Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 8, Issue, 10, pp.40334-40338, October, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

STANDARDIZATION OF ONE-STEP SINGLE TUBE RT-PCR FOR RAPID DETECTION OF ALL CIRCULATING STRAINS OF DENGUE VIRUSES

[1]Sulochana Kaushik, [2]Vikrant Sharma, [3]Dhruva Chaudhary, [3]Sanjeev Nanda, [1]Jaya Parkash Yadav and *,[2]Samander Kaushik

[1]Department of Genetics, Maharshi Dayanand University, Rohtak, Haryana, India
[2]Centre for Biotechnology, Maharshi Dayanand University, Rohtak, Haryana, India
[3]Pt BDS UHS Rohtak, Haryana, India

ARTICLE INFO

Article History:
Received 26th July, 2016
Received in revised form
20th August, 2016
Accepted 18th September, 2016
Published online 30th October, 2016

Key words:

Dengue Virus,
Dengue Hemorrhagic Fever (DHF),
RT-PCR,
Monoplex PCR.

ABSTRACT

Dengue is a viral disease which is spread by a mosquito vector and is prevalent in tropical and subtropical regions. Dengue poses serious health threat to a major portion of population therefore rapid and specific tests are needed to combat the situation. Thus, one-step reverse transcription polymerase chain reaction (RT-PCR) was standardized to detect all the four serotypes of dengue viruses i.e. DENV1 to DENV4. This study was performed on standard strains of dengue viruses. RNA was extracted from these strains and was used to optimize one step RT-PCR. This assay was able to detect all the four serotypes of dengue virus.

Copyright © 2016, Sulochana Kaushik et al. *This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.*

Citation: Sulochana Kaushik, Vikrant Sharma, Dhruva Chaudhary, Sanjeev Nanda, Jaya Parkash Yadav and Samander Kaushik, 2016. "Standardization of one-step single tube rt-pcr for rapid detection of all circulating strains of dengue viruses", *International Journal of Current Research*, 8, (10), 40334-40338.

INTRODUCTION

Dengue is a mosquito born viral infection and prevalent in whole tropical region of world including India. Dengue virus (DENV) is a single stranded RNA virus containing a genome which encodes three structural and seven non-structural proteins (Holmes and Burch, 2000). Dengue viruses are classified into *Flaviviridae* family (Kuhn *et al*., 2001). *Aedes aegipty* and *Aedes albopictus* are responsible for spreading dengue viruses (Chan *et al*., 1971[a]; 1971[b]). Dengue fever can be of three type; dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Clinical symptoms of dengue are variable but majority of patients have severe headache, muscle pains, joint pains and vomiting (Low *et al*., 2011; Endy *et al*., 2011). Sometime dengue is more serious and utmost care should be taken when abdominal pain, bleeding, fatigue and persistent vomiting happen (Guzman *et al*., 2010). Dengue virus is now widespread in more than 100 countries i.e. more than half of globe.

Corresponding author: Samander Kaushik,
Centre for Biotechnology, Maharshi Dayanand University, Rohtak, Haryana, India.

About 2.5 billion people live in these areas are at risk of epidemic dengue. About 100 million cases of dengue fever (DF) and 450,000 cases of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) are reported annually (Monath, 1994; Gubler, 1988; WHO, 2002). Outbreak of dengue also observes in whole India after rainy season excepting some cold reason where mosquitoes are not found. Our national capital is one of the hot-spot for dengue virus outbreak. Delhi situated in the northern part of India where many outbreaks have occurred due to different dengue virus types in 1967, 1970, 1982, 1988, 1996, 2003, 2007 and after 2008 almost every year have outbreak of dengue viruses (Sarkar *et al*., 1964; Balaya *et al*., 1969; Diesh *et al*., 1972; Rao, 1985; Rao, 1987; Kabra, 1992; Broor *et al*., 1997 and Dar *et al*., 2003). All four dengue virus serotypes circulate in India and cause epidemics but only occasional cases of DHF/DSS were reported from Delhi till 1996. A large outbreak of DHF/DSS has occurred in Delhi in 1996 by DENV-2 (Dar *et al*., 2003). Factors believed to cause increase in dengue epidemics are population growth and urbanization, deterioration in water quality, suboptimal waste management, lack of effective mosquito control and human travel (Gubler, 1998).

Dengue fever is difficult to diagnose clinically because it resembles other febrile illnesses that are prevalent in the same regions such as Malaria, Influenza, Leptospirosis, Hantavirus and Chikungunya (Kaushik *et al*., 2007; Carme *et al*., 2009; Santana *et al*., 2010). Several methods have been developed for the confirmation of dengue cases. A primary infection of a dengue virus provide life-long immunity towards that particular serotype but this immunity will not be helpful towards the other remaining DENV serotypes (Murrell *et al*., 2011). Subsequent infection with other serotypes may be more serious.

Major problem in dengue is patient management which can be solved by rapid diagnosis during early phase of infection. Rapid diagnosis can be possible with the help of modern molecular techniques (McBride *et al*., 2000) but there is no treatment even after rapid diagnosis. In the view of rapid expansion of dengue worldwide there should be effective prevention strategies to control the disease. As like other viruses, effective vaccines and antiviral drugs against dengue viruses will be helpful. Although development of vaccine is very difficult against any RNA virus but in case of dengue virus antibody-dependent enhancement (ADE) is another major problem. Development of a vaccine becomes very difficult for Dengue viruses due to ADE. Antibody-dependent enhancement gives serious effect during the secondary infection of dengue viruses with other heterologous strains and may causes DHF/DSS. Therefore vaccine must not only protect against all four dengue serotypes but also avoid inducing the ADE effect which seems to be complicated. Therefore even today there are no effective commercially licensed vaccines available for the treatment of dengue (Kato *et al*., 2010; Chang *et al*., 2011).

In absence of effective treatment early diagnosis may be more helpful. Presently dengue is diagnosis by commercial kit, virus isolation, serological and molecular method. Commercial kits and serological methods have low sensitivity and specificity. Although virus isolation is considered as gold standard and economic but it is also time consuming. Therefore, some more rapid assays are required for dengue identification. Real time reverse transcription polymerase chain reaction (RT-PCR) is a better choice of diagnosis but costly. RT-PCR can be of two types; one step, in this RT and PCR amplification take place in single tube without opening tube; two steps, RT and PCR are two different reactions in two different tubes. One-step reaction reduces the chance of contamination and saves time. Present study entitled "Standardization of One-step Single Tube RT-PCR for Rapid Detection of all Circulating Strains of Dengue Viruses" has been conceptualized for rapid detection of dengue viruses in a single reaction. Rapid, accurate and sensitive diagnoses are crucial for dengue and helpful for patient management and in preventing spread of disease.

MATERIALS AND METHODS

Serotypes of Dengue Virus

There are four serotypes of dengue viruses DEN-1, DEN-2, DEN-3 and DEN-4, circulating worldwide. Reference strains of these dengue serotypes were obtained from the virology laboratory of All India Institute of Medical Sciences (AIIMS), New Delhi, India. Aliquots of these serotypes of dengue virus strains were made and were stored at -70 °C until the RNA extraction.

RNA Extraction

RNA was extracted from single aliquot of each of the four serotypes of dengue viruses in four nuclease free separate tubes by using the commercial viral DNA/RNA Kit (Gene Jet Viral DNA and RNA purification kit) following the manufacturer's protocol.

Standardization of one step RT-PCR for detection of all the four Dengue serotypes

One step RT-PCR was standardized with single step RT-PCR kit (Amresco® Ready One Step RT-PCR Kit) and primers have been taken from common region of all dengue serotypes. Positive and negative controls were also included with every assay.

Primers

Published primers were used in this study and sequences of forward and reverse primer are 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' and 5'-TTGCACCAACAG TCAATGTCTTCAGGTTC-3' respectively (Lanciotti *et al*., 1992). These primers amplify 511bp amplicon from the common and conserved region of core-pre-membrane (CprM) junction all dengue serotypes. Different concentration of both forward and reverse primers varying from 20pM, 25pM, 30pM, 40pM, and 50pM were tried for dengue viruses amplification.

Standardization of one step RT-PCR cycling conditions

First reverse transcription was standardized. Reverse transcription reaction was carried out by incubation at 42°C for 60 to 90 min followed by enzyme inactivation at 95°C for 15 min. The cDNA was prepared in first phase of reaction was used immediately for PCR reaction without opening the tubes. Then PCR was optimized using the following conditions: Denaturation at 94°C for variable time of 20 sec to 1min, Annealing temperature of 50°C to 60°C for varying time ranging from 20 sec to 1 min and extension temperature of 72°C for 20 sec to 1 min while final extension at 72°C for varying time from 7 min to 10 min. The different parameters of denaturation, annealing and extension temperature were tried.

Agarose Gel Electrophoresis

RT-PCR amplicons were visualized on 2% agarose gel. The agarose gel was prepared in 1X Tris, acetic acid and EDTA (TAE) and the amplicons were loaded with the help of 2µl of 1X loading dye buffer in the wells. Electrophoresis was performed for about 1hr at a constant voltage of 80V in running buffer containing 1X TAE and ethidium bromide (1µg/ml). After electrophoresis gels were visualized in a gel documentation system or the gel trans-illuminator (Gel doc™ XR, BioRad, USA). The gels were run along with marker.

Specificity

The specificity of RT-PCR assay was checked with DNA or RNA extracted from the related viruses like JEV, WNV and Chikungunya virus.

Application of one step RT-PCR for rapid detection of dengue viruses on a panel of clinical samples

Standardized assay was applied on a panel of five clinical samples whose results were known. RNA was extracted from all clinical samples. One step RT-PCR assay was applied on RNA extracted from these clinical samples. RT-PCR products were visualized on 2% agarose gel. Gels were run along with marker.

RESULTS

RNA extraction

RNA was isolated from 200µl aliquot of every serotype with the help of Thermo-scientific Verso kit. RNA was eluted in final volume of 50µl at the end. One step RT-PCR for simultaneous detection of all dengue serotypes was standardized by using 5µl RNA from each strain.

Optimal cycling conditions of RT-PCR are given in the Table 1. All four serotypes of dengue virus strains were amplified by standardized one step RT-PCR. After amplification amplicons were visualized on 2% agarose gel. Negative control was also involved in the assay. All four serotypes of dengue virus strains showed amplification band of 511bp while there was no amplification in the negative control. DEN1, DEN2, and DEN4 showed good bands while DEN3 showed poor intensity band (Fig 1). Some non-specific bands also appear in gel, these may be due to the degradation of RNA.

Check the specificity of primers

Specificity of primers was checked. Assay was also applied on other related viruses but both primers were very specific for their target and did not amplify other RNA/DNA present in samples. Only RNA of dengue viruses was amplified by this assay.

Table 1. Optimal cycling conditions of one step RT-PCR

S. No	Parameter	Temperature (°C)	Time in Minutes
1	Reverse Transcription (RT)	42	60
2	Inactivation of RT	95	15
3	Denaturation Temperature	95	01
4	Anneal Temperature	55	01
5	Extension Temperature	72	01
6	Final Extension Temperature	72	07
7	Hold Temperature	04	∞
	40 cycles were repeated of the PCR (3 to 5)		

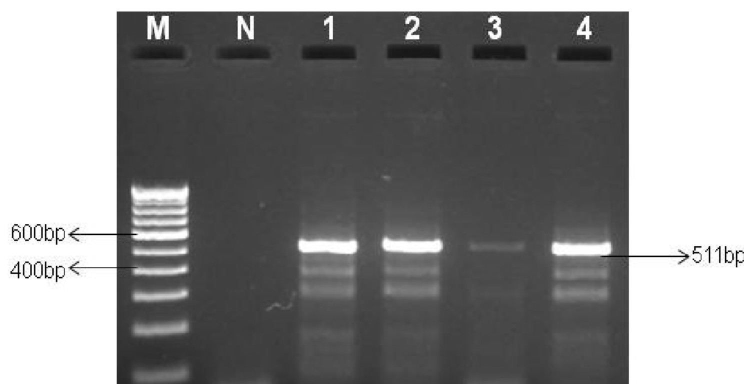

Fig. 1. Standardization of one step RT-PCR assay on serotypes of dengue virus. Lane M, 100bp DNA ladder; lane N, negative control; lane 1-4, dengue virus serotypes 1-4 respectively

Standardization of one step RT-PCR for detection of all the four Dengue serotypes

Assay was standardized with help of single step RT-PCR kit (Amresco® Ready One Step RT-PCR Kit). Single step RT-PCR kit save time and reduce chance of contamination. One step RT-PCR assay was optimized in single tube with common primer. Best result obtain in 25µl optimized reaction mixture containing: 12.5µl of 2X buffer reaction mixture and 50pico moles of each forward and reverse primers and 1µl of enzyme mix (Taq DNA polymerase and Reverse Transcriptase).

Optimal cycling condition used in one step RT-PCR

First RT procedure was performed at 42C° for 60 min to convert RNA into cDNA then RT enzyme was inactivated at 95°C for 15 min. One step RT-PCR was performed with commercial kit and using published primers of dengue viruses which amplify a 511bp product (Lanciotti *et al.*, 1992).

Application of standardized one step RT-PCR on a panel of clinical samples

Standardized one step RT-PCR was applied on a panel clinical samples (whose results were known). Two samples were positive and three samples were negative for dengue viruses. Positive samples showed strong band of 511bp while there was no amplification in the negative samples.

DISCUSSION

Dengue produces a febrile disease that present with undifferentiated symptoms. Recently dengue outbreaks are increasing in various parts of globe and infections are continued to be a major public health problem. According to a WHO report, 500,000 people with DHF require hospitalization each year and about 2.5% of those affected die (WHO, 2009).

Dengue virus is endemic in about 128 countries and more than 2.5 billion or about 40% of world's population lives in area that is on risk (WHO, 2012). According to a study estimate that in 2010 there were 96 million apparent and 296 million unapparent dengue infections worldwide, with more infections in Asia (70%) followed by Africa (16%) and (14%) America (Bhatt *et al*., 2013). Fatalities can be higher in some countries caused by inadequate disease management facilities and without proper treatment DHF fatality rates can exceed 20% (WHO, 2009). In addition, fatality rates reported in hospitalized patients can reach up to 50-60% in dengue patients with complications (acute renal failure, fulminant hepatitis, liver failure, and encephalopathy) (Nimmannitya *et al*., 1987; Seneviratne *et al*., 2006; Lee *et al*., 2009). The increased prevalence of dengue infections worldwide in recent decades and high mortality caused by DHF and DSS highlights the need for more sensitive and specific diagnostic assays such as RT-PCR for detection and typing of DENV. Diagnosis of DENV infection in fatal cases often can be challenging because of unavailability of serum and fresh or frozen specimens.

Furthermore, in patients who die during first week of illness, serology may have limited use because of low levels of IgM antibodies that cannot be detected by serological assays (Halstead, 2007; Bhatnagar *et al*., 2007). On the other hand, in the early viremic stage of DENV infections patients may have higher viral loads. (Laue *et al*., 1999; Singh *et al*., 2006; Tricou *et al*., 2011) The symptoms of dengue are very similar with other infections therefore disease can be confirmed with antigenic or immunologic assay. Presently dengue is diagnosed by virus isolation, antigen detection, ELISA, RT-PCR etc. RT-PCR assay is more sensitive than virus isolation, Virus isolation is although gold standard but time consuming. Virus isolation results need to be confirmed by another assay. Real-time PCR assay is most rapid, sensitive and specific but costly. Conventional RT-PCR is also rapid, sensitive and specific and not much costly. RT-PCR is two type, one step (RT and PCR simultaneously in same tube) and two steps (RT and PCR are two separate reactions). It is reported that a one-step is better than a two-step RT-PCR (de Paula *et al*., 2004). We performed one step RT-PCR for the detection of dengue virus from known strains of dengue virus. Specific primers from CprM genes for dengue virus were used. Standardized one step RT-PCR was applied on a panel of clinical samples whose results were already known. The purpose of applying the standardized assay on the clinical samples was to check the inhibitory components in the blood samples. Our assay is able to detect all the circulating strains of dengue virus simultaneously, therefore equally applicable for other strains of dengue viruses. The present assay can be applied on mosquitoes' samples for surveillance of dengue viruses for early warning. The present assay can up-graded for simultaneously detections of other viruses like Chikungunya, Zika and other flaviviruses with similar clinical symptoms.

Conclusion

Present study entitled "Standardization of One-step Single Tube Multiplex RT-PCR for Rapid Detection of all Circulating Strains of Dengue Viruses" has been conceptualized for rapid detection of dengue virus which is responsible for life threatening infectious diseases. Presently, dengue is diagnosis by clinically, rapid kits, virus isolation, immunological assays and modern molecular assays. These assays have their own limitation and one-step single tube RT-PCR seems to be good alternate in present scenario. In molecular assays PCR is rapid, sensitive and specific and less costly than other assays. Cost of assay is an important factor in developing countries like India. The standardized one step RT-PCR assay for dengue virus can be applied on tissue culture isolates, mosquito's samples, and any type of clinical sample for surveillance of dengue viruses. There is still a need to develop some specific therapeutic agents or vaccines for dengue virus to protect human population.

Acknowledgement

We are highly thankful to the Haryana State Council for Science & Technology (HSCST), Haryana, India for funding this study.

REFERENCES

Balaya S, Paul SD, D'Lima LV, Pavri KM. 1969. Investigations on an outbreak of dengue in Delhi in 1967. *Indian J Med Res.* 57: 767-774.

Bhatnagar, J, Guarner, J, Paddock, C.D., Shieh, W.J, Lanciotti, R.S., Marfin, A.A. *et al*. 2007. Detection of West Nile virus in formalin-fixed, paraffin-embedded human tissues by RT-PCR: a useful adjunct to conventional tissue-based diagnostic methods. *J Clin Virol.* 38: 106-111.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L. 2013. The global distribution and burden of dengue. Nature. 496(7446): 504-7.

Broor, S., Dar, L., Sengupta, S., Chakaraborty, M., Wali, J.P., Biswas, A. *et al*. 1997. Recent dengue epidemic in Delhi, India. In Factors in the Emergence of Arbovirus diseases Edited by: Jean-François Saluzzo, Betty Dodet. *Elsevier Science.*123-127.

Carme, B., Matheus, S., Donutil, G., Raulin, O., Nacher, M., Morvan, J. 2009. Concurrent dengue and malaria in Cayenne Hospital, French Guiana, Emerg Infect Dis. 15(4): 668-71.

Chan, Y.C., Chan, K.L. and Ho, B.C. 1971b. Aedes aegypti (L.) and Aedes albopictus (Skuse) in Singapore City 5. Observations in relation to dengue haemorrhagic fever. Bull WHO. 44(5): 651-658.

Chan, Y.C., Chan, K.L., and Ho, B.C.1971a Aedes aegypti (L.) and Aedes albopictus (Skuse) in Singapore City. 1. Distribution and density. Bull WHO. 44(5): 617-627.

Chang, J., Schul, W., Yip, A., Xu, X., Guo, J.T., Block, T.M. 2011. Competitive inhibitor of cellular alpha-glucosidases protects mice from lethal dengue virus infection. *Antiviral Res.,* 92(2): 369-371.

Dar, L., Gupta, E., Narang, P., Broor, S. 2003. Co-circulation of dengue serotypes, Delhi, India, 2003. Letter to the editor. Emerg Infect Dis. 12(2): 352-353.

De Paula, S.O.D., Lima, C.D.M., Torres, M.P., Pereira, M.R., da Fonseca, B.A.L. 2004. One-step RT-PCR protocols improve the rate of dengue diagnosis compared to two-step RT-PCR approaches. *J Clin Virol.* 30:297-301

Diesh, P., Pattanayak, S., Singha, P., Arora, D.D., Mathur, P.S., Ghosh, T.K. *et al*. 1972. An outbreak of dengue fever in Delhi-1970. J Commun Dis. 4:13-18.

Endy, T.P., Anderson, K.B., Nisalak, A., Yoon, I.K., Green, S., Rothman, A.L. *et al*. 2011. Determinants of inapparent and symptomatic dengue infection in a prospective study of primary school children in Kamphaeng Phet, Thailand. PLoS Negl Trop Dis. 5(3): e975.

Gubler, D.J. 1998. Resurgent vector-borne diseases as a global health problem. Emerg Infect Dis. 4(3): 442-450.

Gubler, D.J. 1998. The arboviruses: epidemiology and ecology. In Dengue Volume II. Edited by: Monath TP. CRC Press, Boca Raton, FL.1988. 223-260.

Guzman, M.G., Hermida, L., Bernardo, L., Ramirez, R., Guill´en, G.2010. Domain III of the envelope protein as a dengue vaccine target. Expert Rev Vaccines. 9(2): 137-147.

Halstead SB. Dengue. Lancet. 2007. 370: 1644–1652.

Holmes EC and Burch SS. The causes and consequences of genetic variation in dengue virus. Trends Microbiol. 2000. 8: 74-77.

Kabra, S.K., Verma, I.C., Arora, N.K., Jain, Y., Kalra, V. 1992. Dengue haemorrhagic fever in children in Delhi. Bull World Health Organ. 70: 105-108.

Kato D, Era S, Watanabe I, Arihara M, Sugiura N, Kimata K, *et al*. Antiviral activity of chondroitin sulphate E targeting dengue virus envelope protein. Antiviral Res. 2010. 88(2): 236-243.

Kaushik, R.M., Varma, A., Kaushik, R., Gaur, K.J. 2007. Concurrent dengue and malaria due to *Plasmodium falciparum* and *P. vivax*. Trans R Soc Trop Med Hyg.. 101(10): 1048-50.

Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E. *et al*. 2001. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell. 108(5): 717-725.

Lanciotti, R.S., Calisher, C.H., Gubler, D.J., Chang, G.J., Vorndam, A.V. 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol.. 30: 545-51.

Laue, T., Emmerich, P., Schmitz, H. 1999. Detection of dengue virus RNA in patients after primary or secondary dengue infection by using the TaqMan automated amplification system. *J Clin Microbiol.*. 37: 2543-2547.

Lee, I.K., Liu, J.W., Yang, K.D. 2009. Clinical characteristics, risk factors, and outcomes in adults experiencing dengue hemorrhagic fever complicated with acute renal failure. *Am J Trop Med Hyg.* 80: 651-655.

Low, J.G., Ong, A., Tan, L.K., Chaterji, S., Chow, A., Lim, W.Y. *et al*. 2011 The early clinical features of dengue in adults: challenges for early clinical diagnosis. PLoS Negl Trop Dis. 2011. 5(5): e1191.

McBride WJH, Belefeldt-Ohmann H. Dengue viral infections; pathogenesis and epidemiology. Microbes and Infect. 2000. 2: 1041-50.

Monath TP. Dengue: The risk to developed and developing countries. Proc Natl Acad Sci, USA. 1994. 91: 2395-400.

Murrell S, Wu SC, Butler M. Review of dengue virus and the development of a vaccine. Biotechnol Adv. 2011, 29(2): 239-247.

Nimmannitya, S., Thisyakorn, U., Hemsrichart, V. 1987. Dengue hemorrhagic fever with unusual manifestations. *Southeast Asian J Trop Med Public Health.* 18: 398-406.

Rao, C.V.R.M. 1987. Dengue fever in India. *Indian J Pediatr.* 1987. 54: 11-14.

Rao, C.V.R.M., Bagchi, S.K., Pinto, B.D., Ilkal, M.A., Bharadwaj, M., Shaikh, B.H. *et al*. 1985. The 1982 epidemic of dengue fever in Delhi. Indian J Med Res.. 82: 271-5.

Santana, V.S., Lavezzo, L.C., Mondin,i A., Terzian, A.C., Bronzoni, R.V., Rossit, A.R. *et al*. 2010. Concurrent Dengue and malaria in the Amazon region, *Rev Soc Bras Med Trop.* 43(5): 508-11.

Sarkar, J.K., Pavri, K.M., Chatterjee, S.N., Chakravarty, S.K., Aanderson, C.R. 1964. Virological and serological studies of cases of haemorrhagic fever in Calcutta. *Indian J Med Res.* 52:684-691.

Seneviratne, S.L., Malavige, G.N., de Silva, H.J. 2006. Pathogenesis of liver involvement during dengue viral infections. Trans R Soc Trop Med Hyg.. 100: 608-614.

Singh, K., Lale, A., Eong, Ooi, E., Chiu, L.L., Chow, V.T., Tambyah, P. *et al*. 2006. Aprospective clinical study on the use of reverse transcription-polymerase chain reaction for the early diagnosis of Dengue fever. *J Mol Diagn.* 8: 613-616.

Tricou, V., Minh, N.N., Farrar, J., Tran, H.T., Simmons, C.P.2011 Kinetics of viremia and NS1 antigenemia are shaped by immune status and virus serotype in adults with Dengue. PLoS Negl Trop Dis.. 5: e1309.

World Health Organization. Dengue and dengue haemorrhagic fever. Fact sheet 2002,117.

World Health Organization. Dengue and Dengue Hemorrhagic Fever. 2009. Fact sheet no. 117. Available at: http://www.who.int/mediacentre/factsheets/fs117/en/index.html.

World Health Organization. WHO Regional Office for South-East Asia. Comprehensive guidelines for prevention and control of dengue and dengue hemorrhagic fever. WHO revised and expanded edition. 2012.

****