

EMERGENCE OF DENGUE VIRUS TYPE-3 IN NORTHERN INDIA

Paban Kumar Dash, Parag Saxena, Ajay Abhyankar, Rakesh Bhargava and Asha Mukul Jana

Division of Virology, Defence Research and Development Establishment, Gwalior, India

Abstract. During the last few decades dengue has reemerged in several parts of Southeast Asia, including India. A major outbreak of dengue infection occurred in northern India during October to December 2003. To determine the etiology, we carried out serological, virological and molecular investigations of this outbreak. A total of 76 dengue suspected patient blood samples were collected from Gwalior, Madhya Pradesh and Delhi, India. Serological investigations carried out using an in-house Dipstick ELISA protocol revealed the presence of anti-dengue antibodies in 53 patients. Twelve of them (22%) had a positive IgM response, indicative of primary infection, and 22 of them (42%) revealed only IgG antibodies, indicative of secondary infection. RT-PCR analysis employing dengue group specific amplicon revealed the presence of dengue specific RNA in four acute phase samples. These four RT-PCR positive samples were further processed for virus isolation in *C_{6/36}* cells and suckling mice, yielding four dengue virus isolates. The Nested PCR analysis employing serotype specific amplicon revealed the presence of dengue-3 specific 389 bp amplicon. This study confirmed the reemergence of dengue virus type-3 in a dominant form in India after a gap of nine years. Earlier, dengue virus type-2 was implicated as the etiology of a major dengue epidemic in Delhi in 1996 and Gwalior in 2001. The implication of dengue type-3 as etiology of a DHF epidemic in neighboring Sri Lanka and Bangladesh recently confirms the reemergence of dengue type-3 as the dominant form on the Indian subcontinent.

INTRODUCTION

Dengue is the second most important mosquito-borne infection, after malaria, in terms of morbidity, mortality, and economic costs, with an estimated 2.5 billion people living in areas at risk. It has now been reported in over 100 countries in tropical and sub-tropical regions of the world. It affects up to 100 million people each year, out of which approximately 25,000 cases progress to dengue hemorrhagic fever (DHF) (WHO, 2002). There are four serotypes of dengue virus (Den-1, Den-2, Den-3, and Den-4) contributing to dengue infections. The four serotypes of dengue virus belong to the family Flaviviridae and the genus *Flavivirus*. All these four dengue viruses are transmitted by *Aedes aegypti*. Dengue virus causes a spectrum of illness ranging from inapparent classical self-limiting dengue

fever (DF) to severe life threatening DHF and dengue shock syndrome (DSS). DF is characterized by sudden onset of fever, headache, retro-orbital pain, myalgia, arthralgia, and rash; whereas, DHF and DSS are characterized by thrombocytopenia, hemorrhage, and plasma leakage, leading to shock. The exact mechanism of DHF and DSS is poorly understood. However, several hypotheses, like antibody dependent enhancement (ADE), in heterotypic secondary dengue infections and the role of a virulent virus and of host factors have been suggested to explain this mechanism (Halstead, 1988; McBride and Bielefeldt-Ohmann, 2000). The risk of DHF is greatly increased when the disease is hyper-endemic with circulation of multiple serotypes in an area (Gubler, 1997). Dengue infection is endemic in many parts of India. Outbreaks have been reported at regular intervals from almost all parts of India. The number of DHF and DSS cases have increased enormously in the last two decades, with the largest and most severe being the 1996 Delhi epidemic, indicating a serious resurgence of dengue virus infection (Lall and Dhanda, 1996; Dar *et al*, 1999).

Correspondence: Dr AM Jana, Division of Virology, Defence Research and Development Establishment (DRDE), Jhansi Road, Gwalior-474002, India.
Tel: +91-751-2233495; Fax: +91-751-2341148
E-mail: amjana@rediffmail.com

The current trends of routine laboratory diagnosis of dengue virus infection include virus isolation, detection of antibody by serodiagnosis and/or molecular detection by the demonstration of viral RNA by RT-PCR (Henchal and Putnak, 1990; Guzman and Kouri, 1996). The isolation of dengue virus is difficult, laborious and requires specialized laboratory facilities. Thus the preliminary diagnosis of dengue infection relies on antibody (IgM and IgG) detection because of short and transient viremia and the difficulty associated with virus isolation. Serodiagnosis is usually made by the demonstration of a four-fold, or greater, increase in the titer of antibodies to one or more dengue virus serotypes in paired serum samples by hemagglutination inhibition (HI) (Clarke and Casals, 1958) and enzyme linked immunosorbent assay (ELISA) (Lam *et al*, 1987; Kuno *et al*, 1991).

There was a major outbreak of dengue infection in northern India from October to December 2003. In the present study, we report the investigation of this outbreak in Gwalior and Delhi city with regard to serosurveillance, virus isolation and molecular diagnosis of the suspected blood samples of febrile patients.

MATERIALS AND METHODS

Blood samples

A total of 64 blood samples from patients suspected of having dengue infection were collected from JA Hospital, Gwalior. An additional 12 blood samples were collected from Hindu Rao Hospital, Delhi. The blood samples were collected in two sets, with and without anticoagulant, for serology and virus isolation, respectively.

Mice

One-day old Swiss albino mice, obtained from the Animal Facilities of our institute were used for the isolation of the dengue virus from suspected blood samples.

Cell-culture

*C*_{6/36} (cloned cells of larvae of *Aedes albopictus*; Igarashi, 1978) cells obtained from the National Center for Cell Science (NCCS), Pune, were maintained in the laboratory by regular sub-culturing. The cells were grown in Eagle's

minimum essential medium (EMEM) (Sigma, USA) supplemented with 10% Tryptose Phosphate Broth (TPB) (DIFCO, USA), 10% Fetal bovine serum (FBS) (Sigma, USA), 3% L-glutamine (Sigma, USA) and gentamicin (80mg/l) (Nicholas Piramal, India).

Virus

Standard dengue virus serotypes 1 to 4 (DEN-1, DEN-2, DEN-3, and DEN-4) obtained from the National Institute of Virology (NIV), Pune were maintained in the laboratory by serial passaging in suckling mice, as well as in *C*_{6/36} cells, which were employed as positive controls to confirm virus isolation and as cocktail antigens in the Dipstick ELISA.

Serosurveillance

All serum samples were tested for the presence of dengue specific IgM and IgG antibodies using the dengue diagnostic kit developed in our laboratory based on the principle of Dot ELISA (Parida *et al*, 2001). Briefly, projections of nitrocellulose (NC) comb (Advanced Microdevices, Ambala, India) were coated with purified cell culture adapted DEN 1-4 cocktail antigen. For the detection of IgM antibodies, IgG antibodies were first removed from patient sera following adsorption with Protein 'A' derived from *Staphylococcus aureus* Cowan I. For the detection of IgG antibodies, patient sera were directly used. Goat anti-human IgM horseradish peroxidase (HRP) and goat anti-human IgG HRP conjugate (Sigma, USA) were used as secondary antibodies for the detection of IgM and IgG antibodies, respectively. The reaction was finally developed by dipping the projections in a substrate solution (phosphate citrate buffer pH 4.5, containing 3, 3'-diamino benzidine, 4-chloro-1-naphthol and hydrogen peroxide). The results were visually recorded as brown dots, indicative of the presence of dengue specific antibodies.

Virus isolation

Suckling mice (*in vivo*). Isolation of the dengue viruses in one-day old suckling mice was carried out following the standard protocol of Gould and Clegg (1991). Briefly, the day-old mice were inoculated with 0.02 ml of plasma (diluted 1:10 in sterile PBS) by intracerebral route, and were observed daily up to the 5th or 6th post-inocula-

tion day (PID) for the appearance of specific clinical symptoms and signs. The day-old mice revealing clinical symptoms were sacrificed and stored at -70°C until further processing. A further three serial blind passages were carried out by inoculating a 20% mouse brain suspension prepared from the above mouse.

Cell culture (*in vitro*). Isolation of viruses from the suspected samples was also attempted in the C_{6/36} cells following the protocol of Yamada *et al* (2002), with some modifications. Briefly, tissue culture flasks (25 cm²) containing preformed monolayers of C_{6/36} cells were adsorbed with 0.5 ml of plasma samples (diluted 1:10 in sterile PBS) for 1 hour at 37°C with intermittent shaking. The inoculum was then replenished with 10 ml of maintenance medium. Suitable healthy cell controls were also kept along side. The cells were then incubated at 32°C and observed microscopically daily for the appearance of cytopathic effects (CPE), if any. On the 6th to 7th PID, the cells were harvested by subjecting them to 3 cycles of freezing and thawing. Subsequently, three serial blind passages were made by using clarified harvested culture supernatant as inoculum.

Virus identification

Identification of the virus isolates obtained from the clinical samples was carried out by RT-PCR followed by Nested PCR by demonstrating the presence of virus specific RNA employing group-specific as well as serotype-specific primers following the protocol of Lanciotti *et al* (1992), with slight modifications.

RT-PCR

Briefly, RNA was extracted from the serum sample and infected mouse brain/C_{6/36} cell culture supernatants using QIAamp viral RNA mini kit (QIAGEN, Germany). Complementary DNA (cDNA) was synthesized in 10 µl of final reaction volume with RT mix comprised of 5X-RT Buffer, dNTPs, RNasin ribonuclease inhibitor, Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) (Promega, USA) at 37°C for 1 hour with dengue virus group specific consensus downstream primer (D2). The amplification of cDNA was carried out in a 50 µl final reaction volume with PCR mix containing dNTPs, *Taq*-

DNA polymerase, 25 mM MgCl₂ in 10x buffer (Promega, USA) using dengue consensus upstream primer (D1) using a thermal cycler (Perkin Elmer, USA). The thermal profile of the PCR reaction was: 1) initial denaturation at 95°C for 2 minutes; followed by 2) 30 cycles of (a) denaturation at 94°C for 30 seconds, (b) annealing at 54°C for 2 minutes, (c) extension at 72°C for 2 minutes, and 3) final extension at 72°C for 10 minutes.

Nested PCR

The primary PCR product obtained from RT-PCR was then further typed by nested PCR employing internal serotype specific primers (TS1/TS2/TS3/TS4) as downstream primer in four different sets of reactions along with D1 as an upstream primer. The rest of the procedure was the same as described in the RT-PCR.

The analysis of the amplified DNA product was carried out in 2% agarose gel for the presence of dengue group specific amplicons and type specific amplicons with respect to each sample in the RT-PCR and nested PCR, respectively.

RESULTS

Outbreak

An outbreak of febrile illness occurred from the 14th of October 2003 to the 11th of December 2003 in Gwalior, Madhya Pradesh, India. A total of 64 cases admitted to different hospitals in Gwalior were referred to our laboratory for serological and virological investigation. An additional 12 clinical samples were collected from Hindu Rao Hospital, Delhi on the 14th of October, 2003 at the height of a major dengue outbreak in Delhi and its surrounding areas. The clinical history revealed that all the patients had suffered from fever ranging from 38.5° to 40°C. A summary of the clinical symptoms and signs is described in Table 1. Most of the common symptoms include headache, myalgia, rash, arthralgia and vomiting. There was also a history of epistaxis, conjunctival hemorrhage, melena and hematuria in some patients. Thrombocytopenia was a common finding and the mean platelet count was 68,000. The trend of the epidemic in Gwalior, as indicated by the epidemic

Table 1
Frequency of symptoms and signs.

Symptoms/signs	No. of patients	Percentage
Fever	64	100
Headache	55	86
Rash	36	56
Myalgia	45	70
Arthralgia	35	55
Backache	32	50
Vomiting	35	55
Epistaxis	10	16
Melena	5	8
Conjunctival hemorrhage	8	12
Abdominal pain	25	37
Hematuria	4	6

Table 2
Age and sex distribution of patients suspected of having dengue infection in Gwalior during October-December 2003.

Age (Year)	No. of patients		
	Male	Female	Total
0-5	10	7	17
6-10	8	12	20
11-15	13	7	20
16-20	2	-	2
>20	3	2	5
Total	36	28	64

curve, showed that the maximum number of cases were reported from the 3rd to the 6th week of the month of November (Fig 1). The number of cases reported in October, November, and December were 23, 35, and 6, respectively. The epidemic affected males and females at a ratio of 1.28:1. Children and adolescents below the age of 15 years were found to be the most affected (89%). The distribution of the disease in terms of the age and sex of the patients is listed in Table 2.

Serology

All 76 serum samples were screened by the Dipstick ELISA test kit developed at our laboratory for the presence of IgM and IgG anti-dengue antibodies. The results showed 53 cases (70%) as serologically positive and 23 cases

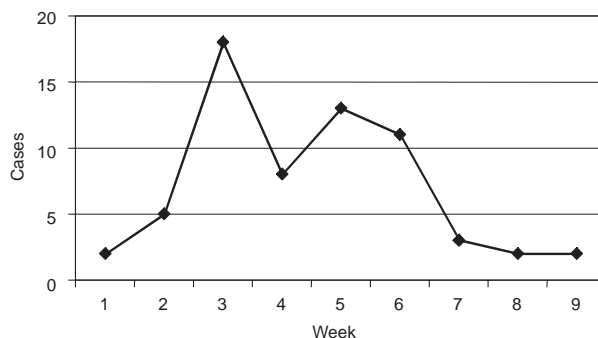


Fig 1-Trend of dengue outbreak in Gwalior from the 14th of October to the 11th of December 2003.

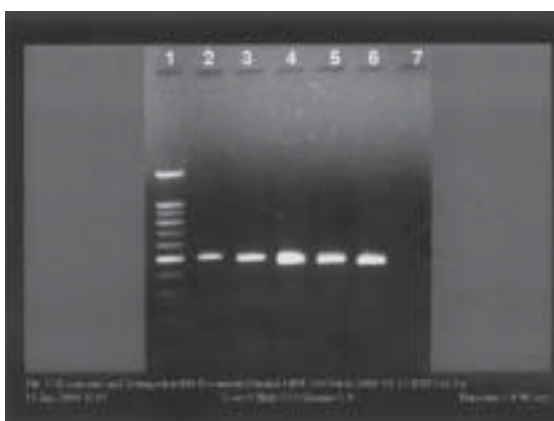


Fig 2-RT-PCR analysis of dengue suspected patient serum samples (lanes 2-5) showing the presence of dengue virus specific 511bp amplicon. Lane 1: 100bp DNA ladder (Promega USA); lane 6: DEN-3 positive control, lane 7: negative control.

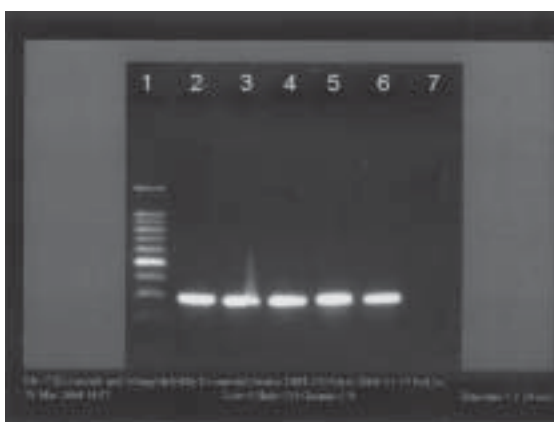


Fig 3-Nested PCR analysis of dengue virus isolates showing the presence of dengue virus serotype -3 specific 289 bp amplicon (lanes 2-5). Lane 1: 100bp DNA ladder; lane 6: positive control; lane 7: negative control.

(30%) as negative. Out of these 53 antibody positive cases, 12 (22%) were found positive for IgM, 22 (42%) for IgG and 19 (36%) had both IgM and IgG antibodies.

RT-PCR

A total of 4 samples (5.3%) were positive for the dengue virus group specific amplicon (511 bp) on 2% agarose gel (Fig 2). However, all these 4 samples were found to be serologically negative.

Virus isolation

All the four RT-PCR positive plasma samples showing the presence of viral RNA were subjected to virus isolation in both one-day-old mice and $C_{6/36}$ cells. Four dengue viruses (2 each from Gwalior and Delhi) were isolated from these four samples in both the mouse and the cell cultures. The presence of virus after each passage level was confirmed by RT-PCR. At first passage level, in one day old mice, the clinical symptoms appeared on 4th post-inoculation day. The clinical picture included flaccid paralysis, followed by death on the 5th to 6th PI day. The cell culture adapted isolates did not reveal any discernable cytopathic effects.

Typing of the isolates

All four isolates were further typed by Nested-PCR employing internal serotype specific primers. The results indicated that all the isolates recovered from the Gwalior and Delhi epidemic belonged to dengue virus type-3 (Fig 3).

DISCUSSION

Dengue is one of the major reemerging viral infections. Outbreaks have dramatically increased in several parts of Southeast Asia, including India. The present outbreak started in Gwalior in mid October, 2003 and continued until early December, 2003. The outbreaks in both Gwalior and Delhi occurred almost simultaneously after a heavy rainy season, during which the temperature and humidity remained favorable for mosquito breeding. The outbreak subsided in December, when the breeding conditions became unfavorable in North India, as the temperature dropped to around 13°C. This is in

agreement with other findings about the occurrence of outbreaks of dengue infection during the post-monsoon period (Lall and Dhanda, 1996; Ram *et al*, 1998; Parida *et al*, 2002).

The study of clinical symptoms revealed that most of the patients were suffering from dengue fever. However, very few DHF cases were also reported. It affected approximately equal numbers of males and females without any appreciable sex bias. Children and adolescents below the age of 15 years were primarily affected, which reaffirms the earlier hypothesis that they belong to the primary risk group for dengue infection in Southeast Asia (Monath, 1994). Dengue is primarily considered as a pediatric disease, and fatal dengue cases have mostly been reported among children in Southeast Asia (Nimmannitya, 1987; Pancharoen *et al*, 2001; Witayathawornwong, 2001; Kabilan *et al*, 2003).

The routine laboratory diagnosis of dengue virus infection is primarily done by the detection of anti-dengue antibodies by serodiagnosis and/or molecular detection by the demonstration of viral RNA by RT-PCR or by the isolation of the virus from the acute phase serum sample (Guzman and Kouri, 1996). A number of serological techniques have been developed for dengue diagnosis. However, μ -capture ELISA (MAC-ELISA), Dipstick ELISA and Immunochromatography based assays have been evaluated and used successfully by various investigators (Cardosa *et al*, 1995; Lam and Devine 1998; Palmer *et al*, 1999; Wu *et al*, 1997, 2000). In the present study, we screened all the serum samples for the presence of IgM and IgG antibodies by a Dipstick ELISA protocol developed at our laboratory. This test was extensively evaluated with field sera collected from different parts of India (Parida *et al*, 2001). One of the most important advantages of this assay system is the higher sensitivity of the detection of IgM antibodies. This was achieved by reducing the presence of abundant IgG in the test sera by pretreatment with Protein 'A'. This protocol can effectively discriminate primary and secondary infection based on the presence of IgM and IgG antibodies. In primary dengue infection, serum IgM antibodies start appearing within 5-7 days of the onset of symptoms and persist for up to

90 days; whereas in secondary infection, there is always a higher rise in IgG antibodies with or without a rise in IgM antibodies. The presence of both IgM and IgG antibodies results from either delayed primary or secondary infection cases (Henchal and Putnak, 1990; Gubler, 1997).

The results of serological tests also supports the dengue viral etiology of the present outbreak. The presence of only IgG antibodies in the majority (42%) of the patients reveals that they were suffering from secondary dengue infection. This is expected, as there was a major dengue outbreak in Gwalior in 2001, which was also extensively investigated by our laboratory (Parida *et al*, 2002). The absence of dengue specific antibodies in large number of patient sera may be attributed either to the presence of undetectable/very low levels of antibodies or to misdiagnosis based on the clinical symptoms. This was expected, as the symptoms of dengue infection are similar to other diseases, such as malaria, typhoid, influenza, rubella, and leptospirosis (WHO, 1997; Ram *et al*, 1998; Harris *et al*, 2000).

RT-PCR is considered a highly reliable diagnostic test when serum/plasma samples are collected during the early febrile phase of dengue infection. In the present study, all the 74 serum samples were subjected to dengue group specific RT-PCR, but we could identify only 4 samples as dengue positive by RT-PCR. This may be due to the fact that most of the patients presented to the hospitals in the post-viremic phase. All four of the RT-PCR positive samples were collected from acute phase febrile patients and did not reveal the presence of antibodies. All 4 positive samples were processed for virus isolation in $C_{6/36}$ cells/suckling mice. Isolation of dengue virus was successful from these four RT-PCR positive samples in both $C_{6/36}$ cells and suckling mice. The isolation was confirmed by RT-PCR, further typing of the isolate, as well as the RT-PCR positive sample, by nested PCR revealed the presence of dengue virus type-3 specific RNA. Isolation and identification of virus from the clinical sample is considered the gold standard and gives a confirmatory diagnosis without ambiguity (Chouhan *et al*, 1990, Vorndam and Kuno, 1997; Yamada *et al*, 2002).

All the four serotypes of dengue viruses were isolated from different parts of India during the dengue outbreak. Previously, dengue virus type-2 had been reported to be the predominant serotype circulating in Northern India (Mahadev *et al*, 1997, Dar *et al*, 1999, Kumar *et al*, 2001). We had also reported the isolation of dengue-2 from Gwalior in 2001 (Parida *et al*, 2002). Recently dengue-3 has been reported as the etiology of the first major DHF outbreak in Bangladesh (Rahman *et al*, 2002). Dengue-3 was also implicated as one of the major serotypes along with dengue-2 in various outbreaks in neighboring Sri Lanka (Messer *et al*, 2002, 2003). The isolation of dengue-3 from the present outbreak in Northern India in 2003 indicates the resurgence of dengue-3. Though dengue-3 had been reported earlier in several outbreaks in West Bengal (1983, 1990), Rajasthan (1985) and Maharashtra state (1994) of India, no new cases had been reported after the 1994 outbreak in the Dhule district of Maharashtra (Mukherjee *et al*, 1987; Chouhan *et al*, 1990, Bhattacharjee *et al*, 1993; Padbidiri *et al*, 1996). The emergence of a newer dengue serotype after an interval always leads to a major outbreak, which is a matter of concern.

In Summary, this study demonstrates that the present outbreak is caused by dengue virus type-3. The reemergence of dengue-3 in a dominant form in different areas of the Indian subcontinent is matter of great concern. Detailed serological and virological studies of the dengue outbreak in endemic areas are warranted to pinpoint the nature of the outbreak and to help to develop effective control and management strategies against this impending dengue menace.

ACKNOWLEDGEMENTS

The authors are thankful to Shri K Sekhar, Director, DRDE, Gwalior for his keen interest and constant support of this study. The authors are also thankful to the Superintendent, JA Hospital, Gwalior and Hindu Rao Hospital, Delhi for providing the clinical samples.

REFERENCES

Bhattacharjee N, Mukherjee KK, Chakravarti SK, *et al*.

- Dengue haemorrhagic fever (DHF) outbreak in Calcutta-1990. *J Commun Dis* 1993; 25: 10-4.
- Cardosa MJ, Baharudin F, Hamid S, Hooi TP, Nimmanitya S. A nitrocellulose membrane based IgM capture enzyme immunoassay for etiological diagnosis of dengue virus infection. *Clin Diagn Virol* 1995; 3: 343-50.
- Chouhan GS, Rodrigues EM, Shaikh BH, *et al.* Clinical and virological study of dengue fever outbreak in Jalore city, Rajasthan 1985. *Indian J Med Res* 1990; 91: 414-8.
- Clarke DH, Casals J. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am J Trop Med Hyg* 1958; 7: 561-73.
- Dar L, Broor S, Sengupta S, Xess I, Seth P. The first major outbreak of dengue haemorrhagic fever in Delhi, India. *Emerg Infect Dis* 1999; 5: 589-90.
- Gould EA, Clegg TCS. Growth, assay and purification of Alphaviruses and Flaviviruses. In: Mahy BWJ, ed. *Virology: a practical approach*. United Kingdom: Oxford University Press, 1991: 43-78.
- Gubler DJ. Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. In: Gubler DJ, Kuno G, eds. *Dengue and dengue hemorrhagic fever*. London: CAB International, 1997: 1-22.
- Guzman MG, Kouri G. Advances in dengue diagnosis. *Clin Diagn Lab Immunol* 1996; 3: 621-7.
- Halstead SB. Pathogenesis of dengue: challenges to molecular biology. *Science* 1988; 239: 476-81.
- Harris E, Videz E, Perez L, *et al.* Clinical, epidemiologic, and virologic features of dengue in the 1998 epidemic in Nicaragua. *Am J Trop Med Hyg* 2000; 63: 5-11.
- Henchal EA, Putnak JR. The dengue viruses. *Clin Microbiol Rev* 1990; 3: 376-96.
- Igarashi A. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. *J Gen Virol* 1978; 40: 531-44.
- Kabilan L, Balasubramanian S, Keshava SM, *et al.* Dengue disease spectrum among infants in the 2001 dengue epidemic in Chennai, Tamil Nadu, India. *J Clin Microbiol* 2003; 41: 3919-21.
- Kumar A, Sharma SK, Padbidri VS, Thakare JP, Jain DC, Datta KK. An outbreak of dengue fever in rural areas of northern India. *J Commun Dis* 2001; 33: 274-81.
- Kuno G, Gomez I, Gubler DJ. An ELISA procedure for the diagnosis of dengue infections. *J Virol Methods* 1991; 33: 101-13.
- Lall R, Dhanda V. Dengue haemorrhagic fever and the dengue shock syndrome in India. *Natl Med J India* 1996; 9: 20-3.
- Lam SK, Devi S, Pang T. Detection of specific IgM in dengue infections. *Southeast Asian J Trop Med Public Health* 1987; 18: 532-8.
- Lam SK, Devine PI. Evaluation of capture ELISA and rapid immunochromatographic test for the detection of IgM and IgG antibodies produced during dengue infection. *Clin Diagn Virol* 1998; 10: 75-81.
- Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol* 1992; 30: 545-51.
- Mahadev PVM, Prasad SR, Ilkal MA, Mavale MS, Bedekar SS, Banerjee K. Activity of dengue-2 virus and prevalence of *Aedes aegypti* in the Chirimiri colliery area, Madhya Pradesh, India. *Southeast Asian J Trop Med Public Health* 1997; 28: 126-37.
- Mc Bride WJH, Bielefeldt-Ohmann H. Dengue viral infections; pathogenesis and epidemiology. *Microbes Infect* 2000; 2: 1041-50.
- Messer WB, Gubler DJ, Harris E, Sivananthan K, de Silva AM. Emergence and global spread of a dengue serotype 3, subtype III virus. *Emerg Infect Dis* 2003; 9:800-9.
- Messer WB, Vitarana UT, Sivananthan K, *et al.* Epidemiology of dengue in Sri Lanka before and after the emergence of epidemic dengue hemorrhagic fever. *Am J Trop Med Hyg* 2002; 66: 765-73.
- Monath TP. Dengue: The risk to developed and developing countries. *Proc Natl Acad Sci USA* 1994; 91: 2395-400.
- Mukherjee KK, Chakravarti SK, Dey PN, Dey S, Chakraborty MS. Outbreak of febrile illness due to dengue virus type 3 in Calcutta during 1983. *Trans R Soc Trop Med Hyg* 1987; 81: 1008-10.
- Nimmannitya S. Dengue hemorrhagic fever in Thailand. *Southeast Asian J Trop Med Public Health* 1987; 18: 291-4.
- Padbidri VS, Mahadev PVM, Thakare JP, *et al.* Virological and entomological investigations of an outbreak of dengue fever in Dhule district, Maharashtra. *Indian J Med Microbiol* 1996; 14: 25-32.
- Palmer CJ, King SD, Cuadrado RR, Perez E, Baum MM, Ager AL. Evaluation of the MRL diagnostics dengue fever virus IgM capture ELISA and the

- PanBio rapid immunochromatographic test for diagnosis of dengue fever in Jamaica. *J Clin Microbiol* 1999; 37: 1600-1.
- Pancharoen C, Thisyakorn U. Dengue virus infections during infancy. *Trans R Soc Trop Med Hyg* 2001; 95: 307-8.
- Parida MM, Dash PK, Upadhyay C, Saxena P, Jana AM. Serological and virological investigation of an outbreak of dengue fever in Gwalior, India. *Ind J Med Res* 2002; 116: 248-54.
- Parida MM, Upadhyay C, Saxena P, Dash PK, Jana AM, Seth P. Evaluation of a Dipstick ELISA and a rapid immunochromatographic test for diagnosis of dengue virus infection. *Acta Virologica* 2001; 45: 299-304.
- Rahman M, Rahman K, Siddque AK, *et al.* First outbreak of dengue hemorrhagic fever, Bangladesh. *Emerg Infect Dis* 2002; 8: 738-40.
- Ram S, Khurana S, Kaushal V, Gupta R, Khurana SB. Incidence of dengue fever in relation to climatic factors in Ludhiana, Punjab. *Indian J Med Res* 1998; 108: 128-33.
- Vorndam V, Kuno G. Laboratory diagnosis of dengue virus infections. In: Gubler DJ, Kuno G, eds. Dengue and dengue hemorrhagic fever. London: CAB International, 1997: 313-34.
- Witayathawornwong P. Dengue hemorrhagic fever in infancy at Petchabun Hospital, Thailand. *South-east Asian J Trop Med Public Health* 2001; 32: 481-7.
- World Health Organization. Dengue and dengue haemorrhagic fever. Fact sheet. 2002: 117.
- World Health Organization. Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2nd ed. Geneva: World Health Organization, 1997.
- Wu SJL, Hanson B, Paxton H, *et al.* Evaluation of a dipstick enzyme-linked immunosorbent assay for detection of antibodies to dengue virus. *Clin Diagn Lab Immunol* 1997; 4: 452-7.
- Wu SJL, Paxton H, Hanson B, *et al.* Comparison of two rapid diagnostic assays for detection of IgM antibodies to dengue virus. *Clin Diagn Lab Immunol* 2000; 7: 106-10.
- Yamada K, Takasaki T, Nawa M, Kurane I. Virus isolation as one of the diagnostic methods for dengue virus infection. *J Clin Virol* 2002; 24: 203-9.