

Full Length Research Paper

Virological diagnosis of dengue fever in Jeddah, Saudi Arabia: Comparison between RT-PCR and virus isolation in cell culture

Esam Azhar^{1*}, Moujahed Kao¹, Matthias Niedrig², Badr Masri¹, Azad Godus¹, Raed Badierah¹, Nassem Khan³, Adnan Almazroo⁴, Ahmad Ashshi⁵ and Ghazi Jamjoom¹

¹Special Infectious Agents Unit, Bio-safety level - 3, King Fahd Medical Research Center, King Abdulaziz University, P. O. Box: 128442, Jeddah-21362, Saudi Arabia.

²Robert Koch-Institute, Viral Hemorrhagic Fever Department, Nordufer 20, 13355 Berlin /Germany.

³Division of Rheumatology, University of Arkansas for Medical Sciences, Mail Slot - 509, 4301 West Markham, Little Rock, AR 72205, USA.

⁴Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.

⁵Medical Laboratory Department, Faculty of Applied Medical Sciences, Umm Al-Qura University, Makkah, Saudi Arabia.

Accepted 27 September, 2010

A total of 233 serum samples were collected from patients presenting to King Abdulaziz University Hospital with suspected cases of Dengue Fever (DF) from 2006 to 2008. Dengue virus was successfully isolated from 70 samples by culture on C6/36 and LLC-MK2 cells; it was then detected by indirect immunofluorescence assay (IFA). The cytopathic effect (CPE) of dengue virus on C6/36 appeared in most of the samples within 1-4 days post-inoculation comparing to 7-12 days on LLC-MK2 cells, and this was characterized by the ability to induce syncytia and multinucleated giant cells. On the other hand, by using RT-PCR technique, 87 (37.3%) samples were positive. All 70 (30.4%) samples with positive cell culture results were detectable by RT-PCR in addition to 17 culture-negative samples were RT-PCR positive. Dengue virus type 1 (DENV-1) was the dominant serotype followed by DENV- 3 and DENV-2, while DENV-4 was not detected in tested samples. These results indicate that DENV-RNA detection by RT-PCR is more sensitive than virus isolation. We suggest that the high sensitivity coupled with the turnaround time, have made the RT-PCR a better choice as a routine test for DENV diagnosis.

Key words: Dengue virus, Dengue fever, RT-PCR, IIF, Saudi Arabia, Jeddah, Dengue diagnosis, virus isolation, virus replication, C6/36, LLCMK-2.

INTRODUCTION

Dengue fever (DF) has emerged as a global health problem; Dengue virus (DENV) causes epidemics throughout the subtropical and tropical regions (Block et al., 1988; Guzman and Kouri, 2003). The disease is caused by a single stranded RNA virus. This virus belongs to the family Flaviviridae and has four closely related but serologically distinct types: DENV-1, DENV-2,

DENV-3, and DENV-4 (Clarke, 2002). All four types are implicated in causing classical dengue fever (DF) or life-threatening syndrome, dengue hemorrhagic fever (DHF), which is characterized by abrupt onset of vascular leakage and the dengue shock syndrome (DSS) in DHF with evidence of poor perfusion, e.g. shock (Gubler, 1997, 1998).

The virus is transmitted to human by bites of infected *Aedes aegypti* and *Aedes albopictus* mosquitoes (Chen and Wilson, 2004). The clinical picture ranges from relatively mild dengue fever, accompanied with characteristic symptoms such as fever, headache, retro-orbital pain,

*Corresponding author. E-mail: esamazhar@yahoo.com. Tel: (+966) 2 640 0000 Ext. 25297. Fax: (+966) 2 640 8443.

arthralgia, rash and myalgia, to severe DHF and DSS.

The primary infection with one of the four Dengue serotype provides lifetime homologous immunity, but only weak cross protection against other serotypes. Secondary infection with a heterologous serotype is implicated in the increased association with DHF/DSS as the result of antibody-dependent enhancement (Guzman and Kouri 1996).

The routine laboratory diagnosis of dengue virus infection is primarily achieved by RT-PCR (Drosten et al., 2002; Poersch et al., 2005; Gomes et al., 2007; Saxena et al., 2008; Yamada et al., 2002) and recently by NS1 dengue antigen Elisa. Serological diagnosis is carried out by detection of IgM and IgG antibodies by ELISA. Isolation of dengue virus in cell culture also plays an important role in dengue detection and serotyping.

The first Isolation of DENV from a fatal case of DHF in adults in Jeddah / Kingdom Saudi Arabia (KSA) was reported after the first dengue outbreak in 1994 by Fakeeh and Zaki (2001). Since then, sporadic dengue cases here continued to be reported from Jeddah and Makkah regions. In 2005/2006 a new outbreak was reported by the Ministry of Health in Saudi Arabia (Ayyub et al., 2006; Khan et al., 2008).

In this paper we report the results of dengue surveillance of serum samples tested from 2006 to 2008 in our Special Infectious Agents Unit, Bio-safety Level 3 at King Abdulaziz University (KAU) in Jeddah using two cell lines C6/36 and LLC-MK2 followed by virus detection using indirect immunofluorescence assay (IFA). In parallel, dengue virus RNA was directly detected in the same serum samples by RT-PCR. DENV typing was carried out by IFA using specific monoclonal antibodies against dengue 1 to 4 and by RT-PCR using serotype-specific primers.

MATERIALS AND METHODS

Cell cultures

A. albopictus mosquito cells line (C6/36) is the most commonly used cell culture system as it provides a relatively rapid, sensitive, and economic means of dengue virus isolation (CRL-1660, ATCC). This cell line was grown in Eagle minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), and 2 mM glutamine under 5% CO₂ at 28°C (Tesh, 1979; Kuno et al., 1985; Diamond et al., 2000; Ter Meulen et al. 2000).

The mammalian rhesus monkey kidney cells, LLC-MK2 were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS, Penicillin (5 U/ml) and streptomycin (5 µg/ml). The cells were incubated at 37°C in 5% CO₂ atmosphere (Guzman et al., 1984).

Patient samples

A total of 233 serum samples were collected from patients hospitalized at KAU in Jeddah between January 2006 and February 2008. All samples were taken from patients 1 to 8 days after onset of suspected dengue symptoms (fever, retro-orbital pains, muscle

pains...etc). Serum samples were tested immediately or stored at -80°C until use. A convalescent sample for viral diagnostic serology could not be collected two weeks after fever onset because the majority of patients did not return for follow-up investigations.

Dengue virus isolation on cell culture

These two cell lines C6/36 and LLC-MK2 have been routinely used in our laboratory for dengue virus isolation. Patient serum was diluted 1:10 with medium and passed through a 0.22 µm filter, 100 µl of the filtered diluted serum was added to 80% confluent monolayer of C6/36 or LLC-MK2 cells in cell culture tubes and incubated for 1h at 28°C for C6/36 cells and at 37°C for LLC-MK2 cells. After adding 1 ml of medium, cultures were incubated and examined daily for appearance of cytopathological effect (CPE).

The CPE of dengue virus in C6/36 appeared between 1 to 4 days post inoculation while in LLC-MK2 around 7 to 12 days. Infected cells with CPE were harvested and tested by IFA using a polyclonal antibody pool. If CPE did not appear after 7 days on C6/36 or after 12 days on LLC-MK2, the infected cells were still harvested and tested by immunofluorescence for identification of DENV. For serotyping dengue virus specific monoclonal antibodies (Robert Koch-Institute in Berlin /Germany) against serotypes 1 to 4 were used with dilution of 1:50 in PBS (Henchal et al., 1983).

Polyclonal antibody pool

Human sera with high antibodies Immunoglobulin G (IgG) titers in capture ELISA (PanBio, Australia), were pooled and filtered with 0.45 µm Nunc Filter. Pooled sera were tested with ELISA and IFA and the optimal dilution for IFA primary antibody was determined (data not shown). This pooled serum was used as internal laboratory reference serum.

Indirect immunofluorescence assay (IFA)

DENV infected C6/36 or LLC-MK2 cells were mixed with non-infected cells and deposited on Teflon coated 8-well slides. The slides were air dried inside a bio-safety cabinet and fixed in chilled acetone/methanol (1:1) for 20 min., the wells were overlaid with 30 µl of specific polyclonal (Serum pool 1:1000 dilution) or monoclonal antibodies against DENV for serotyping. The slides were incubated in a moist chamber at 37°C for 60 min before they were washed three times in PBS. The bound antibody was detected with fluorescein-isothiocyanate (FITC)-conjugated goat anti-human IgG (Sigma, Chemicals Co.) diluted 1:1000 PBS if the first antibody is human serum or goat anti-mouse IgG (Sigma, Chemicals Co.) diluted 1:500 in PBS and 0.2% Evans blue (Sigma Chemicals Co.) if the first antibody is mouse monoclonal antibodies. The slides were washed, mounted with Fluoprep (BioMerieux) and finally, examined under a Leitz fluorescence microscope.

Detection and typing of dengue virus by real time RT-PCR

RT-PCR was performed on the 233 patients' serum samples. The light cycler (Roche v 2.0) was used. Viral genome was amplified using Oligonucleotide primers as described by (Drosten et al., 2002) and PCR products were detected using FAM-labeled hybridization probes, 20 pmol/µl of each primer (TIB-MOLBIOL, DenS2: 5'-GGATAGACCAGAGATCCTGTGT, DenAs1-3: 5'-CATTCCATTTTCTGGCGTTC, DenAs4: 5'-CAATCCATCCATCTT-GCGG-CGCTC) and 10 µM of DENV probe (TIB-MOLBIOL, DenP: 5'-6FAM-CAGCATCATT CAGGCACAGTXX--PH), Specific

Table 1. Characterization of dengue virus strains isolated from dengue patients in Jeddah.

Year	No. of sera tested	Dengue virus isolation in cell culture					Dengue virus Detection by RT-PCR					
		Positive virus isolation (%)	DENV serotypes identified by monoclonal antibodies (%)				RT-PCR positive (%)	DENV serotypes typing by RT-PCR (%)				
			1	2	3	4		1	2	3	4	
2006	199	64	61	1	2	0	78	75	1	2	0	
2007	25	4	2	0	2	0	6	5	0	1	0	
2008	9	2	2	0	0	0	3	2	0	1	0	
Total	233	70 (30.04)	65 (92.8)	1 (1.4)	4 (5.7)	0 (0)	87 (37.3)	82 (94.3)	1 (1.1)	4 (4.6)	0 (0)	

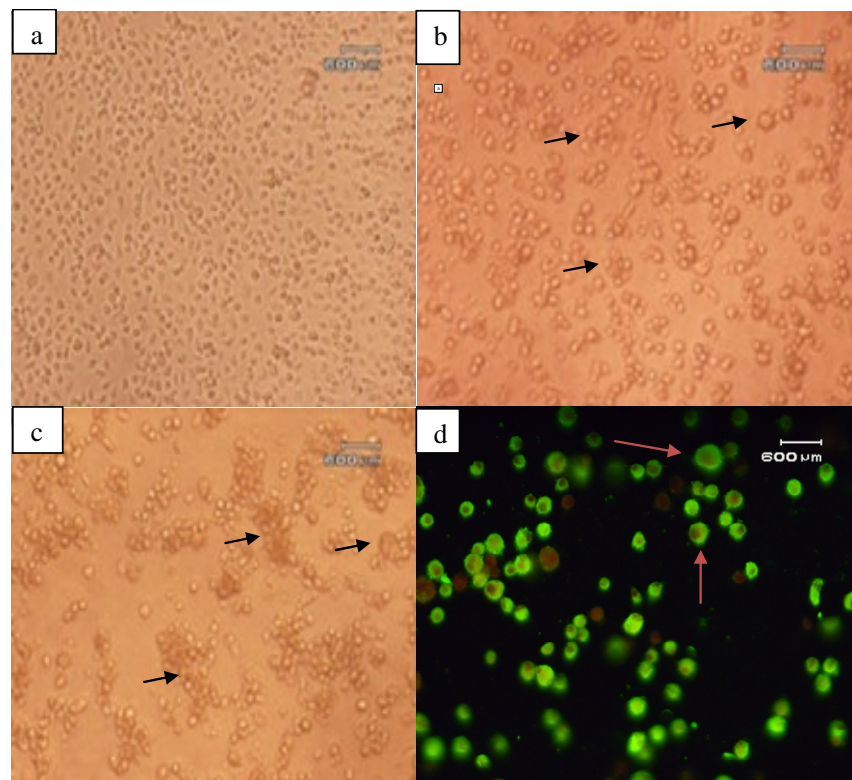


Figure 1. Dengue infected C6/36 cells. a) Monolayer of uninfected C6/36 cells. b) CPE of dengue virus, 1 day after inoculation of C6/36 cells with patient serum. The infected cells became round with light swelling and few aggregations. c) On the second day after virus inoculation, increased cell aggregation and formation of multinucleated giant cells as a result of cell-cell fusion. (arrows). d) Dengue infected C6/36 cells, 5 days after infection stained with specific monoclonal antibodies against serotype 1.

Oligonucleotide with 5' FAM-labeled probe is designed for each type of DENV, preparing 4 tubes for each sample using Qiagen QuantiTect Probe RT-PCR kit.

RESULTS

Dengue virus isolation and CPE characterization

Dengue virus infection was confirmed in 70 out of 233

cases by inoculation in C6/36 cells and in LLC-MK2 cells (Table-1). The CPE of dengue virus in C6/36 appeared between 1 to 4 days post inoculation, but in LLC-MK2 after 7-14 days. CPE in C6/36 cells was characterized as follow: first, the cells became round and swollen, then small aggregates would appear, as shown in (Figure 1b and 1c). Finally, after several days multinucleated giant cells, syncytia, and many degenerated cells and cell debris were observed. Some cells showed necrosis and become detached from the tubes at later stages of

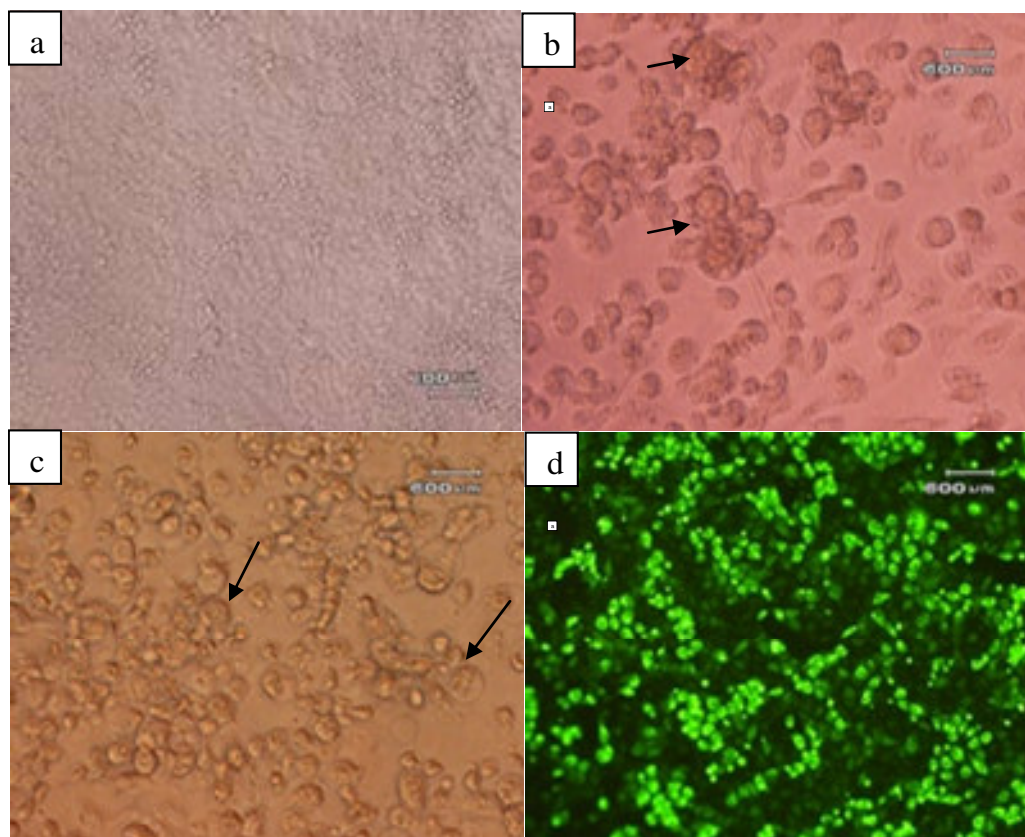


Figure 2. Dengue infected LLC-MK2 cells. a) Confluent monolayer of LLC-MK2 non-infected monkey kidney cells. b) The infected cells became round with light swelling and few aggregations. c) Seven days after inoculation of LLC-MK2 cells with serum from infected patient with DF, diffuse type of CPE typical of DENV all over the monolayer. Infected cells became rounded, swollen, syncytia and multinucleated cells can be seen. Also some degenerated cells (arrows) Giant cell formation and aggregation characterize the LLC-MK2 cells. d) Indirect Immunofluorescent antibody testing with polyclonal antibodies identifying dengue virus in tissue culture of LLC-MK2 cells. Dengue infected LLC-MK2 cell, 10 days after infection using polyclonal antibodies (1:1000).

infection.

The CPE of DENV in LLC-MK2 cells was characterized by the ability of these cells to form multinucleated giant cells and syncytia after 7 to 14 days post inoculation with patient's serum. The rounding and swelling of the giant LLC-MK2 cells were well marked as shown in (Figure 2b and 2c). Infected cells with CPE appearance were harvested and tested by IFA using the polyclonal antibody pool (1:1000). Positive cultures exhibit clear positive cytoplasmic fluorescence (Figure 2d).

Dengue virus typing by IFA using monoclonal antibodies

Figure (1d) shows positive immunofluorescence tested in the cytoplasm with the nucleus laying at the edge of the cell (eccentric). This is typical for the typing results for the three dengue forms in our studies. Typing of 70 isolated dengue virus strains showed that three dengue serotypes

were detected simultaneously in the three year period from 2006 to 2008. Serotype DENV-1 was the main isolated serotype (65 of 70 positive samples, 92.8%), followed by dengue type 3 (4 of 70 positive samples, 5.7%) and dengue type 2 (1 of 70 positive samples, 1.4%) DENV-4 was not found (Table).

Dengue virus detection and typing by real time PCR

Almost all positive sera were detectable after 21 cycles. The results of detecting DENV viral RNA by RT-PCR are shown in Table. Eighty-seven (37.3%) out of the 233 serum samples were RT-PCR positive. These results indicate that RT-PCR has higher sensitivity for virus detection than cell culture. Typing of the viral strains by Real-time RT-PCR with specific primers confirms the results to serotyping by IFA. DENV-1 was also the

dominant serotype detected in 82 patient's samples (94.3%), DENV-2 detected only in one sample (1.1%) and DENV-3 in four patients (4.6%); furthermore, DENV-4 was not detected in any of the tested samples by either technique (Table).

DISCUSSION

Dengue virus isolation on cell culture from patient's serum and mosquitoes remains the "gold standard," but this method is still difficult due to several reasons: 1) The lower sensitivity of cells in comparison with RT-PCR. 2) A longer time needed (1 to 2 weeks) for virus growth, detection by IFA and to identify the isolated virus with serotype-specific monoclonal antibodies, as DENV sometimes requires multiple passages before inducing CPE in the infected cells. 3) The isolation of dengue virus from clinical specimens is frequently possible only during the viraemic-phase (acute dengue fever) which occurs in the first 5 days after onset of the symptoms. 4) The viability of virus isolation depends on the proper handling and prompt delivery of the specimen to the laboratory. 5) The CPE produced in mosquito cell culture by many dengue viruses is difficult to detect, and it can be morphologically variable. 6) The work in a Bio-safety Laboratory Level three (BSL-3) is required for culturing of dengue virus in many countries including Saudi Arabia. Therefore many laboratories in the world do not perform dengue virus isolation in cell culture routinely. In recent years, RT-PCR has proved to be very useful in dengue diagnosis and has been able to detect dengue viruses up to the 8th day after the onset of the symptoms (Yong Yean Kong et al., 2006). Most important, it is rapid and can be done in few hours after receiving the sample; consequently, a proper treatment can be started soon enough (within 4 h) to avoid complications. Finally RT-PCR has demonstrated high sensitivity and specificity (Dorsten et al., 2002). However, the virus isolation in cell culture cannot be replaced completely by RT-PCR, as the isolation of live DENV on cell culture will still remain a valuable tool for further dengue research such as; genome sequencing, preparation of vaccine, immunizing of animals, performing of animal experiments to study the pathogenesis of the disease, and producing of monoclonal and polyclonal antibodies for diagnostic proposes.

In our study the molecular method based on RT-PCR has been compared with the cell culture method, our results have shown that the application of RT-PCR on 70 culture-positive blood samples gave a specificity and sensitivity of 100% and allowed a rapid diagnosis. In contrast, virus isolation was not successful on all RT-PCR positive samples; only 70 (80%) virus isolates were detected from 87 RT-PCR positive serum samples. These results indicate that RT-PCR technique is more sensitive than virus isolation from clinical samples. It

must be emphasized, however, that RT-PCR should not be used as a substitute for virus isolation. The availability of virus isolates is important for characterizing virus strain differences, since this information is critical for viral surveillance and pathogenesis studies.

Dengue virus serotype analysis is important in epidemiological and pathological studies. Three dengue serotypes were detected over the three-year period from 2006 to 2008. Typing of the disease in this study was done using specific monoclonal antibodies and by RT-PCR. We have found no discrepancies between serotypes detected in RT-PCR and IFA. Our dengue typing results indicate that dengue virus types 1, 2 and 3 still circulating in Jeddah since 1994, dengue serotype 1 being responsible for most of our severe DF and DHF during this period (data not shown).

During 1994 to 1997 the study of Fakeeh and Zaki. (2001) had shown that DENV-2 was the most frequent serotype in primary dengue constituting (66.7%) of the positive samples, followed by DENV-1 and DENV-3. Our present paper demonstrates that DENV-1 was the dominant isolate followed by DENV-3 and DENV-2 during study period from 2006-2008. The long-term persistence of dengue virus and continuously circulation of three serotypes 1, 2, 3 since 1994 in Jeddah, with one serotype emerging as the cause of each periodic epidemic probably indicates that the circulating serotype changes over time as has been demonstrated in other endemic situations for dengue viruses (De Simone et al., 2004; Nisalak et al., 2003, Cummings et al., 2004; Timothy et al., 2002).

We think that multiple factors influence dengue transmission and serotype circulation in Jeddah like the cycle of epidemics, the emergence of new dominant serotypes, local environment, vector breeding, the pre-existing dengue serotype specific antibodies in population over time and their capacity to induce epidemics or severe dengue diseases. All these factors required extensive study by concerned Saudi authorities to prevent and minimize future outbreaks among Saudis and local residents.

ACKNOWLEDGMENTS

We are grateful to Ms. Samah Alahmadi for her help in writing the manuscript. We are also thankful to the Physicians of King Abdulaziz Hospital (KAU) for providing us with the blood samples.

REFERENCES

- Ayyub M, Khazindar AM, Lubbad EH, Barlas S, Alfi AY, Al-Ukayli S (2006). Characteristics of dengue fever in a large public hospital, Jeddah, Saudi Arabia. *J. Ayub. Med. Coll. Abbottabad*, 18: 9-13.
- Block J, Kay BH, Hall RA, Gorman BM (1988). Isolation and characterization of dengue viruses serotype 1 from an epidemic in northern Queensland, Australia. *Arch. Virol.*, 100: 3-4, 213-220.
- Chen LH, Wilson ME (2004). Transmission of dengue virus without a

- mosquito vector: nosocomial mucocutaneous transmission and other routes of transmission. *Clin. Infect. Dis.*, 39 (6): e56-e60.
- Clarke T (2002). Dengue virus: break-bone fever. *Nature*, 416 (6882): 672-674.
- Diamond MS, Edgil D, Roberts TG, Lu B, Harris E (2000). Infection of human cells dengue virus is modulated by different cell types and viral strains. *J. Virol.*, 74(17): 7814-7823.
- Drosten S, Gottig S, Schilling S, Asper M, Panning M, Schmitz H, Guenther S (2002). Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. *J. Clin. Microbiol.*, 40(7): 2323-2330.
- Fakeeh M, Zaki AM (2001). Virologic and serologic surveillance for dengue fever in Jeddah, Saudi Arabia, 1994-1999. *Am. J. Med. Hyg.*, 65(6): 764-767.
- Gomes AL, Silva AM, Cordeiro MT, Guimaraes GF, Marques ET Jr, Anath FG (2007). Single-tube nested PCR using immobilized internal primers for the identification of dengue virus serotypes. *J. Virol. Methods*, 145(1): 76-79.
- Guzman G, Kouri G (2003). Dengue and dengue hemorrhagic fever in the Americas: lessons and challenges. *J. Clin. Virol.*, 27(1) 1-13.
- Guzman G, Kouri FG, Soler NM, Morier DL, Vazquez RS (1984). Isolation of dengue virus type 2 from the serum of patients using the suckling mouse and cultured LLC-MK2 cells]. *Rev. Cubana Med. Trop.*, 36(1): 4-10.
- Gubler DJ (1997). Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. In: Gubler DJ, Kuno G., editor. *Dengue and dengue hemorrhagic fever*. London: CAB International.; pp. 1–22.
- Gubler DJ (1998). Dengue and dengue hemorrhagic fever. *Clin. Microbiol. Rev.*, 11(3): 480-496.
- Guzman MG, Kouri G (1996). Advances in dengue diagnosis. *Clin. Diagn. Lab. Immunol.*, 3: 621-7.
- Henchal EA, McCown JM, Seguin MC, Gentry MK, Brandt WE (1983). Rapid identification of dengue virus isolates by using monoclonal antibodies in an indirect immunofluorescent assay. *Am. J. Trop. Med. Hyg.*, 32: 164–169
- Khan NA, Azhar EI, El-Fiky S, Madani HH, Abuljadial MA, Ashshi AM, Turkisatni AM, Hamouh EA (2008). Clinical profile and outcome of hospitalized patients during first outbreak of dengue in Makkah, Saudi Arabia *Acta Tropica*, 105(1): 39-44.
- Kuno G, Gubler DJ, Velez M, Oliver A (1985). Comparative sensitivity of three mosquito cell lines for isolation of dengue viruses. *Bull. World Health Organ*, 63(2): 279-286.
- Poersch CDO, Pavoni DP, Queiroz MH, Borba DL, Goldenberg S, Santos CND, Krieger MA (2005). Dengue virus infections: comparison of methods for diagnosing the acute disease. *J. Clin. Virol.*, 32(4): 272-277.
- Saxena P, Dash K, Santhosh SR, Shrivastava A, Parida M, Rao PI (2008). Development and evaluation of one step single tube multiplex RT-PCR for rapid detection and typing of dengue viruses. *Virol.*, J. 5(1): 20.
- Tesh RB (1979). A method for the isolation and identification of dengue viruses, using mosquito cell cultures. *Am. J. Trop. Med. Hyg.*, 28(6): 1053-1059.
- Ter Meulen J, Grau M, Lenz O, Emmerich P, Schmitz H, Oh F, Jaspert R, Niedrig M (2000). Isolation and partial characterization of dengue virus type 2 and 4 strains from dengue fever and dengue haemorrhagic fever patients from Mindanao, Republic of the Philippines. *Trop. Med. Int. Health*, 5(5): 325-329.
- Yamada K, Takasaki T, Nawa M, Kurane I (2002). Virus isolation as one of the diagnostic methods for dengue virus infection. *J. Clin. Virol.*, 24(3): 203-209.
- De Simone TS, Nogueira RMR, Araújo ESM, Guimarães FR, Santos FB, Schatzmayr HG, Souza RV, Teixeira Filho G, Miagostovich MP (2004). Dengue virus surveillance: the co-circulation of DENV-1, DENV-2 and DENV-3 in the State of Rio de Janeiro, Brazil. *Trans R Soc. Trop. Med. Hyg.*, 98(9): 553-562.
- Nisalak A, Endy TP, Nimmannitya S, Kalayanaroj S, Thisyakorn U, Scott RM, Burke DS, Hoke CH, Innis BL, Vaughn DW (2003). Serotype specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. *Am. J. Trop. Med. Hyg.*, 68(2): 191–202.
- Cummings DA, Irizarry RA, Huang NE, Endy TP, Nisalak A, Ungchusak K, Burke DS, Yamada K, Takasaki T, Nawa M, Kurane I (2004). Travelling waves in the occurrence of dengue hemorrhagic fever in Thailand. *Nature*, Jan 22; 427(6972): 344-447.
- Timothy PE, Nisalak A, Chunsuttiwat S, Libraty DH, Green S, Rothman AL, Vaughn DW, Ennis FA (2002). Spatial and Temporal Circulation of Dengue Virus Serotypes: A Prospective Study of Primary School Children in Kamphaeng Phet, Thailand. *Am. J. Epidemiol.*, 156: 52-59.
- Kong YY, Thay CH, Tin TC, Devi S (2006). Rapid detection, serotyping and quantitation of dengue viruses by TaqMan real-time one-step RT-PCR. *J. Virol. Methods*, 138(1-2): 123-30.