et al., 2013). The speed of the assay was probably due to the use of improved polymerase.

This is the first report for evaluation of RT-LAMP employing NS1 region of viral genome with larger clinical samples size of 300 in a Genie® II flourometer. The performance of the RT-LAMP assay was validated by testing the samples simultaneously by the CDC real time PCR that is most sensitive and specific method for detection and differentiation of the DENV (CDC Dengue). Both the RT-LAMP and the CDC real time assay for detection and differentiation of the DENV showed comparable sensitivity (k = 1.0). The RT-LAMP scored over RT-PCR in terms of sensitivity and specificity

Table 6
Reproducibility of DENV RT-LAMP assay.

Amplification time in (min), T_m (°C)						
Serotype	Run1	Run2	Run3	Run4	Run5	Mean amplification time, SD[a]
DENV-1	12:00,82.4	12:30,82.5	12:15,82.4	12:00,82.4	12:.45,82.5	12:18, 0.2
DENV-2	27:30,84.6	27:00,84.6	27:00,84.6	27:15,84.7	27:45,84.7	27.18, 0.2
DENV-3	18:00,86.1	18:45, 86.1	19.00, 86.0	18:30, 86.1	18:00, 86.1	18.35, 0.4
DENV-4	11:30,88.1	11:15, 88.1	11:30,88.1	11:45,88.0	11:00,88.1	11:24, 0.2

[a] SD—standard deviation.

and reproducibility compared with study done by Teoh et al. In developing countries such as India where dengue is endemic and resources are limited, serological assays are most common methods used to confirm DENV infection. In our study using actual clinical samples, the NS1 antigen by ELISA was positive in 138/250 of patient's samples which were collected in acute phase of illness when antibodies were absent. The RT-LAMP or CDC Real time assay when used in combination with NS1 antigen and anti -dengue IgG and IgM Elisa increased the diagnostic coverage of febrile patients to 96% (240/250). This is in concordance to the study done by Teoh et al. (2013). The most predominant serotype documented in this study was DEN-3 and DEN-2 which belonged to the genotype III and genotype IV respectively. Co-circulation of more than one serotype of DENV is also known to cause hyperendemicity (Dash et al., 2011).

The NS1 serotype specific RT-LAMP assay developed in this study may be utilized as a rapid, simple, easy, cost effective, isothermal, highly sensitive and specific field applicable technique for detection and differentiation of the dengue virus serotypes which overcomes the deficiencies present in existing techniques. Its applicability in tertiary care institutes is emphasized by its ability in viral quantification and evaluation of viraemia in patients. It can suitably be introduced in zonal/peripheral hospitals as an adjunct to existing immunological tests acting as parallel controls. The RT-LAMP assay in OptiGene Genie II instrument may be employed as the new gold standard for timely and accurate diagnosis and serotyping of dengue in the clinical care, disease surveillance, disease prevention, and control activities in endemic countries such as India.

Conflict of interests statement

The authors declare that there is no conflict of interests regarding the Publication of this article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2014.10.005.

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