

Rapid Diagnosis of Dengue Outbreaks in Resource Limited Facilities

ID Khan¹, AK Sahni²

ABSTRACT

Objective: Dengue is a re-emerging public health problem threatening the tropical developing world, mandating rapid diagnosis and supportive management in the absence of licensed vaccines or anti-dengue therapy. Regions endemic to dengue and related viruses are overwhelmed by the sudden surge of cases during outbreaks. It is difficult to justify confirmatory diagnosis of every case using The World Health Organization (WHO) criteria or differentiate it from other concurrent viral illnesses. The study evaluated a rapid, sensitive and specific diagnostic methodology suitable for dengue outbreaks in resource-limited facilities.

Methods: There were one hundred dengue patients as per WHO Criteria, as well as 100 healthy controls from New Delhi, India, were included. Samples collected on the fifth day of onset of fever were tested by lateral flow immunochromatography (LF-ICT), IgM ELISA and reverse transcriptase polymerase chain reaction (RT-PCR) and results were compared. Diagnostic accuracy indices and Kappa analysis were calculated.

Results: The sensitivity, specificity, positive and negative predictive values (PPV and NPV) of non-structural protein 1 (NS1) against RT-PCR was 98.31%, 100%, 100%, 99.3% and strength of agreement was good.

Conclusion: Antigen-based and molecular tests are a better tool for early diagnosis of dengue. The combined LF-ICT kits are highly sensitive, specific, user-friendly, compact, frugal and thus recommended for use in dengue outbreaks, field conditions and as bed-side diagnostic tests, for confirmatory dengue diagnosis. Further studies are required to assess their utility in prognosis, surveillance and establishment of guidelines for dengue outbreaks.

Keywords: Dengue, lateral flow Immunochromatography, rapid diagnosis, re-emerging tropical disease, resource limited facilities, reverse transcriptase, polymerase chain reaction

Diagnóstico Rápido de los Brotes de Dengue en Instalaciones con Recursos Limitados

ID Khan¹, AK Sahni²

RESUMEN

Objetivo: El dengue es un problema de salud pública re-emergente que amenaza a los países tropicales en desarrollo. En ausencia de vacunas con licencia o terapia anti-dengue, se requiere diagnósticos rápidos y tratamientos de apoyo. Las regiones endémicas de dengue y virus relacionados están abrumadas por el repentino aumento de casos durante los brotes. Es difícil justificar el diagnóstico de confirmación de cada caso con criterios de la Organización Mundial de la salud (OMS), o diferenciarlo de otras enfermedades virales concurrentes. El estudio evaluó una metodología adecuada para el diagnóstico rápido, sensible y específico de brotes de dengue en instalaciones con recursos limitados.

Métodos: El estudio incluyó cien pacientes con dengue según criterios de la OMS, así como 100 controles sanos de Nueva Delhi, India. Las muestras recogidas en el quinto día del inicio de la fiebre fueron examinadas mediante la prueba inmunocromatográfica de flujo lateral (PIC-FL), el inmunoensayo ELISA

From: ¹Command Hospital (EC), Kolkata 700027, India and ²Military Hospital, Bhatinda, Punjab, India.

Correspondence: Dr ID Khan, Department of Pathology, Command Hospital (EC), Kolkata 700027, India. Email: titan_afmc@yahoo.com

*IgM, y la técnica de reacción en cadena de la polimerasa con transcriptasa inversa (RCP-TI), comparándose entonces los resultados. Se calcularon los índices de precisión diagnóstica y el análisis de Kappa. **Resultados:** La sensibilidad, especificidad, y los valores predictivos positivos y negativos (VPP y VPN) de la proteína no estructural 1 (NS1) frente a RCP-TI fueron 98.31%, 100%, 100%, y 99.3% respectivamente, y la fuerza de concordancia fue perfecta.*

***Conclusión:** Las pruebas moleculares y las pruebas basadas en antígenos constituyen una mejor herramienta para el diagnóstico temprano del dengue. Los kits combinados de PIC-FL son altamente sensibles, específicos, fáciles de usar, compactos, y frugales, y por ende recomendados para uso en brotes de dengue, condiciones de campo, y como pruebas de diagnóstico a la cabecera del paciente para el diagnóstico de confirmación del dengue. Se requieren estudios adicionales para evaluar su utilidad en el pronóstico, vigilancia, y establecimiento de normas a seguir ante los brotes de dengue.*

Palabras claves: Diagnóstico de dengue, inmunocromatografía de flujo lateral, diagnóstico rápido, enfermedades tropicales re-emergentes, reacción en cadena de la polimerasa con transcriptasa inversa (RCP-TI, instalaciones con recursos limitados

West Indian Med J 2017; 66 (1): 5

INTRODUCTION

Dengue is a re-emerging public health problem threatening approximately half of the world population. Many outbreaks of dengue involving all four serotypes during the past few decades have strained the tropical developing nations' health system worldwide including Asia-Pacific, Africa, Americas and Eastern Mediterranean regions (1). Despite control measures, dengue continues to emerge with increased number of cases, severity, expansion to rural areas and newer geographies.

The associated high morbidity poses threat to international travellers, military deployments and could be a promising bio-weapon potential. Serotype specific immunity conferred with the first infection is not protective against subsequent infections by different serotypes which predispose to severe dengue due to antibody dependent enhancement (2). The absence of licensed vaccines or specific anti-dengue therapy necessitates the need of early, sensitive and specific diagnosis for patient management, prevention of complications, aetiologic investigation and disease control.

The World Health Organization advocates confirmatory diagnosis of dengue through [a] viral isolation or [b] immunoglobulin M/immunoglobulin G (IgM/IgG) seroconversion or [c] four-fold rising titres of IgM/IgG/dengue antigen in paired sera or [d] dengue antigen detection through enzyme linked immunosorbent assay [ELISA] or [e] immunochemistry or [f] immunofluorescence or [g] nucleic acid amplification which are exacting in time, effort and expense (3). Resource limited facilities in regions endemic to dengue and other related viruses are often overwhelmed by the sudden surge of cases during outbreaks and find it difficult to justify diagnosis of every dengue case using the WHO criteria or be able to differentiate it from other concurrent viral illnesses. Most resource limited facilities tend to diagnose and treat dengue based on similar clinical presentation during outbreaks. Related mosquito transmitted co-endemic viral illnesses such as: Japanese encephalitis, west Nile fever, yellow fever, chikun-

gunya fever may create considerable interference with the diagnosis of dengue. Reverse-transcriptase polymerase chain reaction (RT-PCR), virus isolation, IgM/IgG ELISA and other serological tests have been used; however, the quest for the gold standard continues (4). Non-structural protein 1/immunoglobulin M/immunoglobulin G and also the use of lateral flow immunochromatography (LF-ICT) kits have exhibited high sensitivity and specificity (5) and this study intends to evaluate their utility in resource limited facilities.

SUBJECTS AND METHODS

The study included 100 randomized patients experiencing a febrile illness clinically consistent with dengue infection and positive dengue serology or RT-PCR according to the WHO criteria for dengue, as well as 100 randomly selected asymptomatic healthy controls of all age groups between, May 2013 to December 2014. It spanned the post-monsoon season for two consecutive years covering many outbreaks from New Delhi and adjoining areas in India, after approval from the hospital Ethical Committee. The controls were matched for age and gender and selected amongst the attendants of patients. Samples were collected on the fifth day of onset of fever from patients and simultaneously from relatives. Samples were transported to a large tertiary care hospital laboratory within four hours of collection for separation of serum and immediate processing or storage at -70°C until processed. Three experienced technicians blinded to detailed clinical information about the study groups and other test results, independently performed and assessed the tests, which were further assessed by the principal investigator. All samples were tested by lateral flow immunochromatography (LF-ICT), IgM ELISA and RT-PCR and results were compared. Samples were subjected to LF-ICT for detection of NS1 antigen, IgM and IgG antibodies (SD BIOLINE Dengue Duo NS1 Ag + Ab Combo, Standard Diagnostic Inc., Korea) as per manufacturer's protocol. Immunoglobulin M was also detected by IgM capture ELISA Kit from the National Institute of Virology (NIV), Pune, India,

wherein 100 µl of 1:100 dilution of serum and biotinylated flavivirus cross-reactive monoclonal antibody were used under routine ELISA procedure as per manufacturer's protocol. Immunoglobulin M values > 1.1 NIV units were considered positive.

Reverse-transcriptase polymerase chain reaction was preceded by RNA extraction using the QIAamp viral RNA mini kit (Qiagen, Germany) following manufacturer's protocol. For RT-PCR, cDNA was synthesized from viral RNA using reverse antisense primer [5' TTG-CAC-CAA-CAG-TAC-ATG-TCT-TCA-GGT-TC 3', primer length 29 mer] targeting the prM gene, reverse transcriptase of Moloney murine leukaemia virus origin (MMLV-RT) and RNasin® Ribonuclease inhibitor (Promega, USA) following manufacturer's protocol. Reverse-transcriptase polymerase chain reaction was carried out using the cDNA as template, forward sense primer [5' TCA-ATA-TGC-TGA-AAC-GCG-CGA-GAA-ACG-G 3', primer length 28 mer] targeting the capsid gene and thermostable Taq DNA polymerase [Promega, USA] loaded to the thermal cycler [Bio-Rad, USA] (Table 1).

One cycle of initial denaturation was done at 95 °C for two minutes followed by 35 cycles of denaturation, primer annealing and primer extension at 94 °C for one minute, 55 °C for one minute and 72 °C for two minutes, respectively. Final extension was done at 72 °C for 10 minutes. RT-PCR amplicon was detected by 1–2% agarose gel electrophoresis followed by visualization in a Gel Documentation System [Bio-Rad, USA] (6). The reference strains were used as positive controls and DEPC treated water as negative control. Percentages, 95% confidence interval (CI) and Chi-square with $p \leq 0.05$ were used to indicate significance. Diagnostic accuracy indices such as sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated with various pairs of tests. Inter-operator variability was analysed using Kappa statistical analysis which included Cohen's un-weighted Kappa, Kappa with linear weighting, Kappa with quadratic weighting; standard error, 95% CI and frequencies and proportion of agreement. Values < 0 indicated no agreement, 0–0.20 as slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial and 0.81–1 as almost perfect agreement (7).

RESULTS

The age range of dengue patients was from 8 to 75 years, mean 33.13 ± 14.85 and the male: female ratio was 1.7:1. Both age (Chi-square, $p = 0.417$) and gender ($p = 0.615$) were not significant. Out of a total of 100 patients experiencing a febrile illness clinically consistent with dengue infection, 58 serum samples were positive for NS1 antigen, 42 for IgM and 18 for IgG by LF-ICT (Table 2).

Table 2: Results of various diagnostic modalities

Test	Positive (%)	95% Confidence interval
100 dengue patients		
NS1 antigen by LF-ICT	58 (58%)	48.33%–67.67%
IgM by LF-ICT/ELISA	42 (42%)	32.33%–51.67%
IgG by LF-ICT	18 (18%)	10.47%–25.53%
RT-PCR	65 (65%)	55.65%–74.35%
IgM and IgG	18 (18%)	10.47%–25.53%
RT-PCR and NS1	58 (58%)	48.33%–67.67%
RT-PCR and IgM	7 (7%)	2%–2%
RT-PCR and IgG	3 (3%)	-0.34%–6.34%
100 dengue controls – No tests were positive		

NS1: non-structural protein 1; LF-ICT: lateral flow immunochromatography; ELISA: enzyme linked immunosorbent assay; RT-PCR: reverse-transcriptase polymerase chain reaction; IgM: Immunoglobulin M; IgG: Immunoglobulin G

All IgM positive results by LF-ICT correlated with IgM capture ELISA. Reverse-transcriptase polymerase chain reaction revealed 65 positive samples (Fig. 1).

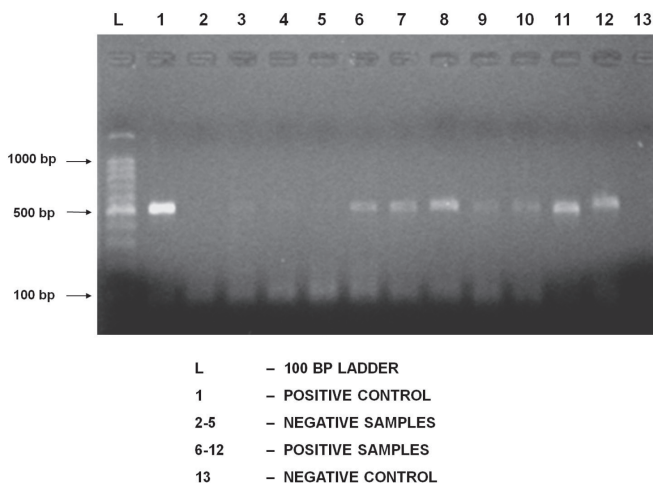


Figure: Reverse transcriptase polymerase chain reaction for dengue.

Table 1: Dengue primer sequences

Primer	DC-1S	DC-2C
Direction	Sense	Antisense
Nucleotide Sequence (5'to 3')	TTG-CAC-CAA-CAG-TAC-ATG-TCT-TCA-GGT-TC	TTG-CAC-CAA-CAG-TAC-ATG-TCT-TCA-GGT-TC
Primer length (mer)	28	29
Product (bp)		511
Annealing Temp (°C)		54

Of the 65 samples positive by RT-PCR, 58 also showed positivity for NS1 antigen, seven also showed positivity for IgM and three also showed positivity for IgG (Table 3).

The specificity and PPV approaching 100% is ideal; however this is an observation with a limited sample size, and is likely to vary in larger studies. Diagnostic case control stud-

Table 3: Diagnostic accuracy of antigen and antibody based tests for dengue

Antigen/antibody	Sensitivity (%)	Specificity (%)	PPV	NPV	Kappa score	Agreement
NS1 antigen (against RT-PCR)	98.31% (96.5%–100.1%)	100%	100%	99.3% (98.1%–100.5%)	0.99	Perfect
IgM antibody (against RT-PCR)	10.77% (6.5%–15.1%)	74.07% (68%–80.1%)	16.67% (11.5%–21.8%)	63.29% (56.6%–69.9%)	Not applicable	Not applicable
IgG antibody (against RT-PCR)	4.62% (1.7%–7.5%)	88.89% (84.5%–93.2%)	16.67% (11.5%–21.8%)	65.93% (59.4%–72.5%)	Not applicable	Not applicable

PPV: Positive predictive values; NPV: negative predictive values; NS1: non-structural protein 1; IgM: Immunoglobulin M; IgG: Immunoglobulin G

Reverse-transcriptase polymerase chain reaction and NS1 test were compared considering RT-PCR as the gold standard confirmatory test. The sensitivity, specificity, PPV and NPV of NS1 against RT-PCR was 98.31%, 100%, 100% and 99.3%. Observed kappa was 0.99, standard error 0.01, 95% CI 0.96, 1 and strength of agreement was perfect. No tests were positive in the 100 healthy controls.

DISCUSSION

The study was conducted during two outbreaks of dengue spanning the post-monsoon season of two consecutive years. Dengue patients were randomly sampled and matched controls were recruited randomly so as to represent similar population from which the positive samples were drawn. Samples were collected on the fifth day of onset of fever, as per history elicited from patients. The LF-ICT kit detecting (NS1) non-structural protein 1, IgM and IgG was compared with RT-PCR and IgM capture ELISA. Non-structural protein 1 is a 55 kDa highly conserved non-structural glycoprotein secreted by virus infected cells in early dengue infection. Non-structural glycoprotein-1 is more sensitive in primary infection early in the course of the disease, corroborates with high viral load and exhibits limited cross-reactivity (8, 9).

Higher levels of viraemia and elevated free secreted NS1 may identify patients at risk of severe dengue (10). As NS1 corresponds to short lived viraemia of early infection, it may be used to predict clinical outcome and may be the test of choice to initiate antiviral therapy, should it become available in future (9–12). Non-structural protein 1 antigen can be employed in both LF-ICT and ELISA formats (9). Other than NS1, NS5 can also be a potential target for early diagnosis of dengue (13). The high sensitivity of both NS1 and RT-PCR and a perfect agreement by Kappa emphasizes the correlation of NS1 positivity with positive results by RT-PCR which corresponds to early viraemia (14, 15).

ies face spectrum and limited challenge bias due to limited sample size as in this study. However, spectrum effects can be advantageous in the early investigation of a new diagnostic test (16). The sensitivity and specificity of the NS1 based dengue tests is reported between 71%–81% and 93%–100%, respectively, and false positives are considered rare, thereby eliminating the requirement of a second sample (8, 9, 12, 14). However, sensitivity of NS1 decreases when initial viral load is low or later in the course of the disease when antibody titres rise and NS1 gets sequestered in immune complexes, and in secondary dengue infection (8, 9). It has been reported that NS1 is detectable with low antibody titres (Haemagglutination Inhibition, HI < 320) while IgM and IgG are detectable with high antibody titres [HI 640–10240] (17). No overlap between NS1 and IgM/IgG antibodies was seen, although NS1 and IgM may be positive with early seroconvert patient where NS1 is declining and IgM/IgG antibodies have started to rise, sometimes representing inadequacy of the test to differentiate between early and late infection.

The sensitivity and specificity of the IgM-based dengue tests is reported between 68%–99% and 46%–98%, respectively, depending upon host humoral immune response (8). The positivity of IgM and IgG in both early and late infection, primary and secondary infection, requirement of paired sera and cross-reactivity with flaviviruses limits their utility as stand-alone tests for dengue. Samples positive either for IgM antibodies alone or both IgM and IgG antibodies may form a complex with viral particles and cause hindrance in viral detection (18). Reverse-transcriptase polymerase chain reaction is one of the most sensitive and specific markers of dengue viraemia seen in early infection, although it may be positive in late infection owing to incomplete neutralization of the virus and persistence of fragmented viral nucleic acid (9). Although sensitivity is greatly reduced late in the course of infection when viraemia subsides, antibodies appear and vascular complications are likely to manifest, it is still considered the gold

standard. However, use is limited by acquisition capacity for sophisticated equipment, reagents, expertise and standardization in resource limited laboratories. Loop assisted isothermal amplification (LAMP), a rapid, quantitative, highly sensitive and specific molecular test, is limited by primer designing and cross-contamination (5, 19–21). More sensitive and real time assays have the advantages of rapidity, quantitative measurement, lower contamination rate, higher sensitivity, specificity and easy standardization. These considerations in nucleic acid amplification techniques have popularized ELISA formats in limited resource facilities. The results of ELISA correlate well with other tests, as also evidenced in this study when all IgM was equally positive by both LF-ICT and ELISA, although false-positive test may be seen due to persistence of antibodies from a recent previous infection with a different dengue serotype or a related endemic flavivirus (8).

The LF-ICT contains dried antigens and colloidal gold-labelled monoclonal antibodies specific for dengue NS1, IgM and IgG on a nitrocellulose strip which are captured by immobilized anti-human globulins to generate a coloured line. Immunoglobulin A may also be included. A combined kit containing all of these increases the applicability in both early and late infections, as well as in secondary dengue infections with high sensitivity, specificity and a low false-positivity rate (5, 7). The high sensitivity, specificity, rapidity, stability, reproducibility and convenience of LF-ICT make it a suitable test in both resource limited and resource rich healthcare facilities. It is compact, faster to manufacture and has a prolonged shelf life. It can be carried out as a single bed-side test, in a single step, requires low sample volume and enables visual interpretation of results. The procedure eliminates washing step, contamination from reuse and sample pre-treatment (22). Resource limited facilities may have erratic electric and water supply, limited equipment and storage facilities, procurement problems and manpower shortages. While these problems need to be addressed before establishment of sophisticated immunology and molecular microbiology laboratories, LF-ICT can be utilized as a first line test in field conditions even outside the laboratory. There is no difference in detection with respect to different dengue serotypes, different viral loads or different clinical presentation (9). The LF-ICT tests have been evaluated at airports to screen for imported dengue cases and also in peripheral/rural areas (23, 24). The applicability of either human, vector or environmental samples in the combined kit enhance its utility as a frugal and robust epidemiological tool for seroprevalence studies, mass screening programmes and surveillance of dengue infections (25, 26). However, sensitivity is more for primary than secondary dengue, serotypes cannot be identified and the results need to be read manually by the operator. The results are qualitative or semiquantitative and a negative result with stand-alone NS1, IgM or IgG makes interpretation difficult and does not rule out dengue infection (5, 9). Further, there may be issues regarding generating a sensitive antibody preparation with good selectivity and covalent attachment may decrease the affinity for the antigen (22).

Simultaneous targeting of NS1, IgM and IgG enhances utility over the entire temporal extent of dengue and is also able to rule out dengue infection (4). Utilising Bayesian latent class models, wherein no test is assumed perfect, the sensitivity, specificity, PPV and NPV of combination NS1, IgM and IgG based tests were found to be 87.0%, 82.8%, 62.0% and 95.2% (4). SD BIOLINE kit, used in the present study, has been reported with a sensitivity, specificity and assay efficiency of 83%–100%, 89%–98% and 91% when NS1, IgM and IgG were simultaneously targeted, giving detection rate comparable to RT-PCR (5, 8, 17, 27, 28). It has been reported to differentiate 71% primary and 83% secondary dengue infections (4, 8, 17, 28). The results of the present study and that of previous studies correlate well in advocating simultaneous targeting of NS1, IgM and IgG under LF-ICT format for first line detection of dengue.

CONCLUSION

The study furthers that antigen-based tests and molecular tests are a better tools for early diagnosis of dengue. Antibody based tests are suited for late infection and can be used for seroprevalence and epidemiological surveillance due to persistence of antibodies. The combination of both antigen and antibody based tests in rapid LF-ICT kits and reference assay formats may prove superior in better differentiation between early and late infection; primary and secondary dengue and epidemiological surveillance. The combined rapid LF-ICT kits are highly sensitive, specific, user-friendly, compact, frugal and thus, recommended for use in dengue outbreaks, field conditions and as bed-side diagnostic tests. Parallel large multicentric studies and meta-analyses are required to assess the potential impact of implementing confirmatory laboratory diagnosis of dengue through combination LF-ICT incorporating NS1, IgM and IgG to optimize diagnosis, indicate prognosis, conduct clinical, laboratory and vector surveillance, assess cost-effectiveness, help establish standard guidelines for dengue outbreaks and furtherance of future developments.

ACKNOWLEDGEMENTS

We thank Hav Inderjit, Hav Vineeth, Hav Prem and Sub Pratap for technical assistance.

REFERENCES

1. Shepard DS, Undurraga EA, Halasa YA. Economic and disease burden of dengue in Southeast Asia. *PLoS Negl Trop Dis* 2013; **7**: e2055.
2. Kurane I, Mady BJ, Ennis FA. Antibody-dependent enhancement of dengue virus infection. *Rev Med Virol* 2005; **1**: 211–21.
3. WHO: Dengue: Guidelines for diagnosis, treatment, prevention and control. WHO; 2009.
4. Pan-Ngum W, Blacksell SD, Lubell Y, Pukrittayakamee S, Bailey MS, de Silva HJ et al. Estimating the true accuracy of diagnostic tests for dengue infection using Bayesian latent class models. *PLoS One* 2013; **8**: e50765.
5. Wang SM, Sekaran SD. Early diagnosis of dengue infection using a commercial dengue duo rapid test kit for the detection of NS1, IgM, and IgG. *Am J Trop Med Hyg* 2010; **83**: 690–5.
6. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: A Laboratory Manual*. 2nd edition 1989. Cold Spring Harbor Laboratory Press, USA.

7. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics*. 1977; **33**: 159–74.
8. Guzman MG, Kouri G. Advances in dengue diagnosis. *Clin Diagn Lab Immunol* 1996; **3**: 621–7.
9. Callahan JD, Wu SJ, Dion-Schultz A, Mangold BE, Peruski LF, Watts DM et al. Development and evaluation of serotype and group-specific fluorogenic reverse transcriptase PCR (TaqMan) assays for dengue virus. *J Clin Microbiol* 2001; **39**: 4119–24.
10. Shu PY, Chang SF, Kuo YC, Yueh YY, Chien LJ, Sue CL et al. Development of group and serotype specific one-step SYBR Green I based real-time reverse transcription PCR assay for dengue virus. *J Clin Microbiol* 2003; **41**: 2408–16.
11. Kuno G. Universal diagnostic RT-PCR protocol for arboviruses. *J Virol Methods* 1998; **72**: 27–41.
12. Leone G, van Schijndel H, van Gemen B, Kramer FR, Schoen CD. Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. *Nucleic Acids Res* 1998; **26**: 2150–5.
13. Sudiro TM, Ishiko H, Green S, Vaughn DW, Nisalak A, Kalayanaraj S et al. Rapid diagnosis of dengue viraemia by reverse transcriptase polymerase chain reaction using 3-noncoding region universal primers. *Am J Trop Med Hyg* 1997; **56**: 424–9.
14. Osorio L, Ramirez M, Bonelo A, Villar LA, Parra B. Comparison of the diagnostic accuracy of commercial NS1-based diagnostic tests for early dengue infection. *Virol J* 2010; **7**: 361.
15. Blacksell SD, Jarman RG, Bailey MS, Tanganuchitcharnchai A, Janjaroen K, Gibbons RV et al. Evaluation of six commercial point-of-care tests for diagnosis of acute dengue infections: the need for combining NS1 antigen and IgM/IgG antibody detection to achieve acceptable levels of accuracy. *Clin Vaccine Immunol* 2011; **18**: 2095–101.
16. Whiting P, Rutjes AW, Reitsma JB, Glas AS, Bossuyt PM, Kleijnen J et al. Sources of variation and bias in studies of diagnostic accuracy: a systematic review. *Ann Intern Med* 2004; **140**: 189–202.
17. Posthuma-Trumpie GA, Korf J, van Amerongen A. Lateral flow immunoassay: its strengths, weaknesses, opportunities and threats. A literature survey. *Anal Bioanal Chem* 2009; **393**: 569–82. <http://www.ncbi.nlm.nih.gov/pubmed/18696055>
18. Hang VT, Nguyen NM, Trung DT, Tricou V, Yoksan S, Dung NM et al. Diagnostic accuracy of NS1 ELISA and lateral flow rapid tests for dengue sensitivity, specificity and relationship to viraemia and antibody responses. *PLoS Negl Trop Dis* 2009; **3**: e360.
19. Shu PY, Yang CF, Kao JF, Su CL, Chang SF, Lin CC et al. Application of the Dengue Virus NS1 Antigen Rapid Test for On-Site Detection of Imported Dengue Cases at Airports. *Clin Vaccine Immunol* 2009; **16**: 589–91.
20. Dussart P, Petit L, Labeau B, Bremand L, Leduc A, Moua D et al. Evaluation of two new commercial tests for the diagnosis of acute dengue virus infection using NS1 antigen detection in human serum. *PLoS Negl Trop Dis* 2008; **2**: e280. <http://www.plosntds.org/article/info%3Adoi%2F10.1371%2Fjournal.pntd.0000280>
21. Valdez SJJ, Ruiz AD, Vázquez RCS, Calzada GN, Guzmán TCM. Evaluation of the SD Dengue Duo diagnosis system for detection of NS1 protein and IgM and IgG dengue antibodies. *Rev Cubana Med Trop* 2012; **64**: 27–34.
22. Chaterji S, Allen JC, Chow A, Leo YS, Ooi EE. Evaluation of the NS1 rapid test and the WHO dengue classification schemes for use as bedside diagnosis of acute dengue fever in adults. *Am J Trop Med Hyg* 2011; **84**: 224–8.
23. Sahni AK, Grover N, Sharma A, Khan ID, Kishore J. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) for diagnosis of dengue. *MJAFI* 2013; **69**: 246–53.
24. Young PR, Hilditch PA, Bletchly C, Halloran W. An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. *J Clin Microbiol* 2000; **38**: 1053–7.
25. Vøge NV, Sánchez-Vargas I, Blair CD, Eisen L, Beaty BJ. Detection of dengue virus NS1 antigen in infected *Aedes aegypti* using a commercially available kit. *Am J Trop Med Hyg* 2013; **88**: 260–6.
26. Vaughn DW, Green S, Kalayanaraj S, Innis BL, Nimmannitya S, Suntayakorn S et al. Dengue viraemia titer, antibody response pattern, and virus serotype correlate with disease severity. *J Infect Dis* 2000; **181**: 2–9.
27. Libraty DH, Young PR, Pickering D, Endy TP, Kalayanaraj S, Green S et al. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue haemorrhagic fever. *J Infect Dis* 2002; **186**: 1165–8.
28. Rodriguez LMA, Ligonio AR, Encina JLR, Martinez-Cazares MT, Parissi-Crivelli A, Lopez-Monteon A. Expression, purification, and evaluation of diagnostic potential and immunogenicity of a recombinant NS3 protein from all serotypes of dengue virus. *J Trop Med* 2012; **2012**: 956875.