Short Report: A Comparative Study of Clinical Features between Monotypic and Dual Infection Cases with Chikungunya Virus and Dengue Virus in West Bengal, India

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Abstract. Chikungunya virus (CHIKV) and dengue virus (DENV) are circulating individually in the state of West Bengal, India. However, after 1965 the dual-infection caused by both viruses had not been recorded until 2010. In 2010, an investigation of the febrile cases was carried out to confirm the involvement of both viruses simultaneously. A total of 550 blood samples were tested for the detection of immunoglobulin M (IgM) antibody against both CHIKV and DENV. Serology by the enzyme-linked immunosorbent assay method confirmed that 131 (23.8%) and 104 (18.9%) patients had IgM antibody against CHIKV and DENV, respectively, whereas 68 (12.4%) had IgM antibodies against both CHIKV and DENV. Fever, joint pain, rashes, headache, myalgia, and nausea/vomiting are the common features in the case of both monotypic and dual-infection. Severe arthralgia and swelling of joints were common only in CHIKV-positive cases and abdominal pain was mainly associated with DENV infection. Diarrhea was reported only by the dual-infected patients (16.2%).

Arthropod-borne viruses or arboviruses are one of the major public health problems worldwide. Out of many arboviruses, chikungunya virus (CHIKV) and dengue virus (DENV) are the two most rapidly spreading arboviruses. The CHIKV belongs to the genus Alphavirus of the Togaviridae family, whereas DENV belongs to the family Flaviviridae and genus Flavivirus. To date, both CHIKV and DENV are co-circulating in India and Southeast Asia.1 Both viruses are the RNA virus and the diseases caused by them are transmitted to humans by the vector mosquitoes Aedes aegypti and Aedes albopictus.2 Both diseases have some common signs and symptoms that include fever, rashes, joint pain, nausea, headache, and vomiting.

In India, CHIKV was first recorded in West Bengal in 1963–65 along with the dengue outbreak.3 Chikungunya cases were not recorded in West Bengal after that outbreak, however, until 1973, several outbreaks caused by CHIKV were recorded from other states of India.4 After that, the virus disappeared from India.5 In 2005–2006, CHIKV outbreaks were reported from many states of India, including West Bengal.6 Dengue is one of the rapidly spreading infections affecting 50 million people per year.7 In India, DENV was first isolated in Kolkata in 1824,8 however, the outbreak caused by DENV was first recorded in Kolkata in 1965.

Co-circulation of CHIKV and DENV is not uncommon in South-East Asia.9–11 In India, concurrent isolation of CHIKV and DENV had been reported since 1964 from different States.12 In 2010, a hospital-based study revealed co-circulation of CHIKV and DENV in some areas of West Bengal, India with high morbidity.13 The aim of our work was to study the socio-demographic features of dual-infected cases, suffered from both CHIKV and DENV infection simultaneously and to compare the clinical features between the monotypic and dual-infected patients in West Bengal, India. For this purpose, in 2010, a study was conducted from the suspected cases, referred by the district health authority and by the clinicians of different hospitals. Samples were collected from the patients admitted with high fever (> 39°C) and any two of the following symptoms, i.e., rashes, joint pain, swelling of joints, nausea/vomiting, headache, myalgia, and retro-orbital pain. The local hospitals reported the absence of bacterial etiology and parasites in the blood samples. Informed consent was obtained from the patient or from the parents or legal guardians of minors before the collection of samples. A total of 550 samples were referred by the clinicians to our department for detection of CHIKV or DENV with detailed socio-demographic information and clinical history. Written consents were obtained before collection of the samples. Leukocyte counts of the patients were 3.5 x 10⁹/L–5 x 10⁹/L and the platelet counts were 105 x 10⁹/L–160 x 10⁹/L. The sera were separated from the clotted blood samples and stored in aliquots at −80°C for further use.

All of the samples were subjected to an enzyme-linked immunosorbent assay (ELISA) test to detect the presence of immunoglobulin M (IgM) antibodies against both CHIKV and DENV by IgM antibody-capture (MAC)-ELISA kits.14 The kits were purchased from the National Institute of Virology, Pune, India.15 Optical density (OD) was measured at 492 nm using an ELISA reader. The normal deviate test was performed to compare the data. The Z-values were calculated manually. The P value < 0.05 was considered significant.

Out of 550 samples, 131 (23.8%) and 104 (18.9%) samples were positive to IgM antibody against only CHIKV and DENV, respectively, whereas, 68 (12.4%) samples were positive to IgM antibody against both CHIKV and DENV. No cross-reactivity was observed between the two viruses.

For the reverse transcription-polymerase chain reaction (RT-PCR) test, viral RNA was isolated from all the samples by using the Qiagen viral RNA isolation kit (Qiagen, GmbH, Hilden, Germany). The RT-PCR test was performed following the cost-effective RT-PCR method for detecting both CHIKV and DENV.13 The DENV typing was performed by using nested PCR with genotype-specific primers.14 Out of 550 samples tested, both DENV and CHIKV were detected in 24 samples; of which 18 samples contained DEN-2 serotype and 6 samples contained DEN-3 serotype. The IgM antibody against DENV or CHIKV was not detected in these 24 samples. The CHIKV RNA was detected in another seven
Table 2
Clinical characteristic of co-infected patients referred from different medical colleges and hospitals in West Bengal, India in 2010

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Chikungunya cases (%)</th>
<th>Dengue cases (%)</th>
<th>Co-infected cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N = 131)</td>
<td>(N = 104)</td>
<td>(N = 68)</td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>131 (100)</td>
<td>104 (100)</td>
<td>68 (100)</td>
</tr>
<tr>
<td>Joint pain</td>
<td>92 (70.2)</td>
<td>45 (43.3)</td>
<td>53 (77.9)</td>
</tr>
<tr>
<td>Rash</td>
<td>52 (39.7)</td>
<td>19 (18.3)</td>
<td>40 (58.8)</td>
</tr>
<tr>
<td>Headache</td>
<td>53 (40.5)</td>
<td>60 (57.7)</td>
<td>28 (41.2)</td>
</tr>
<tr>
<td>Myalgia/body ache</td>
<td>40 (30.5)</td>
<td>46 (44.2)</td>
<td>7 (10.3)</td>
</tr>
<tr>
<td>Itching</td>
<td>23 (17.6)</td>
<td>12 (11.5)</td>
<td>8 (11.8)</td>
</tr>
<tr>
<td>Nausea/vomiting</td>
<td>27 (20.6)</td>
<td>41 (39.4)</td>
<td>6 (8.8)</td>
</tr>
<tr>
<td>Joint swelling</td>
<td>85 (64.9)</td>
<td>4 (3.8)</td>
<td>8 (11.8)</td>
</tr>
<tr>
<td>Arthralgia/difficulty in movement</td>
<td>79 (60.3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>0</td>
<td>25 (24.2)</td>
<td>1 (1.5)</td>
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<tr>
<td>Retro-orbital pain/redness of eyes</td>
<td>0</td>
<td>8 (7.7)</td>
<td>4 (5.8)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0</td>
<td>0</td>
<td>11 (16.2)</td>
</tr>
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</table>

Regarding the monthly distribution of co-infected cases, the highest number of cases was found in the month of October (43.3%) followed by the month of November (31.3%) (Figure 2). The stagnant fresh water during the rainy seasons (June to September) favored the breeding of the vector mosquitoes. Therefore, the co-infected cases attained its peak in the month of October, which is the post-monsoon period.

In West Bengal, the first CHIKV outbreak was recorded during 1963 to 1965 in Kolkata (formerly Calcutta) along with the outbreak of DENV. After 1965, CHIKV totally disappeared from this region. In 2006, after a gap of 40 years, the virus again reappeared. It has been observed by us that by 2010 CHIKV had gradually grabbed almost every district of this state by replacing the Asian genotype to Central/East African genotype (unpublished data).

The state of West Bengal is an endemic zone of DENV. Several outbreaks have been reported from this region. Although both viruses individually affected a large number of people of this state, after 1965, the dual infection caused by both viruses were recorded in 2010 from this region. The possible reason for dual infection may be because in West Bengal and in India the mosquitoes *Ae. aegypti* and *Ae. albopictus* are abundantly present and are also the vectors for CHIKV and DENV. *Aedes aegypti* are predominated mainly in the urban areas, whereas *Ae. albopictus* can survive in both rural and urban environments. The vectors can carry both of the virus, which might have facilitated the spreading of the dual infection in both rural and urban regions.

Some man-made situations such as urbanization, industrialization, and deforestation result in vector shuffling in many places. An illustration of the monthly distribution of immunoglobulin M (IgM)-positive cases in West Bengal in 2010 is shown in Figure 2.

Figure 2. Monthly distribution of immunoglobulin M (IgM)-positive cases in West Bengal in 2010.
Rapid spread of chikungunya virus following its resurgence during 2006 in West Bengal, India

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ABSTRACT

Re-emergence of chikungunya virus (CHIKV) in West Bengal was detected after almost 40 years when an outbreak of fever occurred in Baduria village (West Bengal, India) in October 2006. The symptoms of CHIKV infection are similar to those of dengue virus (DENV) infection. Serum samples were tested for detection of IgM antibody to CHIKV and DENV and the aetiologic agent was detected as CHIKV, RT-PCR was carried out for confirmation of CHIKV infection. By 2009, CHIKV had spread rapidly within ten districts of West Bengal. Middle-aged women (age group 31–40 years) were predominantly affected. Here we report the analysis of 2134 serum samples collected during 2006–2009 from the different districts of West Bengal, among which IgM antibody to CHIKV and DENV was detected in 403 and 199 samples, respectively. This report highlights the gradual dominating activity of CHIKV with dengue-like clinical features in dengue-endemic regions such as West Bengal.

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1. Introduction

Chikungunya virus (CHIKV) is an enveloped, positive-strand RNA virus belonging to the genus Alphavirus of the Togaviridae family. Infection with CHIKV results in chikungunya fever. Aedes aegypti and A. albopictus are the main vectors of CHIKV in Asia and the Indian Ocean region.1 Following transmission, CHIKV replicates in the skin and spreads to the liver and joints.2 The incubation period of the virus is 2–4 days, followed by a sudden onset of disease with no prodromal phase. The acute phase of the disease lasts for 3–10 days with high fever, rigors, headache, photophobia and a petechial or maculopapular rash, but the convalescent phase usually lasts from weeks to months with accompanying joint pain and swelling of joints; sometimes it may last for a year or more.3 Sporadic cases of neurological complications due to CHIKV have also been reported.4

CHIKV was first isolated in Tanzania in 1953.5 In India, the first CHIKV outbreak was recorded during 1963–1965 in Kolkata (formerly Calcutta)6 along with an outbreak of dengue fever. In this epidemic, approximately 100,000 cases occurred, with 500 hospitalisations and 200 deaths. Following this epidemic, CHIKV totally disappeared from this region,7 although reports of CHIKV infection from other states of India were observed up until 1973.8 All of the isolated virus up to this period belonged to the Asian genotype.9 West Bengal is a well known dengue-endemic zone. The first epidemic of dengue fever in West Bengal was reported by Silar et al. in 1872 in Kolkata.10 However, the aetiological

headache, photophobia and a petechial or maculopapular rash, but the convalescent phase usually lasts from weeks to months with accompanying joint pain and swelling of joints; sometimes it may last for a year or more.3 Sporadic cases of neurological complications due to CHIKV have also been reported.4

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backache; and retro-orbital pain. All of the clinical specimens were transported to the ICMR Virus Unit, maintaining the cold chain. Serum was separated from the collected blood samples and stored at -80°C until use.

2.7. Serological detection

Infection with either CHIKV or DENV produces similar clinical symptoms. DENV is also endemic in West Bengal. For serological detection purposes, all of the serum samples received during the study period were subjected to MAC-EUSA concurrently for the detection of IgM antibody to CHIKV and DENV following the manufacturer's protocol. MAC-EUSA kits were purchased from the National Institute of Virology (NIV) (Pune, Maharashtra, India). These kits follow the IgM antibody-capture ELISA technique using specific antigens for CHIKV or DENV. The optical density at 405 nm was measured using an ELISA reader (Titertek Multiskan Plus Model 314; Lambysystems, Helsinki, Finland).

2.8. Isolation of virus

A control strain of CHIKV (GenBank accession no. EF027140.1) was purchased from the NIV and was revived in the C6/36 mosquito cell line in a tissue culture system. Diluted control (100 µl) was inoculated in a monolayer growth of the C6/36 cell line in 25 cm² tissue culture flasks (Nunc, Roskilde, Denmark) and adsorbed for 2 h at 28°C with 5% carbon dioxide in an incubator. Attempts were also made to isolate the virus from 72 selected ELISA-negative samples from patients with a history of ≤2 days of fever and chikungunya-like illness in 24-well tissue culture plates (Nunc).

The wells were washed with 1x PBS (Gibco BRL—Invitrogen, Grand Island, NY, USA) and then Eagle's minimal essential medium (Gibco BRL) was added supplemented with 10% fetal bovine serum (Gibco BRL) and penicillin-streptomycin antibiotic mixture (Pen Strep; Gibco) and incubated under the same condition in an incubator. Wells were observed regularly for the appearance of cytopathic effect (CPE) up to 7 days. On appearance of the CPE, the tissue culture fluid was centrifuged at 1000 x g for 10 min at 4°C to remove cell debris. The supernatant of the tissue culture fluid (STF) was used for further studies.

2.9. Molecular detection

The STF of those samples that produced CPE were selected for RT-PCR along with the STF of the control strain. RNA was extracted from STF using a QIAamp RNA Viral Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocol. RT-PCR was carried out with a QIAGEN OneStep RT-PCR Kit using the primer pair CPN1F 5' (GAACAGATGGCCACTCACACC-3') and CPN1R 5' (CTCTTATACGGCGGTGTGATG-3') and 5 µl of total RNA. RT-PCR was performed with reverse transcription (50°C for 20 min) followed by inactivation of the reverse transcriptase enzyme (95°C for 15 min) and 35 cycles of PCR, which included denaturation at 95°C for 30 s, annealing at 55°C for 32 s and extension at 72°C for 45 s. A final extension step was carried out at 72°C for 2 min.

### Table 1

<table>
<thead>
<tr>
<th>District</th>
<th>2007</th>
<th>2008</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>District-wise distribution of chikungunya virus IgM-positive cases in West Bengal, India, during 2006-2009</td>
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</tbody>
</table>
In 2006, 31 chikungunya cases were detected by ELISA from West Bengal after almost four decades. The outbreak was also confirmed by RT-PCR. The first outbreak that occurred at Baduna (North 24 Parganas) is anticipated to have been introduced by travellers as it is very close to Kolkata International Airport. Owing to the absence of any herd immunity in the population, the disease spread rapidly across the state. It is evident from the study that all districts of the South Bengal region were affected by CHIKV infections by the year 2008. In 2009, CHIKV-positive cases were recorded from North Bengal, i.e., from Malda and Dakshin Dinajpur (Table 1). This observation indicates that since the re-emergence of CHIKV in West Bengal in 2006, CHIKV-positive cases have significantly increased from 20.8% to 44.1% during the 4 years of the study.

West Bengal is a well-known endemic zone for DENV and many epidemics have been documented from this state. The last epidemic of dengue occurred in Kolkata in 2005. A total of 2019 blood samples were referred to the ICMR Virus Unit during the period 24 August to 3 October 2005 for confirmation of DENV infection, of which 946 samples were positive for IgM antibody against DENV (S. Chatterjee, unpublished data). In the case of chikungunya, the first outbreak occurred in 1963–1965 and then after a span of around 40 years CHIKV-positive cases were again reported from Baduria (West Bengal) in 2006. The striking findings of this study indicate that since 2006, with the resurgence of CHIKV, DENV cases are decreasing with the increased number of chikungunya cases (Figure 1). From the calculated P-values, it can be concluded that in 2006 the number of dengue cases was significantly higher than chikungunya cases, whereas during 2007–2009 the number of chikungunya cases was significantly higher (Table 2).

This rapid spread of CHIKV has become a problem from a public health point of view. This work only reports referred cases and represents the tip of the iceberg in the population. Total cases are much higher than those dealt with. On the other hand, the clinical features of the chikungunya cases at the acute stage are accompanied by severe joint pain, a short period or absence of rashes, with or without swelling of the joints, which almost mimics dengue.
Increasing trend of Japanese encephalitis cases in West Bengal, India – a threat to paediatric population

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ABSTRACT

Objective: To detect the Japanese encephalitis virus (JEV) as the etiologic agent from the acute encephalitis syndrome (AES) cases mainly amongst the children and young adults from vaccinated and non-vaccinated districts of West Bengal. Methods: For the detection of JEV, a total of 828 sera were referred from vaccinated and non-vaccinated districts of West Bengal during 2005-2011. Japanese encephalitis (JE) positive cases were confirmed by ELISA and RT-PCR method. Results: Out of 828 cases, 245 samples were positive by ELISA method and 46 samples were positive by RT-PCR method. Out of 291 total positive cases, 162 (55.6%) were below 20 years of age. Initially in 2005, JE cases were highest amongst the children and young adults (0-20 years). After vaccination, although the JE cases declined gradually in the vaccinated districts, but again from 2010, JE cases from the said age group showed an increasing trend from those districts. JE cases were also reported from other endemic zones of this state, which were still non-vaccinated.

Conclusions: In West Bengal, JE cases are still predominated among children and young adults till the year 2011. Mass scale vaccination programme and investigation on the circulating strains are essentially required to find out the reasons of increasing tendency of JE cases in this state.

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1. Introduction

Japanese encephalitis (JE) is one of the major public health problems in many parts of South East Asia. About 3 billion people live in countries where the Japanese encephalitis virus (JEV) is endemic. In Asia, annual incidence of the disease is about 50000 cases1. Every year, 10000-15000 people die due to this disease and about 15 000 people, who survive, develop neuro-psychological complications2,3. JE is endemic in many parts of India including the state of West Bengal4,5. In India, the first case of JE was recorded from Vellore, Tamil Nadu in 19556. Until 1970, JE was confined within southern part of India. In West Bengal, the first major outbreaks of JE occurred in the districts of Bankura and Burdwan in 19737, where about 700 cases and 300 deaths occurred. From 1978 to 2007, 103 389 cases and 33729 deaths due to JEV infection were recorded from various parts of India8. Every year, 1500-4000 cases are reported from this country9.

Studies done so far revealed that, JE was common amongst the children10,11. In India, JE cases have been recorded mainly amongst the children from different states like West Bengal, Uttar Pradesh and Tamil Nadu, affecting the children between 5 and 15 years12-14. However, in areas where JEV is recently introduced, adults are also getting the infection. In children, JE accounted for over half the cases of encephalitis and was associated with a mortality rate of 39%; over half of the survivors were left with permanent neurological sequelae15. Children up to 10 years of age are the most vulnerable group for JEV infection due to either absence or low protective immunity against the virus in them; 700 million children live at risk to this disease16,17. There are currently 3 vaccines available for the prevention of JE. In spite of vaccination programme in the last six decades, 10.5 million children have been infected of which 3 million deaths have occurred18. In 2005, over 5000 cases and 2000 deaths occurred due to JEV infections in India19.

In India, in the year 2006, children belonged to the age group of 1-15 years were vaccinated with live JE vaccine of SA-14-14-2 strain, manufactured by Chengdu Institute of Biological Products, Chengdu, China20. For this purpose, most affected areas of some states were selected; it included seven districts of UP, two districts of Assam and one district

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each from West Bengal and Karnataka. In 2007 and 2008, vaccination programme had been initiated in Andhra Pradesh, Bihar, Haryana, Maharashtra and Tamil Nada.

In spite of vaccination programme, cases and deaths due to JEV in them being reported every year. In this study we aimed to detect the JE cases mainly among the children from the vaccine covered districts as well as from other districts where vaccination has not yet been done.

2. Materials and methods

2.1. Study area

West Bengal is located at the eastern region of India (Figure 1). Kolkata formerly Calcutta is the capital of this State is also one of the metropolitan cities of India. The state has an area of 88,722 km². At January 2011, the population of the state is 91,347,736 of which 10,112,599 (11.07%) belong to the age group 0-6 years. Agriculture is the main economic source of this state.

Except the northern hilly region, other parts of this state are warm and humid for the maximum time of the year. The main seasons are summer, monsoon, autumn, late autumn and winter. The summer lasts from mid-March to mid-June, with the temperature ranging from 38 °C to 45 °C. The monsoon arrives by the middle of June and lasts until September.

2.2. Vaccination programme

In West Bengal, JE vaccination programme was initiated in 2006 along with some other states of India. The vaccine was given to the children of Burdwan district, belonging to the age group of 1-15 years in the year 2006. In 2007 and 2008, vaccine was administrated to the children of Birbhum and West Midnapore districts respectively, whereas the district Howrah and Hooghly were covered by JE vaccine in the year 2009. In the year 2010 and 2011, no vaccination campaign had been initiated.

2.3. Selection criteria

Patients admitted with acute encephalitis syndrome (AES) in different medical and district hospitals, with high fever (>39 °C) and any two of the following symptoms, i.e. headache, neck rigidity, vomiting, unconsciousness, convulsions, seizure, stupor, delirium, altered sensorium or presence of Kerning’s sign, were considered for this study. Informed consent was obtained from the parents or legal guardians of minors before the collection of the samples. Cerebral malaria and bacteriological antedote were ruled out by the clinicians of the hospitals concerned.

2.4. Sample collection

During the study period from 2005-2011, a total of 828 AES cases were referred by the clinicians from different districts hospitals of West Bengal to our department for detection of JEV in them with detail socio-demographic information and clinical history. From 2006, the vaccine was given to the children between 1 year and 15 years of age, by the government. In the year 2010, the children up to 15 years of age would attain the age of up to 20 years. So in this study, the cases up to 20 years of age were considered. The study was duly approved by the research ethics committee of ICMR Virus Unit, Kolkata.

2.5. Sample processing and analysis

The collected blood samples and CSF were transported maintaining the cold chain system to this unit. Sera were separated and the samples were stored at -80 ° C until testing.

For diagnosis of JEV infection, enzyme-linked immunosorbent assay (ELISA) was performed. The kit was purchased from National Institute of Pune, India. The kit follows IgM antibody captured ELISA technique using specific antigen for JE. Optical density was measured at 492 nm using an ELISA reader (Titertek Multiskan Plus, Lab systems Finland, Type - 314).

For the detection of actual JE positive cases, ELISA negative samples were further screened for the isolation of viral RNA followed by RT-PCR test. Viral RNA was isolated by using Qiagen viral RNA isolation kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer’s protocol. The RT-PCR test was performed following the cost effective RT-PCR method. The PCR products were run on 1.5% agarose gel.

A. Results

Results of agarose gel (1.5%) electrophoresis showing JE specific band at 306 bp (Figure 1).
Table 3

District wise distribution of Japanese encephalitis positive cases in West Bengal, India.

<table>
<thead>
<tr>
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<td>13</td>
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<td>88</td>
<td>48</td>
<td></td>
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</table>

The vaccinated districts are marked as *.

Table 2

Age and sex wise distribution of Japanese encephalitis cases in West Bengal, India from 2005-2011.

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Sex</th>
<th>2005 Total</th>
<th>In children</th>
<th>2006 Total</th>
<th>In children</th>
<th>2007 Total</th>
<th>In children</th>
<th>2008 Total</th>
<th>In children</th>
<th>2009 Total</th>
<th>In children</th>
<th>2010 Total</th>
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<td>9</td>
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<td>3</td>
<td>5</td>
<td>7</td>
<td>29</td>
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<td></td>
<td></td>
<td></td>
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<td>11-20</td>
<td>Male</td>
<td>17</td>
<td>3</td>
<td>2</td>
<td>6</td>
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<td></td>
<td></td>
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<td>0</td>
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<td>3</td>
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<td>3</td>
<td>11</td>
<td>28</td>
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<td>5</td>
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</tr>
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<td>0</td>
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<td>4</td>
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<td></td>
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</table>

Out of total 291 positive cases, 162 cases (55.6%) were below 20 years of age. In 2005 and 2006, about 92.7% and 88.8% JE positive cases belonged to that age group. In 2007, 2008, 2009 and 2011 JE positive cases amongst children and young adult of upto 20 years of age were 42.1%, 30.9%, 44.8%, 42.8% and 54.5%, respectively (Figure 2).

4. Discussion

JE is considered as a serious disease due to its complexity and lack of any specific treatment. Pig-mosquito-pig and bird-mosquito-bird cycle is responsible for the maintenance of the virus in nature. Man is the 'dead end' host. The virus causes large epidemics at intervals and has been endemic in many rural areas of West Bengal. Although the JE cases are being reported at the tip of iceberg in this state, but still a huge number of positive cases has been found from the year 2009. In endemic areas young and young adult age groups (≤ 20 years) are mainly affected. The economy of most of the people in the rural areas is mainly dependent on cultivation. Moreover, people of that age group usually take active part in crop field for the cultivation. The vector usually breeds in the stagnant water in the paddy field and the majority from this age group gets exposed to the vector directly. To raise the economic standard; poorer section of the community has accepted piggery and mini poultry as an accelerated source of income. The domestic bird, animal and cattle happen to be the reservoir of JEV12M. The majority...
of the people of these districts live under the same roof with those animals, which bring animals in close association with human being, and thus the community becomes exposed to JEV infection through mosquitoes. Here household crowding, low socioeconomic status and lack of air conditioning appear to be the risk factors for acquiring JEV.

Effective vaccine against JEV is now available. Due to the widespread use of JE vaccine JE cases has been declined in China, Korea, and Japan[20]. The targeted age group for this study was below 20 years age during the period of study, maximum numbers of JE cases amongst the children and young adults were detected in the year 2005 followed by 2006. In this state, the vaccination programme was initiated in 2006. In our study, although in some districts of West Bengal like Burdwan, Birbhum, Midnapore (W), Howrah and Hooghly, the vaccine was given up to 15 years of age between the year 2006 and 2009, but in the year 2010 and 2011, JE cases were reported amongst the children and young adults from those vaccine covered districts. Again from the year 2009 onwards, high number of positive cases was reported from those vaccine covered districts. It might be due to partial vaccination in those districts although the possibilities of the change in genotype of the circulating strains can not be excluded. Recently in Uttar Pradesh, India genotype I strain of JEV was isolated from Gorakhpur, a JE endemic region of the state[19]. The children and young adult population of this region was vaccinated with SA-14-14-2 strain of JEV genotype III. But, with the introduction of genotype I in this region, the circulating strain affected good number of people of this region.

The maximum numbers of positive cases were found from the districts of Midnapore (East), Malda, Murshidabad, Nadia and South 24 Parganas, where no vaccination has yet been initiated. In 2011, JE cases were recorded from the district of Jalpaiguri and Coochbehar, which are located in the hilly and cold climatic regions. These two districts are adjacent to the state of Assam, where an outbreak of JEV had been recorded in July 2011[22]. Possibly, the JE affected people of those two districts were infected by the vector mosquitoes that migrate from the neighboring state.

An intense molecular study on the circulating strain of JE is required to find out the reasons of increasing tendency of JE cases. Immediate mass vaccination with a booster dose is required to all the children of the highly endemic regions of this state. Not only the children and young adults, but the adults were also affected by JEV recently. Government should think about the vaccination programme in adults as early as possible. Otherwise the increasing trend of JEV activity in the state of West Bengal, India will become a serious public health threat in near future.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We express sincere gratitude to the Officer-In-Charge, ICMB Virus Unit, for allowing us to carry out the work in this department. We also gratefully acknowledge the help we received from NIV, Pune, India for providing us the ELISA kits for the detection of IgM antibody to JEV. The enthusiastic help obtained from the doctors of the Medical Colleges and District Hospitals, for providing us the clinically suspected samples for this study, is gratefully acknowledged.

References


Mass scale screening of common arboviral infections by an affordable, cost effective RT-PCR method

Debjani Taraphdar, Arindam Sarkar, Shyamalendu Chatterjee*

Indian Council of Medical Research Virus Unit, Kolkata

ARTICLE INFO

Objective: To develop a rapid, cost effective RT-PCR method for the mass scale diagnosis of such diseases at the viremia stage to find out the actual disease burden in that area. Methods: For this purpose, cases with the history of only short febrile illness were considered. Thus 157 samples with the history of dengue/chikungunya like illness and only 58 samples with a history of acute encephalitis syndrome (AES) were selected. Results: Out of 157 samples, 42 and 74 were detected as dengue and chikungunya, respectively and out of 58 AES cases only 23 could be detected as Japanese encephalitis by this RT-PCR method. Conclusion: This cost effective RT-PCR method can detect the total positive cases that remain undetected by ELISA method. Moreover, this method is capable to detect the viral RNA from patients' sera even after the appearance of IgM antibody at one fifth costs as compared with the other commercially available kits.

1. Introduction

Arboviral arthropod borne infections are one of the most important public health problems all over the world including India. The members of this group have the ability to replicate both in arthropods and vertebrate. The former one has a greater chance to act as a vector for transmission of the viruses than the later one. In most instances, the virus is maintained in nature between the vector and animal host. Like in the case of Japanese encephalitis, pig-mosquito-pig and bird-mosquito-bird cycle is responsible for the maintenance of the virus in nature. Man is the "dead end" host[1]. Sometimes, a human-vector-human cycle may occur where the reservoir may be in either humans or the vector, like in yellow fever, dengue and chikungunya cases.

The members of the genus Flaviviruses and Alphaviruses of the arboviruses accounts for important diseases in India[2]. The major arboviral infections that affect a significant portion of Indian populations every year are Japanese encephalitis, dengue and presently chikungunya cases. Japanese encephalitis virus is a member of the genus Flavivirus of the family, Flaviviridae, transmitted to humans by mosquitoes (Cidex sp.) between wild and domestic birds and pigs[3]. Japanese encephalitis is numerically one of the most important causes of viral encephalitis worldwide, with an estimated 50 000 cases and 15 000 deaths annually. Most of China, Southeast Asia, and the Indian subcontinent are affected by the virus, which is spreading at an alarming rate. The mortality rate of this disease ranges from 0.3%–60%[4]. The morbidity especially in children and young adults aged 2–15 years is very high, although elderly people are not spared, if affected. It has been reported that those who recover have neurological sequelae in about 30% of cases[5]. Japanese encephalitis is numerically one of the most important causes of viral encephalitis worldwide, with an estimated 50 000 cases and 15 000 deaths annually. Most of China, Southeast Asia, and the Indian subcontinent are affected by the virus, which is spreading at an alarming rate. The mortality rate of this disease ranges from 0.3%–60%[4]. The morbidity especially in children and young adults aged 2–15 years is very high, although elderly people are not spared, if affected. It has been reported that those who recover have neurological sequelae in about 30% of cases[5]. Since the isolation of this virus in Japan in 1935, it has spread all over the world including India and has become a major public health problem[6]. In India, the existence of
Japanese encephalitis virus was first reported serologically in 1954\(^7\). However, the disease was first recognized in India at Vellore in 1957\(^2\). Since then, outbreaks at different years have been reported in different states\(^9\). In West Bengal, the first major outbreak of Japanese encephalitis took place in the year 1973 in the district of Burdwan and Bankura where more than 700 cases and 300 deaths have been reported\(^10\). The recurrence of Japanese encephalitis epidemic in the district of Bankura and Burdwan in the year 1974\(^11\) and again in the year 1987 and 1988, in the district of Burdwan\(^12\), has been well documented.

Dengue virus, also a member of the genus Flavivirus with its four sero types is now classified within the Flaviviridae family\(^13\) and transmitted mainly by *Aedes aegypti* mosquitoes. Among the four sero types, infection with any of them generally leads to a mild self limiting febrile illness i.e. dengue fever. Its typical symptoms include headache, a characteristic skin rash, joint pain and body ache. A more severe form of the disease involving vascular and haemostatic abnormalities leads to dengue hemorrhagic fever and dengue shock syndrome, which is responsible for a high mortality rate, especially in children\(^14\). Dengue virus is responsible for a growing health problem in the tropical and sub tropical countries. The global incidence of dengue fever and dengue hemorrhagic fever has increased dramatically in recent decades\(^15\).

In India dengue was first isolated in 1946, and many epidemics have since been reported\(^16\)-\(^18\). Dengue hemorrhagic fever was first reported in Calcutta, West Bengal, in 1963 again in 1964 and subsequently in Visakhapatnam in 1969 and in Jalore, Rajasthan in 1985. Delhi has experienced the major epidemic of dengue hemorrhagic fever in 1996\(^17\)\(^18\). In addition, outbreaks have also been reported at regular intervals from different states of India\(^19\)-\(^21\). In West Bengal, recurrent outbreaks of dengue were reported in 2002, 2004 and 2007 from West Midnapore\(^22\) and from Siliguri in 2009\(^23\).

Chikungunya virus is one of the major viral pathogens throughout the world, causing severe morbidity in developed and developing countries. It is a mosquito borne virus, of the genus *Alphavirus*, which is transmitted to humans by *Aedes* mosquitoes\(^24\). Chikungunya virus causes an illness with symptoms almost similar to dengue fever except the prolonged arthralgia which persists for months to years\(^25\), even after the disappearance of other clinical illnesses including the fever.

Chikungunya virus was first isolated from Tanzania in 1953\(^26\). In Asia, the first chikungunya outbreak was documented in Bangkok, Thailand in 1958, followed by other countries like Cambodia, Vietnam, Laos, Myanmar, Malaysia, Philippines, and Indonesia\(^27\). In India, the virus was first isolated in 1963–1965 in Calcutta\(^28\). The last outbreak of chikungunya virus infection occurred in India in 1971\(^29\). After that, no reports of such illness are available. After a long gap, the present outbreak was first reported from the southern Indian state of Andhra Pradesh in November–December 2005\(^30\). An estimated 1.38 million people across southern and central India developed symptomatic disease during 2005 and 2006. By now it has affected a major part of the country very rapidly.

Japanese encephalitis, dengue and chikungunya are mosquito borne diseases. In case of Japanese encephalitis the disease is transmitted by the *Culex* mosquitoes, which breed in the stagnant water of the paddy field during monsoon and post monsoon period. But in the case of dengue and chikungunya, *Aedes* mosquitoes, mainly the *Aedes aegypti* plays the role of vector in India, which is the day biter, domestic and peridomestic in nature and breeds in the household containers. In all the cases, the vector density rises after the monsoon period. Not only that, people of the rural areas take up piggery and poultry as their economic support. These could be the amplifying hosts or reservoirs of some arboviral infections. For these reasons, the epidemics of those arboviral infections occur every year in India. To diagnose all these cases from the viremia stage, we aimed to develop and standardize a rapid, sensitive method at a low cost, for early confirmation of the cases to control the epidemic as well as to reduce the mortality rate, with a better patient care.

The reverse transcriptase polymerase chain reaction (RT–PCR) is one of the methods for the diagnosis of many diseases\(^30\). Many cases are remaining undiagnosed as most of them are subclinical with a history of mild and short febrile illness. All these cases can be detected by RT–PCR method to ascertain the total number of cases in the affected area. Here, we report a single step RT–PCR method for the mass scale diagnosis of common arboviral infections in West Bengal, India. This method is rapid and equally sensitive to detect the virus in the patient sera at an affordable cost, with a great public health importance.

2. Materials and methods

2.1. Clinical samples

A total of 472 acute blood samples were referred from different medical college hospitals and from district hospital in West Bengal from August 2010 to December 2010. Among them, 391 samples from the patients with high fever, rashes, joint pain/swelling and vomiting were selected for detection of dengue and chikungunya infections, if any. Rest of the 81 samples with the history of acute encephalitis syndrome were considered for Japanese encephalitis diagnosis. In all the cases, steady cold chain conditions were maintained during transport and repeated freeze–thawing was avoided for the protection of viral RNA.
2.2. Serology

For the detection of IgM antibody, ELISA test was performed, according to manufacturer's protocol. The kits were purchased from National Institute of Virology (NIV), Pune, India.

2.3. RNA extraction

The samples, having a history of short febrile illness up to 48 h, were considered for the isolation of viral RNA. In this process, some IgM antibody positive samples were also included. RNA was extracted from the sera, using QIAamp® RNA viral kit (QIAGEN Inc., Valencia, CA), according to manufacturer's protocol.

2.4. RT-PCR

2.4.1. One step kit

To compare our single step method, two kits were used i.e. Qiagen one step RT-PCR kit and Access Quick RT-PCR system (Promega, USA), following the respective manufacture's protocol using RNA (50 pg to 1 ng) and 0.6 μM of the primer pairs. For Japanese encephalitis detection, newly constructed Japanese encephalitis virus specific forward primer 5’ CGA GAA CTT GGA VGA CTC ATT GA-3’ and reverse primer 5’- ATT GCC CAT GGT GAG ACA –3’ were used, whereas, chikungunya specific CNPlF 5’-GAA ATT GAT CCC GAC TCA ACC ATC CAC ATT-3’ and CNPlR 5’-GCT TTA ATC GCC TGG TGC TAT AGC-3’ and dengue specific primers 5’-ACA TGG TCT GAC CAA GAA ACC G-3’ and 5’-TCT GCC AGC CAG TCA ATG TCT CTA GGT TC-3’ were used for its viral RNA detection.

2.4.2. Our one step protocol

One step RT-PCR method was standardized using 24 μL dH₂O, 2 μL of 10X PCR buffer, 0.65 mM dNTP (Invitrogen), 1.4 mM MgCl₂, 0.1 X L of 0.1 M DTT (Promega, USA), 0.125 μM primer pairs each, 0.5 U of AMV RT (Promega, USA), 0.5 U of Taq polymerase (Applied Biosystems) and 50 pg to 1 μg of RNA. The PCR cycle was set by initial heating at 42 °C for 1 h and 15 min at 94 °C. The reaction was then run for 35 cycles at 94 °C for 30 sec, 54 °C for 1 min, and 72 °C for 1 min, followed by an additional elongation for 3 min at 72 °C.

2.5. Cost calculation

The cost per reaction mixture was analyzed for each method. As the same amount of template was used in every method, so it was excluded in the calculation. In our one step method, 0.25 μM primer pairs were used in comparison to the kit based methods, where 0.6 μM primer pair was used (recommended). Considering the usage of the less quantity of the primers in our method, the unit cost was calculated excluding the cost of the primers.

3. Results

3.1. Serology

Out of 391 samples, 95 samples were positive to IgM antibody to dengue and 154 samples were reactive to chikungunya IgM antibody. Out of 81 acute encephalitis syndrome cases, 40 were Japanese encephalitis ELISA positive (Table 1).

<table>
<thead>
<tr>
<th>Method</th>
<th>No. tested</th>
<th>Positive to Dengue</th>
<th>Positive to Chikungunya</th>
<th>Positive to JE</th>
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<tbody>
<tr>
<td>ELISA</td>
<td>391</td>
<td>95</td>
<td>154</td>
<td>81</td>
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<tr>
<td>RT-PCR</td>
<td>157</td>
<td>42</td>
<td>74</td>
<td>58</td>
</tr>
</tbody>
</table>

JE: Japanese encephalitis.

Table 2

Comparative analysis of the unit costs of the newly developed procedure with the commercial kits.

<table>
<thead>
<tr>
<th>Name of the kit</th>
<th>Cost of reagents (USD)</th>
<th>Total cost (USD)</th>
<th>Unit cost of reagent (USD)</th>
<th>Unit cost per method (USD)</th>
</tr>
</thead>
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<td>Qiagen RT PCR kit</td>
<td>–</td>
<td>373.01</td>
<td>–</td>
<td>14.92</td>
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<tr>
<td>Access Quick RT-PCR kit</td>
<td>–</td>
<td>297.76</td>
<td>–</td>
<td>14.89</td>
</tr>
<tr>
<td>Our developed method</td>
<td>–</td>
<td>594.78</td>
<td>–</td>
<td>2.58</td>
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<tr>
<td>Materials used</td>
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<td>130.97</td>
<td>2.10</td>
<td>–</td>
</tr>
<tr>
<td>DTT (50 μL)</td>
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<td>0.01</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AMV RT (200 U)</td>
<td>228.84</td>
<td>0.38</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Taq pol (1000 U)</td>
<td>171.28</td>
<td>0.09</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

3.2. PCR result

Out of 157 screened patient samples, only 42 and 74 samples produced dengue and chikungunya specific prominent band, respectively in 1.5% agarose gel, which amply proved those infections in them (Table 1 and Figure 1). Seven samples were also dengue IgM positive out of those 42 RT-PCR positive samples. A total of 23 samples produced Japanese encephalitis specific band by RT-PCR method out of 58 screened samples, of which four samples were Japanese encephalitis IgM reactive. In case of chikungunya, RNA could not be detected in IgM positive samples.
3.3 Cost calculation

The calculated costs per reaction were summarized in Table 2. It is clear from the table that the cost per reaction of our developed one step method was the lowest as compared with the commercially available kits.

4. Discussion

The spread of these arboviral diseases throughout the country has become a major public health problem. As the vector density increases in the monsoon and post monsoon period, the transmission rate of the diseases rises sharply, which in turn increases the morbidity/mortality rate in West Bengal as well as in India. A huge number of febrile cases are referred to our laboratory. To assess all the cases, an economically feasible and potential process is required.

RT-PCR is one of the methods for the diagnosis of several viral diseases. It has also been successfully implemented for the detection of the members of the Flavivirus group[33], WHO still considers RT-PCR as a standard method, for the detection arboviral infections. So early detection of Japanese, dengue and chikungunya viruses in clinical samples collected from different districts of West Bengal can control the spread of the diseases, which in turn will minimize the disease burden of that area. For this purpose, we conducted a validation study of PCR based detection of Japanese encephalitis, dengue and chikungunya viruses in clinical samples collected from different districts of West Bengal. This method is rapid, sensitive and cost effective, and can be employed for large scale detection of all the fever cases.

In our developed one step method, the cost of the test per sample claims almost one fifth in comparison with the widely available commercial kits like Qiagen one step RT-PCR kit and Access Quick kit. Although the initial investment of our developed one step method is higher, but for the mass scale screening of all the fever cases in the affected area, it ultimately lowers down the cost per reaction. Moreover, this method can detect the viral RNA at the late viremic stage, when the IgM antibody has already ushered in.

Hence, this cost effective, short term one step method constitutes a new report for the screening of endemic arboviral diseases by diagnosing all the positive cases and thus helps to measure the actual disease burdens which in turn have the great public health importance to control the epidemic of the respective diseases by adopting necessary measures.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We express our sincere gratitude to all the staff members of ICMR Virus Unit, for their constant help and assistance to carry out the laboratory investigations and compilation of data. We also gratefully acknowledge the help we received from N.I.V, Pune for providing us the specific ELISA kits for the detection of IgM antibody. The enthusiastic help obtained from the doctors of the Medical Colleges and District Hospitals, for providing us the clinically suspected samples for this study, is gratefully acknowledged. We are indebted to the Officer-In-Charge, ICMR Virus Unit, for allowing us to carry out the work in this department. We received all sorts of financial help from the Department of Science and Technology, Govt. of West Bengal, India (Sanction No. 755/Sanc./ST/P/FS&T/9G–27/2007, dated 26.02.2008) to carry out the work in the ICMR Virus Unit, Kolkata, India.
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Molecular evidence for the occurrence of Japanese encephalitis virus genotype I and III infection associated with acute Encephalitis in Patients of West Bengal, India, 2010


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Molecular evidence for the occurrence of Japanese encephalitis virus genotype I and III infection associated with acute Encephalitis in Patients of West Bengal, India, 2010

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Abstract

Background

Japanese encephalitis virus (JEV), a mosquito-borne zoonotic pathogen, is the sole etiologic agent of Japanese Encephalitis (JE); a neurotropic killer disease which is one of the major causes of viral encephalitis worldwide with prime public health concern. JE was first reported in the state of West Bengal, India in 1973. Since then it is being reported every year from different districts of the state, though the vaccination has already been done. Therefore, it indicates that there might be either partial coverage of the vaccine or the emergence of mutated/new strain of JEV. Considering this fact, to understand the JEV genotype distribution, we conducted a molecular epidemiological study on a total of 135 serum/cerebrospinal fluid (CSF) samples referred and/or collected from the clinically suspected patients with Acute encephalitis syndrome (AES), admitted in different district hospitals of West Bengal, India, 2010.
Findings

JEV etiology was confirmed in 36/135 (26.6%) and 13/61 (21.3%) 2–15 days’ febrile illness samples from AES cases by analyzing Mac-ELISA followed by RT-PCR test respectively. Phylogenetic analysis based on complete envelope gene sequences of 13 isolates showed the emergence of JEV genotype I (GI), co-circulating with genotype III (GIII).

Conclusion

This study represents the first report of JEV GI with GIII, co-circulating in West Bengal. The efficacy of the vaccine (derived from JEV GIII strain SA-14-14-2) to protect against emerging JEV GI needs careful evaluation. In future, JE outbreak is quite likely in the state, if this vaccine fails to protect sufficiently against GI of JEV.

Keywords

Acute encephalitis syndrome, Japanese Encephalitis Virus, Genotype I, Genotype III, West Bengal

Background

The mosquito-borne Japanese encephalitis virus (JEV) is an enveloped, positive-sense single-stranded RNA virus, member of the genus Flavivirus under the family Flaviviridae [1]. JEV is the sole etiologic agent of Japanese Encephalitis (JE); a neurotropic killer disease being one of the major causes of viral encephalitis in human. Since the isolation of this virus in Japan in 1935 [2], it has spread worldwide becoming a major public health problem. Worldwide case-fatality rate of JE was recorded to be 30% approximately with 30-50% of survivors developing permanent neurologic deficit/sequelae [3].

Recent studies have shown that the envelope (E) gene is an established phylogenetic marker for JEV, since this region is free from selective pressure that supports obscure long-term evolutionary relationship [4]. Altogether 5 distinct genotypes have been identified among the JEV strains [5] of which genotype III (GIII) is mostly circulated in the Southeast Asian countries, including Japan, South Korea, China, Taiwan, Vietnam, Philippines, and India [6]. However, it was recently documented that GIII is replaced by genotype I (GI) in South Korea, Thailand and China [7]. Though GIII is predominant in India, GI has been introduced in the country recently [7]. In 1973 JE outbreak was first recorded in the districts of Burdwan and Bankura in West Bengal where 700 cases and 300 deaths were reported [8,9]. Thereafter, several JE outbreaks took place in the state [10-12]. Every year sporadic JE cases are being reported indicating its endemicity in this state despite the vaccination programme undertaken by the State Health Department, Government of West Bengal [13]. In addition, the geographic features, environmental factors and socio-economic status of this state also favor JEV transmission [14]. Moreover, the reports of JE incidences in the state are the indications of either partial coverage of the vaccine or the emergence of mutated/new strain of JEV. Genetic variation of JEV circulating in West Bengal has not yet been investigated and hence to ascertain the same a molecular epidemiological study was undertaken.
Materials and Methods

A total of 92 serum and 43 cerebrospinal fluid (CSF) samples were referred and/or collected from 135 clinically suspected pediatric-adolescent (0–20 years old) and adult (≥21 years old) individuals with Acute encephalitis syndrome (AES), showing high grade fever (≥39°C) for 2–15 days including any two of the following symptoms, viz. headache, vomiting, stupor, delirium, abnormal movements, presence of kernig’s sign, convulsions, neck rigidity, altered sensorium, unconsciousness admitted in 8 different district hospitals, West Bengal during the period from July to December in 2010 (Figure 1).

Figure 1: Map of West Bengal showing the location of sample collection areas

All the samples were tested for IgM antibody against JEV by using IgM antibody-capture (Mac) ELISA kit (National institute of virology, Pune, India), according to the manufacturer’s protocol.

Only 61 JEV IgM negative samples with a history of ≤3 days’ illness were screened and 200 µl of each of them were used for virus isolation on C6/36 cell line according to the standard protocol [13]. The tissue culture fluids were collected from the samples producing prominent cytopathic effect (CPE) and subjected for RNA extraction by QIAamp RNA viral kit (Qiagen, GmbH, Hilden, Germany), following the manufacturer’s protocol.

To identify the isolates as JEV, reverse transcription-PCR (RT-PCR) was carried out with the extracted RNA (50 pg to 1µg) by Qiagen one step RT-PCR kit (Qiagen, GmbH, Hilden, Germany), in accordance with the manufacturer’s specifications, using 0.6 μM of primer pairs [13] that specific for structural E gene sequence of JEV. The PCR products were separated by electrophoresis on 1% agarose gel, stained with ethidium bromide.

RT-PCR amplicons were purified using the Qiagen gel extraction kit (Qiagen, GmbH, Hilden, Germany), according to the manufacturer’s protocol, followed by direct sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), as per the manufacturer’s instructions and the products were analyzed using an automated DNA sequencer, 3130XL Genetic Analyzer (PE Applied Biosystems, Foster city, CA, USA). The 1,500 nucleotides generated complete sequences of the JEV E gene that were edited and corrected using the Finch TV software (http://www.geospiza.com). Multiple sequence alignment and phylogenetic analysis were performed by using CLUSTALW (www.ebi.ac.uk/Tools/clustalw2/index.html) and MEGA version 5.0 software (www.megasoftware.net). The phylogenetic tree was constructed by the neighbor-joining method, tested with Kimura 2-parameter model.

Results

Out of 135 samples, only 36 (26.6%) samples were reactive to JEV specific IgM antibody, of which 23 (63.8%) and 13 (36.1%) samples were CSF and serum respectively. Only 61 of the remaining 99 JEV IgM negative samples having the history of ≤3 days of febrile illness were selected and subsequently subjected to tissue culture resulting in 19 samples producing prominent CPE of which only 13 (21.3%) samples were identified as JE positive by RT-PCR method, consisting of 8 (61.5%) from CSF and 5 (38.4%) from serum.
We have a total of 49 (36 IgM + 13 RT-PCR positive) JE cases (36.2%) of which 30 (19 IgM + 11 RT-PCR positive) were pediatric-adolescent (61.2%) and remaining 19 (17 IgM + 2 RT-PCR positive) were found to be adult cases (38.7%). Moreover, the occurrence of JEV infection was recorded during the month of July to December with the maximum number of cases (46.9%) observed in the month of September.

The Figure 2 shows the phylogenetic tree derived from 13 E gene sequences of JEV isolates along with 41 previously published JEV strains, including 12 from India and 29 from worldwide (Table 1). Dendrogram showed 2 E gene sequences of the isolates (GenBank: JN703381, JN703382) belonging to GI and comprising 89%-91% nucleotide (nt) identity with 11 E gene sequences of other isolates (GenBank: JN968468- JN968477, JN189785) belonging to GIII (Figure 2). Moreover, these 2 GI E gene sequences showed 99% nt similarity with each other and were most similar (96%) with Japanese GI strain Ishikawa (GenBank: AB051292), followed by 94%-95% nt similarity with Indian isolate JEV-GKP-094054 (GenBank: HM156572). Eleven GIII E gene sequences showed 97%-99% nt similarity with each other and 93%-98% nt similarity with other Indian GIII strains, having the highest similarity (97%-98%) with Indian P20778 strain (GenBank: Z34096).

Figure 2 Phylogenetic analysis of 13 JEV isolates in serum/CSF samples from AES patients, West Bengal. The Phylogenetic tree was generated by Neighbor-Joining (NJ) method, tested with Kimura 2-parameter model using the complete envelope (E) gene nucleotide sequences of 13 Japanese encephalitis virus (JEV) isolates from hospitalized acute encephalitis syndrome case-patients in West Bengal, India, 2010, with reference to other 41 prototype JEV strains from worldwide. The Murray valley encephalitis virus strain (MVEV-1-51) was used as out group for generating the rooted tree. The robustness of dendrogram was evaluated by 1000 bootstrap pseudo replicates. Bootstrap values (>50% of replicates) were shown in corresponding nodes. Horizontal branch lengths are proportional to genetic distance and vertical branch lengths have no significance. Each taxon is named systematically by mentioning the accession number, strain name, country of origin and year of isolation. The isolates’ sequences used in this study were marked with filled circle and triangle symbols.

Genotypes are indicated on the right. Scale bar indicates nucleotide substitutions per site

Table 1 Background information of selected strains/isolates of JEV referenced in this study

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Discussion

JEV infection is considered as a prime issue on public health concern in West Bengal. The present study reveals that 36/135 (26.6%) and 13/61 (21.3%) samples were positive to JE by Mac-ELISA and RT-PCR method respectively. This observation is the proof of JEV infection in recent time and to detect the total number of JE cases, ELISA negative acute samples (from ≤ 3 days' febrile illness) should be confirmed by RT-PCR test.

JE incidences (61.2% vs. 38.7%) were higher in pediatric-adolescent age group than adult because pediatrics were infected possibly due to lack of immunity and adolescents were directly exposed to the mosquito vector (Culex sp.) bite, as they usually took active part in cultivation in crop-fields where vectors usually breed. In the present study, JE was found to occur in the monsoon period with the maximum number of cases in September when the Culex mosquitoes breed in the paddy fields covered with stagnant rain water.

However, we found that 86 [(99-61)+(61-13)] samples with a history of 2–15 days’ illness were true JE negative possibly due to either mishandling of samples which damaged the IgM antibody/the viral titre or the presence of another etiology responsible for AES.

In our previous reports we have achieved 36 JEV isolates [13] belonging to GIII whose E gene sequences were submitted to NCBI GenBank database like GenBank: JN189782, JN189783, JN189784 and HQ891146 etc. (unpublished data). The present study, therefore, constitutes the first report on E gene based phylogenetic analysis of the JEV isolates from AES cases of West Bengal where JEV GI has emerged very recently, co-circulating with JEV GIH.

Our study reveals that 2 isolates (GenBank: JN703381, JN703382) belonging to GI of JEV from the coastal district Midnapore (22.25°N 87.65°E and 23 meters above sea-level) where this GI might be transmitted from other part of India [7] or by the travellers returning from JE endemic countries, possibly with JEV infection [15]. On the other hand, this district has got many Lakes, swamps, forest and rice fields which provide a wintering and staging grounds for several migratory birds. Such areas are also very suitable for breeding and survival of mosquitoes. In view of these conditions, GI of JEV might have been introduced into West Bengal through migratory birds or cyclonic wind-blown mosquitoes from newer geographic region [16,17]. However, it is still unknown as to how JEV GI has emerged in West Bengal exactly. Therefore, further studies to determine the role of travelers, migratory birds and wind-blown mosquitoes in JEV transmission are required.
The State Health Department of Government of West Bengal undertook the vaccination programme against JE in Midnapore in 2008 [13] using live attenuated JE vaccine derived from GI III strain SA-14-14-2. In this connection, the efficacy of the vaccine to protect against GI of JEV needs careful evaluation. In near future, there is a chance for an impending threat of JEV outbreak in this region/state, if this vaccine fails to protect sufficiently against GI of JEV.

Competing interests

The authors declare that they have no competing interests.

Authors’ contribution

AS and SCHAT conceived the study, the design, and drafted the manuscript. AS and DT carried out serology and molecular work. AS, DT, SKM, SCHAK and SCHAT contributed to the data analysis and data interpretation. All authors read and approved the final manuscript.

Ethical approval

The study was duly approved by the joint ethical committee of ICMR (Indian Council of Medical Research) virus unit and NICED (National Institute of Cholera and Enteric Diseases), Kolkata, India.

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References


Figure 6.
Serological and molecular diagnosis of Japanese encephalitis reveals an increasing public health problem in the state of West Bengal, India

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ABSTRACT
Japanese encephalitis (JE), a neurotrophic disease, was first recorded in the state of West Bengal, India in 1973. Since then JE is being reported every year from different districts. With a view to identify the JE cases accurately, a study was undertaken to detect the Japanese encephalitis virus (JEV) as the etiologic agent from the acute encephalitis syndrome (AES) cases and to identify its distribution in different districts. We report the results of 513 blood or cerebrospinal fluid samples referred/collected from the hospitalized AES cases. The samples were initially subjected to Mac-ELISA test followed by reverse transcriptase (RT)-PCR for the detection of IgM antibodies and the JEV genome, specific to E gene, respectively. Out of 513 samples referred/collected, 139 (27.1%) samples were reactive to JE IgM antibody. The remaining 374 samples were screened to select those which had a history of illness with a duration of <3 days. Only 147 samples were selected and tested, out of which 36 (24.5%) isolates were achieved and those were RT-PCR positive against the control JEV strain. Detection of IgM antibody to JE and the RT-PCR result confirms the active circulation of JEV in different districts of West Bengal and needs to be monitored carefully.

Keywords: Acute encephalitis syndrome Japanese encephalitis virus Mac-ELISA RT-PCR West Bengal

1. Introduction

Japanese encephalitis (JE) is a killer disease caused by the mosquito-borne Japanese encephalitis virus (JEV), a member of the genus Flavivirus under the family Flaviviridae. It is one of the major causes of severe neurotrophic disease which includes fever, aseptic meningitis, acute flaccid paralysis or classic meningomyelencephalitis in humans. Since the isolation of this virus in Japan in 1935, it has spread worldwide and has become a major public health problem. All over the world, approximately 50,000 cases with a mortality rate of about 25% are reported annually, and nearly 50% of the cases, especially children (aged 2–15 years), survive with persistent neurological deficit and/or psychological sequelae.

In India, the existence of JEV was first reported serologically in 1954, however, the disease was first recognized at Vellore in 1955. Since then, epidemics of JE in different states have been recorded. In West Bengal, the first major outbreak of JE took place in 1973 in the districts of Burdwan and Bankura where more than 700 cases and 300 deaths have been reported. Since then many outbreaks have been reported, every year sporadic cases are continuously being reported from different districts of West Bengal. Many JE cases remain undiagnosed as most infections are subclinical. In the present study, we aimed to detect the JE cases amongst the patients with acute encephalitis syndrome (AES). Infection of JEV can be detected directly or indirectly by a number of methods. The IgM specific capture ELISA is the method for diagnosis of many diseases including JE as a primary screening as well as confirmatory test. The ELISA negative samples which were collected in the early clinical phase, were further subjected to the
reverse transcriptase (RT)-PCR for the diagnosis of the etiologic agent. For this purpose, samples, after collection were preserved maintaining the cold chain. Therefore in the present study, serum and cerebrospinal fluid were preferred as the clinical specimens for serological and molecular diagnosis of JEV infection in the suspected cases.

2. Materials and methods

2.1. Patients and clinical specimens

Different district hospitals, two medical colleges and Infectious Disease & Bacteriological Hospital in Kolkata, West Bengal, were selected for this study from 2005–2009. Patients admitted with AES, having high grade fever (≥39°C) for 2–15 days with any two of the following symptoms: headache, vomiting, unconsiousness, convulsions, abnormal movements, stupor, delirium, altered sensorium, neck rigidity, presence of Kerning's sign, apasia, drowsiness and rigor, were considered for this study. Details of the clinical event, investigations, treatment given and the prognosis of the patients during hospitalization were provided by the concerned clinicians. A short case history along with the results of CSF study of each case was recorded. Most of the patients had moderately high sugar levels (45–65 mg/dL), slightly higher protein levels, varying from 50–70 mg/dL, and a white blood cell (WBC) count of ≥6x10⁶/L. Cerebral malaria and bacteriological etiology were ruled out by the concerned clinicians. A short case history along with the investigations, treatment given and the prognosis of the patients during hospitalization were provided by the concerned clinicians.

Informed consent was obtained before the collection of samples. A total of 513 blood/CSF samples were collected and/or referred from the clinically suspected AES cases in sterile gel-line vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA) and also in sterile test tube (Gujarat Borosil Ltd., Bharuch, Gujarat, India) by vein or lumbar puncture and transported on dry ice to the Indian Council of Medical Research (ICMR) Virus Unit, Kolkata, where the serum was separated and both the serum and CSF were stored at −80°C till tested.

2.2. Virus strain

JEV P20778 strain (GenBank Accession No. AF080251) was obtained from the National Institute of Virology (NIV), Pune, and was used as a positive control throughout the study. The virus was reconstituted and inoculated on Aedes albopictus C6/36 mosquito cell lines (obtained from National Centre for Cell Science [NCCS], Pune) in minimal essential medium (MEM; GIBCO BRL-Invitrogen, Grand Island, NY, USA) and also in sterile test tube (Gujarat Borosil Ltd., Bharuch, Gujarat, India) by vein or lumbar puncture and transported on dry ice to the Indian Council of Medical Research (ICMR) Virus Unit, Kolkata, where the serum was separated and both the serum and CSF were stored at −80°C till tested.

2.3. Isolation of virus

Attempts were made to isolate the virus using C6/36 cell lines. For this purpose, only IgM negative to JEV samples with a history of ≤3 days fever along with any two of the symptoms stated earlier, were selected. Out of 513 samples, only 147 samples were thus screened. 200 µl of selected serum/CSF samples were spread over the monolayer of C6/36 cell line and allowed to adsorb for 120 minutes in an incubator at 28°C under 5% CO₂ concentration. After adsorption, the excess sample materials were discarded and 1 ml MEM supplemented with 2% FBS and PenStrep were added in 24 well tissue culture plate (Tarsons Products Pvt. Ltd., Kolkata, West Bengal, India) and were incubated again at 28°C under 5% CO₂ concentration. It was observed regularly for the appearance of cytopathic effect (CPE) up to 7–8 days. For those samples which did not produce CPE, the tissue culture fluids were again passaged up to five times to facilitate the isolation of the virus, if any. After the appearance of CPE, the tissue culture fluid was collected by centrifugation at 1000 g for 5 minutes and the supernatants were kept in aliquots at −80°C till isolation of RNA, followed by RT-PCR test. Non-infected C6/36 cell culture was used as a negative control.

2.4. Serology

For the detection of JEV IgM antibody in the collected samples, ELISA tests were performed with the kit, obtained from the NIV following the prescribed protocol.

2.5. RNA extraction

Those serum/CSF samples that produced prominent CPE and the JEV P20778 strain (used as the positive control throughout the study), were subjected for RNA isolation, using 140 µl of tissue culture fluid. The QiAamp® RNA viral kit (Qiagen, GmbH, Hilden, Germany) was used, following the manufacturer's protocol.

2.6. Reverse transcriptase PCR for envelope gene of Japanese encephalitis virus

For the detection of JEV by RT-PCR method, both serum and CSF samples with a history of ≤3 days fever were selected. Qiagen one step RT-PCR kit (Qiagen) was used according to the manufacturer's protocol, using RNA (50 pg to 1 µg) and 0.6 µM of primer pairs; forward primer: JEnvF (w) 942-ACCATCCTCGCTGTTGGTCGCT-965 and reverse primer: JEnvR (w) 2506-CTTG TGATGCTCAATGGCACATCCAGTGTCA-2477 which anneal to the conserved region of structural envelop protein (E) specific for Japanese encephalitis virus.

3. Results

3.1. Serology

Out of 513 samples collected, only 139 (27.13%) were reactive to JEV IgM antibody by ELISA method and had a
Figure 1. Age and sex distribution of Japanese encephalitis IgM positive patients, 2005–2009.

Figure 2. IgM positive Japanese encephalitis cases, 2005–2009 by month.

3.2. Tissue culture

Well defined CPE was observed in the C6/36 cell line, by the control strain and only 36 out of 147 acute samples. All

Figure 3. IgM positive Japanese encephalitis cases in each year from 2005–2009.

these samples were non-reactive to IgM antibody to JE by the ELISA method.

3.3. PCR result

All the 36 tissue culture isolates (24.5%) from 147 acute samples produced a prominent band at 1.5 kb in 1% Agarose gel (Figure 4).

4. Discussion

In India, JEV is a public health problem and mainly affects the young and young adults up to the age of 20 years. The under reporting of JE cases from JE endemic areas to the nearby hospitals does not rule out the JE occurrence since many patients have to go to the local doctors for immediate treatment because of the poor communication facility between the hospitals and their residence. Such patients only receive clinical diagnosis with no diagnostic facility and are not referred for further services. In the present study, out of 513 samples tested, only 139 (27.1%) were reactive against IgM antibody to JE and had a history of illness for ≥10 days, indicative of active immune response at this stage of illness. This observation is proof of infection in the immediate past. The remaining 374 samples were IgM negative. Although, JE cases have been observed from all age groups, the highest numbers of positive cases have been recorded in the age group 0–10, followed by 11–20, in the male individuals (Figure 1), which corroborates with earlier studies. Only a few cases were detected in the age groups of 21–30 and above (elderly/higher age groups).

The highest number of cases in the 0–10 age group is possibly due to their lack of immunity. Moreover, the male individuals in the age group 15–20 usually take active part in cultivation of crop fields. The vector usually breeds in the stagnant water in the cultivation fields and the majority from this age group are directly exposed to the vector. The low number of JE cases in the higher age group is possibly due to the development of immunity, either by sub clinical infections or due to earlier vaccination.
The monthly distribution of JE cases from 2005-2009 (Figure 2) reveals the highest number of JE cases in September, followed by October and November which are almost at the end of the monsoon season and the prime time for cultivation of paddy. In these months, all the paddy fields are covered with stagnant rain water, the preferred breeding place of Culex. In this study, the second bout of JE cases was observed in December which has been documented earlier. This is another cultivation period of paddy and the same environment facilitates the spread of the disease.

The results by district indicate the absence of JE cases in the northern region which is surrounded by hills and experiences moderately cold climatic condition. Moreover, rain water is less likely to stagnate in the hill areas, reducing the breeding opportunities of the vector mosquito. An effective vaccine against JEV is now available. The vaccinations against JE were undertaken in some of the JE affected districts (i.e., Hoogly and Howrah in 2009, Paschim Midnapore in 2008, Birbhum in 2007 and Burdwan in 2006: JEV status according to Department of Health & Family Welfare) by the State Health Department, Government of West Bengal. It was observed that JE cases were declining in those districts. Even then, the reports of JE cases from those districts indicate that there might be either partial coverage of the vaccine or the emergence of mutated strain of JEV, which is under investigation by sequencing method, followed by phylogenetic analysis. This study also confirms 60 IgM positive cases (43.1%) of JE from those districts which have not been covered with the vaccine against JEV.

A total of 147 samples, having the duration of fever ≤ 3 days (recognized as the vireemic stage), were screened for the isolation of the virus. These were inoculated in the tissue culture system. Only 36 samples produced prominent CPE, characterized by cell rounding followed by early detachment of cells in comparisons with control upon tissue culture, and were considered positive. All these 36 isolates were RT-PCR positive against the JEV specific E gene primer. There is no doubt about the specificity of the primer pair as these were designed on the basis of the conserved region sequence of structural envelop protein for JEV and had produced a good result.

4.1. Limitations

A total of 513 samples were made available for the purpose of this study, of which 139 were positive to JE IgM antibody by ELISA test. The remaining 374 samples were non-reactive to JE IgM antibody. These were screened for the purpose of virus isolation, followed by RT-PCR. Selection was restricted only to those samples having a history of ≤ 3 days of febrile illness. Only 147 samples were thus selected. The remaining 227 samples were ELISA negative, although collected from AES cases who had a history of illness for more than three days.

In this process, only 36 isolates were achieved by tissue culture system. Those were confirmed as JEV isolates by RT-PCR test against control strain. The remaining 111 samples did not produce any CPE during virus isolation in the tissue culture method. Therefore, a total of 338 (227+111) samples with AES for 2-15 days' illness were true JE negative. The possible reasons for the samples to become JE negative might have been either due to the presence of a different etiological agent other than JEV or due to improper transportation, preservation and repeated freezing-thawing responsible for damaging the IgM antibody or the viral titre.
5. Conclusions

The year-wise record of our investigation reveals a steady increase of IgM positive cases up to 2009, indicating a possible public health threat in the near future. As no systematic and extensive studies have yet been carried out in this region, our study may very well be considered as a pioneer attempt of work in the region/State.

Authors' contributions: AS and SChat participated in the conception and design of the study; AS and DT carried out the immunnoassays and molecular test; AS, DT, SKM, Schak and SCbat analysed and interpreted the data and drafted the manuscript; SChat revised the article critically for intellectual content. All authors read and approved the final manuscript. SChat is guarantor of the paper.

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Conflicts of interest: We declare that we have no conflicts of interest.

Ethical approval: The study was duly approved by the ethical committee of ICMR virus unit, Kolkata, India.

References


Influence of socio-economic status and environmental factors on serologically diagnosed Japanese encephalitis cases in the state of West Bengal, India during 2005-2010

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ABSTRACT

Objectives: The main aim of the current study is to examine the influence of socio-economic status and environmental factors on serologically diagnosed Japanese encephalitis cases in the state of West Bengal, India during 2005-2010. Materials and methods: A total of 648 blood/CSF specimens were collected and/or referred from the suspected AES cases, admitted in the different medical colleges and hospitals of the state during the year of 2005-2010. These specimens were subjected to JE Mac ELISA to determine the actual JE case amongst these AES. The association of the socio-economic status and environmental factors with the serologically diagnosed JE positive cases was studied by a statistical analysis through Normal Deviate test or Z test. Result: Out of 648 specimens, only 175 (27.0%) specimens were reactive to JE IgM antibody, of which 60.0% were from the male individuals and 40.0% from the female population. Major cases were observed in the age group of 0 - 10 years; followed by 11 - 20 years. Regarding literacy, only 58.3% cases had no education and 41.7% were from the literate with varying level of education, i.e., from primary level to post graduate level. A total of 65.7% cases were from low income group whereas 34.3% cases were from high income group. Regarding house type, 62.3% cases lived in mud house and 37.7% cases lived in the brick house. In most of the cases (74.3%), persons were living in close proximity to rice fields/lakes/ponds. 69.7% cases were found to occur in the monsoon period whereas 30.3% cases were reported in the pre-monsoon period. Conclusion: Our study concludes that socio-economic status and environmental conditions were statistically significant contextual risk factors for serologically diagnosed JE incidences in West Bengal where JE is proved to be endemic in nature and such study constitutes a new report of this kind in the region.

Keywords: Japanese Encephalitis; Socio-Economic Status; Acute Encephalitis Syndrome (AES); West Bengal

1. INTRODUCTION

Japanese encephalitis (JE) is a neurotrophic killer disease caused by the mosquito-borne Japanese encephalitis virus (JEV), a member of the genus flavivirus under the family flaviviridae [1]. It is a disease of major public health importance due to its high epidemic potential, high case fatality rate and neurological sequelae among survivors. Approximately 3 billion people (60% of the world’s population) live in J.E. endemic regions [2]. The worldwide incidental scenario of JE is 30,000 - 50,000 cases per year, the estimated mortality is 10,000 per year, whereas about 30% of survivors develop serious permanent neuropsychiatric problem [3].

In a zoonotic cycle, JEV is transmitted by vector mosquitoes (Culex sp.) [4] between wild/domestic birds and pigs; where birds act as reservoir host [5] and pigs act as amplifying host [6]. Pig-mosquito-pig and bird-mosquito-bird cycle is responsible for the maintenance of the virus in nature. Man is the “dead end” host [7,8].
JE was first recognized in Japan and since the isolation of this virus in Japan in 1935; it has spread all over the world including India and has become a major public health problem.

In India, the existence of JEV was first reported serologically in 1954 [9]. However, the disease was first recognized in India at Vellore in 1955 [10]. Since then, epidemics of JE in different states have been recorded [11,12]. From 1978 to 2007, 103,389 cases and 33,729 deaths due to JEV infection were recorded from various parts of India [13].

In West Bengal, the first major outbreak of JE took place in the year 1973 in the district of Burdwan and Bankura where more than 700 cases and 300 deaths have been reported [14-16]. Since then many out breaks have been reported [17-19]. Though, the vaccination programme against JE has been conducted by the State Health Department, Govt. Of West Bengal in the different districts of West Bengal, still now every year sporadic cases are continuously being reported [20], which proves the endemicity of JE in this state.

Previous reports have been suggested that Socio-economic status, demographic variables and environmental factors play an important role in the spread JE along with the population dynamics of the vector mosquitoes [21,22].

Here, we report a five years’ (2005-2010) study to correlate the influence of Socio-economic status and Environmental factors on serologically diagnosed Japanese Encephalitis cases in the state of West Bengal.

2. MATERIALS AND METHODS

2.1. Study Area

The study was under taken in the state of West Bengal, situated at the eastern part (23°00′N, 87°00′E) of the India [23]. The state has an area of 88,752 km² [24]. The population of West Bengal is 91,347,736 [25]. Vast paddy fields with 3 - 4 inches stagnant water (serves as congeval home for mosquito breeding) is the common scenario in the districts of the central and southern part of the state. The people belong to rural area of the state are economically backward. Agriculture is the main economic source and most of the people of the rural area in this state, are involved in agricultural practices. The field labourers of the rural area mostly belong to the economically backward classes. To raise their economic status, they usually taken up piggery and mini-poultry in their own hut, commonly share the habitat with human population [24].

Except the northern hilly region, other part of this state is warm and humid for a maximum time of the year. Here, the main seasons are summer, monsoon, autumn, late autumn and winter. The summer lasts from mid-March to mid-June, with the temperature ranging from 38°C to 45°C. The monsoon arrives by mid-June and continues up to the month of September. The agricultural activities are at their peaks during the post-monsoon phase which persists between middle of September to the end of November of the year [26].

2.2. Patients and Clinical Specimens

The study group, i.e., the patients, clinically diagnosed as Acute Encephalitis Syndrome (AES), having high grade fever (≥39°C) for ≥10 days with any two of the following symptoms, viz. headache, vomiting, unconsciousness, convulsions, abnormal movements, stupor, delirium, altered sensorium, neck rigidity, presence of Kernig’s sign; admitted to the different medical colleges and district hospitals were considered for this study period during 2005-2010. Details of the patients were provided by the concerned clinicians of the above said respective hospitals.

Infected consents were taken in prescribed proforma from the relatives of the patients before the collection of the blood/CSF specimens. A total of 648 blood/CSF specimens (2 - 5 ml) were collected and/or referred from the above said clinically suspected AES cases in sterile vacutainers or test tubes by vein puncture/lumber puncture. All the specimens were transported to the ICMR (Indian Council of Medical Research) Virus Unit, Kolkata, for the detection of IgM antibody against JEV, maintaining the cold chain using vaccine carriers. After receiving the blood/CSF specimens along with the duly filled consent forms and the particulars of the patients from the above said hospitals, serum was separated from the clotted blood and finally, both the sera and the CSF were transferred to sterile aliquots and stored at 4°C and/or -80°C till tested. A register was maintained in which the particulars of the patients, i.e., name, age, address, financial status, residential and environmental condition along with disease character was entered.

2.3. Specimens Testing

For the detection of IgM antibody against JEV in the collected specimens, M antibody captured Enzyme-linked Immunosorbent (Mac ELISA) was performed using the kit that was procured from National Institute of Virology (NIV), Pune [27,28]; following the prescribed protocol. OD was measured at 450 nm using an ELISA reader (Titertek Multiskan Plus, Lab systems Finland, Type-314).

2.4. Statistical Method

To evaluate or correlate the impact of Socio-economic status and Environmental factors on serologically diagnosed Japanese Encephalitis cases; statistical analysis

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was performed using Normal Deviate test or Z test. For testing the significance of proportion of incidences among the all IgM positive cases, the hypothesis followed below:

H₀: P = 0.5 (null hypothesis);

Against, H₁: P > 0.5 (alternative hypothesis)

where, P = proportion of the cases in the more susceptible group in the parent population.

3. RESULTS

Out of 648 specimens, only 175 (27.0%) specimens were reactive to JE IgM antibody by ELISA method, among them 105 (60.0%) cases were from male individuals and 70 (40.0%) cases were female population (Table 1, Figure 1). In addition, in most of the male & female IgM positive cases, i.e., 77 out of 175 (44.0%) belonged to the 0 - 10 years age group and 42 out of 175 (24.0%) were in the age group of 11 - 20 years. (Table 1, Figure 1).

So far literacy is concerned, only 102 (58.3%) out of 175 cases were from illiterate group and 73 (41.7%) cases were literates having varying level of education, i.e., from primary level to post graduate level (Tables 2 and 3, Figure 2).

### Table 1. Age group & Sex wise distribution of JE IgM positive cases from the year of 2005-2010.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age group (in years)</th>
<th>Sub total</th>
<th>Z value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 - 10</td>
<td>46 (26.28%)</td>
<td>105 (60.00%)</td>
<td>2.65</td>
</tr>
<tr>
<td>Male</td>
<td>11 - 20</td>
<td>24 (13.71%)</td>
<td>8 (4.57%)</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>21 - 30</td>
<td>7 (4.00%)</td>
<td>7 (4.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31 - 40</td>
<td>8 (4.57%)</td>
<td>7 (4.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>41 - 50</td>
<td>8 (4.57%)</td>
<td>7 (4.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt; 51</td>
<td>12 (6.85%)</td>
<td>7 (4.00%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>31 (17.71%)</td>
<td>66 (37.71%)</td>
<td>115 (65.71%)</td>
<td>4.15</td>
</tr>
<tr>
<td></td>
<td>18 (10.28%)</td>
<td>6 (3.42%)</td>
<td>60 (34.28%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 (2.28%)</td>
<td>4 (2.28%)</td>
<td>66 (37.71%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 (3.42%)</td>
<td>129 (74.38%)</td>
<td>175</td>
<td>3.25</td>
</tr>
</tbody>
</table>

### Table 2. Distribution of JE IgM positive cases against socio-economic status and environmental factor.

<table>
<thead>
<tr>
<th>Variables (N = 175)</th>
<th>No. of IgM +ve cases</th>
<th>Total No. of IgM +ve cases</th>
<th>Z value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Educational Status</td>
<td>Illiterate</td>
<td>102 (58.28%)</td>
<td>175</td>
<td>2.19</td>
</tr>
<tr>
<td></td>
<td>Literate</td>
<td>73 (41.71%)</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>Economic Status</td>
<td>Low Income Group</td>
<td>115 (65.71%)</td>
<td>175</td>
<td>4.15</td>
</tr>
<tr>
<td></td>
<td>High Income Group</td>
<td>60 (34.28%)</td>
<td>175</td>
<td>3.25</td>
</tr>
<tr>
<td></td>
<td>Mud house</td>
<td>109 (62.28%)</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brick house</td>
<td>66 (37.71%)</td>
<td>175</td>
<td>6.42</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>129 (74.38%)</td>
<td>175</td>
<td>5.2</td>
</tr>
<tr>
<td>Breeding &amp; staging site for Mosquito/hind in rice fields/lakes/ponds Near patients' house</td>
<td>No</td>
<td>45 (25.71%)</td>
<td>53 (30.28%)</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>Pre-monsoon</td>
<td>53 (30.28%)</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-monsoon</td>
<td>122 (69.71%)</td>
<td>175</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Literacy level in respect to JE IgM positive cases from the year of 2005-2010.

<table>
<thead>
<tr>
<th>Literacy level</th>
<th>No of IgM +ve JE cases</th>
<th>Total no. of IgM +ve JE cases in literates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>22 (30.13%)</td>
<td>73</td>
</tr>
<tr>
<td>Lower secondary</td>
<td>28 (38.38%)</td>
<td>73</td>
</tr>
<tr>
<td>Secondary</td>
<td>14 (19.17%)</td>
<td>73</td>
</tr>
<tr>
<td>Higher secondary</td>
<td>5 (6.84%)</td>
<td>73</td>
</tr>
<tr>
<td>Graduate</td>
<td>3 (4.10%)</td>
<td>73</td>
</tr>
<tr>
<td>Post graduate</td>
<td>1 (1.36%)</td>
<td>73</td>
</tr>
</tbody>
</table>

As regards the financial status, 115 (65.7%) JE IgM positive cases were from Low income group (income level < 5000/month) whereas only 60 (34.3%) cases were detected from high income group (income level > 5000/month) (Table 2, Figure 3). 109 out of 175 (62.3%) cases were found from the people living in the mud house, significantly higher in comparison to 66 (37.7%) cases from the people living in the brick house (Table 2, Figure 3).

So far the location of the residence of the JE victimized people is concerned, 130 out of 175 (74.3%) cases, i.e., the greater IgM positive cases were from the persons residing in houses near to the rice fields, the breeding sites of JE mosquito vector as well as adjacent to lakes/ponds considered as the staging site of house hold foods or birds and remaining 45 (25.7%) cases were from the persons in the area far away from the rice fields/ lakes/ponds (Table 2, Figure 3).

As to the seasonal variations, most of the IgM positive 122 out of 175 (69.7%) cases were seen in the monsoon and post-monsoon period (June to November) whereas only 53 (30.3%) cases were found in the pre-monsoon period (January to May) (Table 2, Figure 3).

4. DISCUSSION

JE has become a major public health threat in West Bengal due to its complexity and lack of any specific treat-ment. Mainly, it is a rural disease and appeared in the form of large epidemics at intervals and has become endemic in many rural areas of West Bengal.

Effective vaccine against JEV is now available. Due to the widespread use of JE vaccine JE cases has been reduced in China, Korea, and Japan [29]. Although in some districts of West Bengal like Burdwan, Birbhum, Midnapore (W), Howrah and Hooghly, the vaccine was given by the State Health Department, Govt, of West Bengal [20], still sporadic cases are continuously being reported in every JE season.

In the present study, out of 648 samples (referred AES cases) tested, only 175 (27.0%) samples were reactive against IgM antibody to JE and had the history of illness for >10 days, indicative of active immune response at this stage of illness [30,31]. This observation amply proves the infection in the immediate past.

In our study, the total number of male individuals is significantly (P < 0.01) higher (60.0%) than that of females (40.0%) amongst the JE IgM positive AES cases (Table 1, Figure 1). Although, JE cases have been observed from all the age groups, highest numbers of JE positive cases have been recorded in the age group of 0 - 10 years (paediatric age group), followed by the age group of 11 - 20 years (young and young adult age group) [35], in both male and female individuals (Table 1, Figure 1). The highest number of cases in the 0 - 10 age group is possibly due to the lack of immunity in them [36]. Moreover, the male individuals in the age group of 15 - 20; usually take active part in crop field for the cultivation. The vector usually breeds in the stagnant water in the cultivation field and the majority from this age group gets exposed to the vector directly.
Only a few cases were detected in the elderly age groups (Table 1, Figure 1). This low number of JE IgM positive cases in the higher age group is possibly due to the development of immunity, either by sub clinical infections or due to the earlier vaccination, in them.

It is interestingly noted that during the study period, number of the IgM positive cases, i.e., 102 (58.3%) out of 175 cases belonged to the illiterates and was found to be significantly (P < 0.05) higher than the cases 73 (41.7%) cases of the literate group of community (Table 2, Figure 2). In addition, amongst the literates, the lower educational level like primary, lower secondary and secondary constituted the maximum number (87.7%) of the IgM positive cases while the higher educational level like higher secondary, graduate or post graduate professional group was least (12.3%) affected (Table 3, Figure 2). It is worthy to mention that the illiterate rural people and literates with short of education were unaware of the disease and its preventive measures. Therefore, illiteracy or short of education is likely to play a great role to manifest the disease in this state.

The economy of West Bengal is mainly dependent on cultivation and the villagers have mostly taken up cultivation as the source of income. To raise the economic standard; poorer section of the community has accepted piggery and mini poultry as an accelerated source of income. Our study also reveals that majority of IgM positive cases, i.e., 115 out of 175 (65.7%) belonged to the low income group (income level ≤ 5000/ month) and was to be found significantly (P < 0.01) higher than that of the high income group (income level > 5000/ month) comprising 60 (34.3%) JE IgM positive cases (Table 2, Figure 3), mostly these low income group of rural people adopt cultivation as the main source of income. To raise their economic status; they usually take up piggery [17,37] and mini-poultry in their own hut; commonly share the habitat with human population. It is worthy to mention that the stagnant water of paddy field affords a very congenial home for breeding of Culex mosquitoes [14] that act as a vector for JEV [4]. On the other hand, pigs, domestic birds like ducks, fowls which are known to be the favorable source for the maintenance of JEV in nature. Thus, this low income group community people become directly or indirectly exposed to JEV infection and this kind of data also satisfies that low economic status is one of the important risk factors in relation to JE incidences, corroborating with the earlier observation [38, 39].

According to our study, 109 number of IgM positive cases (62.3%) originated from the mud houses was significantly (P < 0.01) higher than that of 66 number of IgM positive cases (37.7%) from the brick houses (Table 2, Figure 3). Our study pointed out that the house type (i.e., made up of mud or brick) is another contextual risk factor in relation to the JE positive cases. This factor very much depends on the economic status. The persons living in brick houses belonged to high income group where as in case of low income group people living in unhygienic condition in mud houses with household crowding and lack of proper ventilation appear to be the risk factor for acquiring JE. This observation is in tune with earlier observations in other studies [38].

Previously, we have mentioned that the Culex mosquitoes are the vectors that breed in rice field and birds are the reservoir for JEV transmission. The residences of the incidental JE victimized people being in the close proximity to the breeding or staging site, i.e., rice fields, lakes or ponds for mosquitoes and marsh/migratory birds were likely to have a great influence on the disease manifestation. In this context, we came across a good number of IgM positive cases, i.e., 130 (74.3%) belonging to those patients whose residents were much closer to the rice fields, lakes or ponds. These cases were significantly (P = 0) higher than those of the 45 cases (25.7%) of such victims whose residences were far away from the rice fields or lakes (Table 2, Figure 3).

In regard to the seasonal variations, monsoon and post-monsoon period make an influence to accelerate JE incidences. Most of the IgM positive cases, i.e., 122 out of 175 (69.7%) cases found in the monsoon and post-monsoon period, were significantly (P < 0.00001) higher whereas only 53 (30.3%) cases were found in the pre-monsoon period (Table 2, Figure 3). It is worthwhile to mention that the monsoon and the post-monsoon period are the prime season for cultivation of paddy. At that time, all the paddy fields are covered with stagnant rain water for the need of the crops, which are preferred by the member of the Culex for breeding place [14] and mosquito density begins to rise with extensive paddy planting.

5. CONCLUSION

Now JE is the most common form of sporadic encephalitis in our state and should not be ruled out first before considering the other viral causes. In this paper, an attempt has been made to evaluate a great influence of socio-economic status and environmental factors on JE incidences in the state of West Bengal. Our study has clearly established the existence of JEV etiology in the sporadic JE incidences by performing antibody captured ELISA method against the JEV specific antigen.

JE risk was significantly associated with the rural residents living in close proximity to irrigated rice fields (preferred breeding place for vector mosquito of JE) and pig-rearing places. To avoid the risk of JE, these rural residents should take personal protection by using of mosquito repellent, insecticide-treated bed nets [40] and deet-permethrin soap [41].
quito bite or vector exposure. In addition, the larvae of the vector mosquito should be controlled by some biological control strategies [42]. On the other hand, to overcome the risk of JE, rural residents should introduce either rearing of pigs in the modern farms separate from their housing [43] or rearing of cattle (considered as dead-end host with low viremia) [6] as a income source instead of pigs to reduce the chances of disease transmission [44]. Moreover, awareness programmes should be arranged to educate the illiterate residents about the disease and its horrible consequences so that they are eager to get them vaccinated against the disease.

Finally, based on this study, it is concluded that the socio-economic status and environmental conditions were statistically significant contextual risk factors for serologically diagnosed JE incidences in West Bengal. As no such study has yet been carried out, it constitutes a new report of this kind in the region.

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Research Article

Molecular Typing of Dengue Virus Circulating in Kolkata, India in 2010

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Dengue is one of the major public health threats in Kolkata. Every year, blood samples with dengue-like illness are referred to us from different medical colleges and hospitals in Kolkata for the detection of dengue infection in them. In 2010, a total of 378 samples were referred to us for that purpose. All the samples were tested for the detection of IgM antibodies by ELISA method, followed by RT-PCR test for the detection of serotypes. Only 173 samples were ELISA positive. Out of 378 samples, 108 were RT-PCR positive. Out of 108 samples, 74 samples had monotypic infection with different serotypes of DENV and 33 samples had dual infections with DENV-2 and DENV-3. Only one sample had the infection with DENV-1, DENV-2, and DENV-3. DHF was found mainly among the patients, infected with multiple dengue serotypes. Only 3 dengue monotypic infected patients had suffered from DHF.

1. Introduction

During the past few decades, dengue fever has gradually become one of the leading causes of morbidity and mortality in tropical and subtropical areas throughout the world [1]. The Dengue virus (DENV), a mosquito-borne member of the family Flaviviridae, circulates as four distinct serological types DENV 1, DENV 2, DENV 3, and DENV 4. Over all, two-fifth of the world population are living in areas, at risk for dengue [2-4]. These four serotypes offer cross protection to a mild self-limiting febrile illness (dengue fever, DF). A more severe form of the disease, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), is responsible for high mortality rate, especially in children [5]. It has been estimated that about 50 million cases of DF occur annually, with 10,000 infant deaths due to DHF/DSS. DHF/DSS has been postulated to result from immune enhancement after a second heterologous DENV infection [6].

In India, DENV was first isolated in 1946 and many outbreaks have been reported [7-10]. DHF was first reported in Calcutta (Kolkata), West Bengal in 1963 [11], again in 1964 [12]. Since then, there are numerous studies from the Indian subcontinent investigating DHF in various parts of the country [13-22]. But there are no studies investigating the overall prevalence of the dengue serotype circulating in the endemic zone, apart from the epidemic outbreak. The purpose of this paper is to present a comprehensive report on the diagnosis of dengue infection amongst the febrile cases, available from January to December, 2010, in the city of Kolkata and also to identify the serotype presently circulating in this region.

The incidence of DF in the rural West Bengal is on the increase and is spreading to geographic regions not previously affected [23,24]. It is widely blown that dengue is endemic in Kolkata. The city has experienced several dengue episodes in the past centuries [25]. Antibodies against Group B-arthropod-borne viruses in more than 80% of the Kolkata population have been recorded almost fifty years back, and that is too possibly due to the infection by dengue viruses [26].

The present study aimed to identify the serotypes of DENV in the population of Kolkata as well as to study the sociodemographic status in relation to DENV infection.

2. Materials and Methods

2.1. Study Area. Kolkata is one of the biggest metropolitan cities in India. The present population of Kolkata is 44,86,679
of which 23,62,662 are males and 21,24,017 are females [27]. The city has an international sea and airport and one rail way station (Sealdah) which is busiest in the world. The rail road of this station covers a number of districts, situated at the border of Bangladesh. Adjacent to the city, there is a thickly populated town, Howrah, which has one of the biggest terminating railway stations of the Eastern India. These two rail stations are the gateway of this city. The monsoon begins in June and persists up to the end of October.

2.2. Patients and Clinical Specimens. Cases were mainly referred from outpatient department (OPD) and indoor of LD & B. G Hospital, attached to this unit, from different medical colleges as well as other hospitals in Kolkata along with a short history of the patients. A good number of cases were referred to us by the private practitioners also. In the matter of selection of dengue fever (DF) cases, the following criteria were initially considered: (1) high fever; (2) headache; (3) retro-orbital pain; (4) nausea/vomiting; (5) malaise/pain; (6) generalized skin rashes [28]. In the present study two or more of these criteria, apart from fever, were fulfilled. The possibilities of bacterial and prokaryotic etiology in the collected samples were excluded through investigations at the respective hospitals.

2.3. Serology. Virus Unit. Sera were separated from the collected blood samples as well as from four different DENV serotype strains, which were used as positive control. Viral RNA was isolated by using QiaGen viral RNA isolation kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol.

2.4. RNA Extraction. To study the molecular typing of DENV, attempts were made to isolate the RNA from all the samples as well as from four different DENV serotype strains, which were used as positive control. Viral RNA was converted to a DNA copy (cDNA) prior to enzymatic DNA amplification by the use of reverse transcriptase (RT) and the DENV downstream consensus primer D2-5’-TTCACCCAAAGCTCAATGCTTCCAGTTC-3’ homologous to the genomic RNA of the four serotypes. Subsequent Taq polymerase amplification was performed on the resulting cDNA with the upstream dengue virus consensus primer D1-5’-TCAATATGCCTAAACGGCGGAAGACC-3’. Target RNA was amplified in 25 μl volumes containing the following components: 800 mM deoxynucleotide triphosphates (dNTPs), 8 mM dithiothreitol, 0.24 μM each of primers D1 and D2, 0.5 U of AMV RT (Promega, Madison, WI, USA), and 0.625 U of Dreamtaq DNA polymerase (Fermentas Inc., USA). The reactions were allowed to proceed for 1 h at 42°C and then to proceed with 95°C for 3 minutes for initial denaturation followed by 35 cycles of denaturation (95°C for 30 sec), primer annealing (55°C for 1 min), and primer extension (72°C for 2 min) along with final extension (72°C for 5 min).

DENV serotyping was conducted by second-round amplification (nested PCR) initiated with 10 μl of diluted material (1:100 in sterile distilled water) from the initial amplification reaction. The total 20 μl of reaction mixture was prepared using 2 μl of diluted first PCR products, 0.8 mM dNTPs, 0.5 U of Dreamtaq DNA Polymerase, and 0.3 μM of primer D1 and 0.3 μM of dengue virus type-specific primers: TS1 5’-CGTCTCAGTGATCCGGGGG-3’, TS2 5’-CGGCCAAGGGCCATGAACAG-3’, TS3 5’-TAACATCATGAGCAAACAGGCAGC-3’, and TS4 5’-CTCTGCTGGTTGCTTTAACAAGAGA-3’. Dithiothreitol and AMV RT were eliminated. The samples were subjected to initial denaturation (95°C for 3 min) followed by 20 cycles of denaturation (95°C for 30 sec), primer annealing (55°C for 1 min), and primer extension (72°C for 2 min) along with final extension (72°C for 5 min). The PCR products were analyzed by running a 1.5% agarose gel stained with ethidium bromide.

3. Results

3.1. Serology. Out of 378 samples collected, only 173 samples were reactive to dengue IgM antibody by ELISA method. Maximum numbers of IgM positive cases were observed in the age group of 6–10 years in both male and female patients (Figure 1). Females were more affected (45.3%) than the males (45.1%). As regards the seasonal prevalence, it is evident from the result that although sporadic cases obtained throughout the year, dengue cases started from the month of July and attained maximum number of cases in the month of November (Figure 2).
3.2. PCR Result. Out of 378 samples, 108 were RT-PCR positive. Seventy-four samples (68.5%) had the DENV infection by single different serotypes, of which 7 samples (9.5%) had the monotypic infection with DENV-1. 45 samples (60.8%) had the monotypic infection with DENV-2 and 22 samples (29.7%) had the monotypic infections with DENV-3 serotype. Both DENV-2 and DENV-3 serotypes were detected in 33 samples (30.6%). Only one sample had all the three serotypes, that is, DENV-1, DENV-2, and DENV-3. No DENV-4 serotype was detected in those samples. Out of 22 DHF cases, 3 were found with single-serotype infection and 16 cases with dual infections of DENV 2 and DENV 3. Only a female patient of 24 years of age had the infection with DENV-1, DENV-2, and DENV-3 serotypes at a time and also suffered from DHF. Clinical data regarding the death of the DHF cases was inadequate to reach any conclusion about the severity of illness. The specimen containing DENV-1, DENV-2, and/or DENV-3 is identified by the detection of a DNA band of 482, 119, or 290 bp in size, respectively, on 1.5% agarose gel, loaded with nested PCR products along with positive controls, stained with ethidium bromide (Figure 3).

4. Discussion

The monitoring of DENV activity is required for public health importance, as the dengue fever and DHF/DSS are increasing worldwide and are spreading in the places, where it was previously not reported. The first isolation of DENV serotype 1 and 4 was reported from India in 1964 [31, 32] and serotype 3 in 1968 [18]. Although concurrent infection with more than one serotype of DENV in the same individual is uncommon, high percentage of concurrent infections with different DENV serotypes had been detected at an outbreak in Delhi, India, in 2006 [33]. Kolkata is a dengue endemic zone; frequent outbreaks of DF and DHF have been occurring since last centuries. In Kolkata, dengue was first documented in 1824 and several epidemics took place in the city during the years 1836, 1906, 1911, and 1972, affecting 40% of the city people [34]. The last large-scale dengue outbreak has been recorded in the year 2005 [35]. In the rural areas of West Bengal, dengue is gradually spreading and establishes new reports [23, 24]. No continuous monitoring of the molecular detection of the dengue serotype has yet been attempted in the city of Kolkata, either in epidemic or in sporadic dengue outbreaks.

Out of 378 blood samples, initially 173 (45.76%) cases were reactive to dengue IgM antibody. Age-wise distribution revealed that the highest number of dengue cases were detected in the age group of 0-10 years, followed by 11-20 years and above (Figure 1). In the highest age group (above 50 years), the number of positive cases were too small and only 16.6% dengue IgM positivity was found in the male individuals. In all the age groups, females were more affected than the males (Figure 1). As the vector mosquitoes

![Figure 1: Age and sex wise distribution of dengue IgM-positive cases in 2010.](image1)

![Figure 2: Monthly distributions of IgM-positive dengue cases in 2010.](image2)

![Figure 3: Results of dengue-specific RT-PCR followed by second-round nested PCR of RNA samples, showing band in 1.5% agarose gel electrophoresis stained with ethidium bromide. Lane L: 100 bp DNA ladder. Lane S1-S8: negative sample. Lane S9, S10 and S20: sample positive for DENV-2 (119bp), Lane S11-S13 and S16-S19: sample with dual infection of DENV-2 (119bp) and DENV-3 (290bp), Lane S15: sample with concurrent infection of DENV-1 (482bp), DENV-2 (119bp), and DENV-3 (290bp), and Lane C: negative control.](image3)
(Aedes sp.) are domestic and peridomestics in nature, the females get more exposure than the males, as most of the time they reside inside the house. During the year-round study, although small number of samples from suspected dengue cases were referred to us during the period from January to May, only a few were positive to dengue IgM antibody by ELISA method. It is evident from our study (Figure 2) that the dengue cases actually started from the month of June and attained its peak in the month of November during this year, which is the post-monsoon period. It may be explained by the fact that the stagnant fresh water during the rainy seasons (June to October) favoured the breeding of the vector mosquitoes.

For the molecular detection, RT-PCR was performed with all samples, of which 118 samples were positive by that method. Forty-one samples were both ELISA and RT-PCR positive. A total of 74 samples had the monocryptic infections, involving DENV 1, DENV 2, and DENV 3, of which DENV 2 was predominated. All these were IgM negative. Conversely, 33 samples had the dual infections with DENV 2 and DENV 3, of which all the samples were IgM reactive. Only one sample produced prominent band against DENV 1, DENV 2, and DENV 3 and also contained IgM antibody. The detection of the viral RNA in presence of the IgM antibody may be explained by the fact that, due to the consecutive infection, the IgM detected in those samples possibly appeared due to the initial infection, which is evident by the intensity of the bands in gel electrophoresis (Figure 3). The hemorrhagic manifestation was found mainly among the patients, infected with multiple dengue serotypes. Only 3 dengue monocryptic infected patients had suffered from DHF. DHF cases were observed mainly among the young and young-adult age groups (0-30 years) which might be due to the absence of immunity against all serotypes of DENV in them.

Although the increasing trend of cocirculation of multiple DENV serotypes suggests that Kolkata is becoming a hyperendemic state, a large-scale monitoring on the circulating strains of DENV is highly required to draw a definite conclusion.

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References


Sero diagnosis of dengue activity in an unknown febrile outbreak at the Siliguri Town, District Darjeeling, West Bengal

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Abstract

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Objective: To investigate the outbreak of unknown fever at Siliguri town, Darjeeling District on request from the State Health Department, Government of West Bengal. Methods: Investigations were made to the affected wards. Sub Divisional Hospital and the nursing homes of Siliguri Town. Duration of illness was 3-5 days. Interesting observations were made in some cases which had gastrointestinal disorders with high serum glutamate pyruvate transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) levels. A total of 69 blood samples and 7 throat swabs (in Minimum Essential Media) were collected and brought to the ICMR Virus Unit, Kolkata for analysis. Mosquitoes from different affected areas were collected for the identification of the definite vector.

Results: Amongst the 69 blood samples, 42 (60.86%) were positive to IgM antibody against dengue virus by Mac enzyme-linked immunosorbent assay (ELISA) test. No IgM antibody to Japanese encephalitis virus was detected among the collected blood samples. Based on the clinical symptoms, presence of IgM antibody to dengue virus and identification of Aedes mosquito, it amply proves that the illness of those cases were due to dengue virus infection.

Conclusions: Based on clinical-epidemiological observations of the investigations the possibility of a communicable disease of viral origin, the detection of IgM antibody and the identification of Aedes aegypti, and the potential circulation of dengue virus in Siliguri town for the first time were all suggested.

1. Introduction

Dengue virus with its four sero types is now classified within the flaviviridae family. Among the four sero types, infection with any of them generally leads to a mild self-limiting febrile illness [dengue fever (DF)]. A more severe form of the disease involving vascular and haemostatic abnormalities leads to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which is responsible for a high mortality rate, especially in children. Dengue virus is responsible for a growing health problem in the tropical and sub tropical countries. It is one of the most important among human Arbovirus infections. The global incidence of DF and DHF has increased dramatically in the recent decades. In India dengue was first isolated in 1946, and many epidemics have since been reported. DHF was first reported in Calcutta, West Bengal, in 1963 again in 1984 and subsequently in Visakhapatnam in 1969 and in Jalaur, Rajasthan in 1985. Delhi has experienced major epidemic of DHF in 1996. The epidemic began in mid August and lasted up to November of that year. In addition to that, out breaks have also been reported at regular intervals from Maharashtra, Punjab, Tamil Nadu, and Madhya Pradesh.

Here, we report an outbreak of DF occurred in Siliguri town, Dist-Darjeeling from October-November 2005, which was intimated to us by the Director of Medical Education and Director of Health Services, Government of West Bengal, for necessary virological investigations. The purpose of this paper is to present a comprehensive report on the results of investigations of the disease from its first appearance up to the period of investigation which was from 18th to 22nd November 2005. Although the outbreak has tolled only one death, but no ease of hemorrhagic manifestation was reported.

2. Materials and methods

According to the epidemiological information, this outbreak had two types of clinical cases at different periods. In the first type, mainly affecting children in whom mild dengue like features was predominant, started during
the middle of July and had its peak in August and then gradually declined. There was no mortality in this group. In the second phase, (present investigation) showing an almost classical dengue like syndrome (without any shock and hemorrhage), started from 1st November and had its peak in the middle of November and then gradually declined. During this phase of illness, all the age groups (7 to 56) irrespective of sex differentiation, were affected and only one death was recorded. In the 2nd episode, all parts of the town were more or less affected.

Cases for investigation included those admitted to the Siliguri SD Hospital, as well as those who attended the Outpatient Department (OPD) of that hospital. During this investigation, the local nursing homes were also visited to examine and evaluate the fever cases, attending the OPD and admitted into those nursing homes. From 2nd November 2005 to 19th November 2005, daily distribution of unknown fever cases attending the OPD of Siliguri SD Hospital, could be collected. In addition to that, a good number of cases were referred to us by the local practitioners for proper investigations. In the matter of selection of cases the following criteria were taken into consideration: a) high fever, b) headache, c) joint pain, d) body ache, e) malaise, f) vomiting or with nausea, g) generalized skin rash, h) spontaneous hemorrhage with or without circulating failure. In the present investigation, two or more of these criteria, apart from fever, were considered. The possibility of bacteriologic and prokaryotic etiology in the collected sample was excluded through investigations at the local hospitals/nursing homes. Attempts were made to collect paired sera, after 15 days, from the positive cases and a total of 41 convalescent blood samples could be collected. All the cases, blood samples were collected by venous puncture.

Sera were separated from the collected blood samples and transported on wet ice to the virus unit, Calcutta, where they were stored at -80°C until testing. All the sera were tested within 1-2 months from the date of collection. A total of 69 acute samples were collected from the cases of the affected area and screened for the presence of dengue/Japanese encephalitis (JE) IgM antibodies by IgM capture enzyme-linked immunosorbent assay (ELISA) (MAC-ELISA), using a kit (Prepared by National Institute of virology, Pune, India), following the prescribed protocol. optical density (OD) (in full name) was measured at 492 nm using an ELISA reader (Titertek Multiskan Plus, Lab systems Finland, Type- 314).

Attempts were made to isolate the virus in sucking infants (2-3 days old) Swiss mice, with the sera collected during the acute stage of the disease. The undiluted serum was inoculated intracerebrally in 1 L of 2-3 days old Swiss albino sucking mice and observed for 15 days. The brains of the sick mice were harvested and were inoculated into nutrient broth to exclude any bacterial contamination/infection. If the mice did not show any illness, another blind passage was made. The acute phase sera were also inoculated into Vero cell cultures with two blind passages and observed for 15 days for cytopathic effects. On the other hand, hemagglutination inhibition (HAI) tests were done with the acute and convalescent sera for the presence of IgM antibody titre, if any, and also for the identification of the type of the virus, using the method described by Clark & Casals. In interpreting serological results, the standard criteria were followed. The samples which were positive to dengue virus by MAC-ELISA method were subjected to HAI test against the four types of dengue antigen i.e. Den-1, Den-2, Den-3 and Den-4 for the identification of the sero type of the circulating strain. All the samples inhibited agglutination against Den-2 antigen. For confirmation of the etiologic agent, the convalescent sera along with the acute samples were subjected to HAI test against dengue and JE antigen.

Samples were considered positive if they had a fourfold rise of titre in the convalescent paired sera.

Mosquitoes, resting inside the houses of the affected areas were collected and after identification, the pools of separate species of mosquitoes were ground in bovalbumin-phosphate saline containing antibodies. After centrifugation, the supernatant was inoculated asexptically into Vero cell line for isolation of virus.

A total of 69 acute samples could be collected from the cases of the affected area and screened for the presence of dengue/JE IgM antibodies by IgM capture ELISA (MAC-ELISA), using a kit (Prepared by National Institute of virology, Pune, India), following the prescribed protocol. OD was measured at 492 nm using an ELISA reader (Titertek Multiskan Plus, Lab systems Finland, Type- 314).

3. Results

Daily distribution of unknown fever cases at the OPD of Siliguri SD Hospital, were presented in Figure 1. Out of 69 acute samples, dengue virus specific IgM antibodies were observed only in 42 (60.86%) samples. IgM antibody to JE virus was not observed in any of the samples, collected from the acute cases. The maximum IgM positivity (72.41%) was observed in the age group of 11-20, followed by the age group of 0-10 (68.42%). In the age group of 21-30 and 31-40 the IgM antibody to dengue virus were 41.66% and 33.33%, respectively. In the upper age group no IgM antibody to dengue or JE virus was observed. Although attempts were made to isolate the virus from the acute samples and also from the mosquito pools, the inoculated mice and the cell culture did not produce any illness and cytopathic effect respectively and no virus could be isolated from any of them. The HAI test was used to detect rise of antibodies to dengue, if any, and also for the identification of the sero type of the circulating virus.

A total of 29 (33.77%) samples, out of 41 convalescent sera, revealed fourfold rise of antibody titre only against dengue antigen. Although the highest IgM antibody positivity (72.41%) to dengue was observed in the age group of 11-20 years, it was very close to the age group of 0-10, where the dengue positivity was 68.42%. An antibody to dengue was 41.66% and 33.33% in the age group of 21-30 and 31-40, respectively. No IgM antibody to dengue was observed in the age group of 41 and above. No samples were positive to JE antibodies alone, in the present investigation. Only 5 (17.24%) convalescent sera showed flavivirus Group reaction and had sharing with antibody titre to JE and dengue antigens. The rest of the 24 (74.13%) convalescent samples did not produce any rise of antibody titre either to dengue or to JE viruses. For the isolation of virus, from the acute sera and from the mosquito pools, the inoculated mice and the cell culture did not produce any illness and cytopathic effect, respectively.

A total of 96 mosquitoes which were collected from the affected areas, only 11 were identified as Aedes sp. and these were from Gurung basti and Khal Para, the most affected area.
Outbreaks of dengue have been recorded in India on several occasions, although no such outbreak has yet been recorded from the district of Darjeeling or from its sister town of Siliguri. In this study, all the cases were classical DF and there were no hemorrhagic manifestations except occasionally a few Petechiae on the skin. This outbreak of dengue in Siliguri town occurred during the monsoon season (September–November), which is similar to most of the previous outbreaks in India. The highest no. of cases were reported during mid-November which is similar with the previous reports of other different out breaks in India. The detection of IgM antibody to dengue virus in the sera samples, collected from the affected area, by ELISA test clearly proves that the febrile illness was due to the infection of dengue virus in the recent past. On the other hand, rise of antibody titre in the convalescent sera, as revealed by the HI test against dengue antigen, again confirms the etiologic agent, of the febrile illness, was dengue virus. The serological result reveals that the acute samples inhibited agglutination against dengue-2 antigen and all the convalescent sera had the four fold rise of dengue antibody titre in them. In the older age group, group B flavivirus reaction indicates, their exposure to JE elsewhere in the remote past. So on the basis of laboratory investigation, epidemiological report and the identification of Aedes mosquitoes in the affected area confirms that the febrile illness was due to dengue-2 virus. In this outbreak, the young and young adult age groups were largely affected, which is similar to the observation of other dengue outbreak by other workers.

The identification of the Aedes mosquitoes collected from the affected area like Gurung basti and Khal para, the most affected area of the town Siliguri, amply proves the spread of the disease by the vector (Ae. albopictus: the proven vector of dengue in suburban areas.


References

Investigations of Recurrent outbreaks of unknown fever, establish rural dengue activity in West Midnapore, a costal district in West Bengal, India.

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Abstract

Title: Investigations of Recurrent outbreaks of unknown fever, establish rural dengue activity in West Midnapore, a costal district in West Bengal, India.

Background: In the year 2002, an investigation was conducted for the diagnosis of an unknown fever outbreak in the District of West Midnapore, West Bengal; on request of the Director of Medical Education, Govt. of West Bengal. In the year 2004, again unknown fever outbreak took place in some other area of same district and that too was investigated on request of the State Medical Department. The recurrence of same episode in the year 2007 was investigated by the local medical team and the collected serum samples were sent to us for laboratory based conformation.

Methods and findings: During the first episode (2002), a total of 1158 fever cases were reported with 7 deaths, which spread all over the district since 2nd week of May till 15th July, 2002. In the second episode (2004), 792 cases were reported and 2 deaths were recorded. A total of 781 acute sera samples could be collected during these three consecutive out breaks. The samples were collected from the patients with clinical features of fever with head ache, body ache, nausea, retro orbital pain, abdominal pain and rashes of duration 2-7 days, which is closely related with the symptoms of dengue infection. Only 195 convalescent sera were made available. Acute samples were tested for the presence of dengue specific IgM antibodies by ELISA method. There was a significantly higher incidence of fever cases in children belonging to the age group up to 10 years. No virus could be isolated from the acute sera collected from fever cases. The results of serological survey showed the presence of IgM antibodies to Dengue virus in only in 446 (57%) of the acute cases. Amongst the 195 convalescent sera, four fold rise of HAI Antibody titre to Dengue virus was observed only in 77 (39%).

Conclusion; Analysis of the epidemiological and serological findings of different years revealed that the out breaks were due to Dengue infection. Children up to 10 years of age in this district were mostly affected during these out breaks.

Key words: Midnapore, West Bengal, Dengue Virus

Introduction

Dengue viruses are the members of the genus Flavivirus of the family Flaviviridae, consists four antigenically distinct Serotypes, which do not offer cross protection. Dengue is an important mosquito borne disease in the world in terms of morbidity, mortality and economic cost [1], especially in the tropics, with more than 2/5th of the world population living in areas at risk for dengue [2-4]. Infection with any one of the four types leads to a mild, self limiting febrile illness (dengue fever, DF). A more severe form of the disease, dengue haemorrhagic fever (DHF)/ dengue shock syndrome (DSS), is responsible for high mortality rate, especially in children [5]. Dengue has now become a regular occurrence worldwide including America, Africa, Asia and the South Pacific, where the vector of the disease is wide spread. The incidence of DF is on the increase and spreading to geographic regions not previously affected. Movement of Dengue between different geographic areas and also from urban to rural is an important element in the epidemiology of the disease. From being a sporadic illness, epidemics of dengue have now become a regular occurrence all over the world. In India Dengue was first isolated in 1946 and many epidemics have since been reported [6-9].

The clinical features of dengue virus infection range from nonapparent infection through dengue fever (DF) and the more severe dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) [10-12]. DHF/DSS, although common in South East Asia, has also been reported in India during Dengue epidemics. DHF was first reported in Calcutta, West Bengal, in 1963 [13] again in 1964 [14] and subsequently in different states of India [15-22].

Laboratory diagnosis of a recent dengue virus infection may be done by detection of the virus in patient’s blood, either by virus isolation in...
susceptible cell cultures [3] or by detection of the viral RNA by reverse transcriptase-polymerase (RT-PCR) chain reaction based techniques [23-24]. These processes are very specific and should be performed within 48 hrs following the onset of illness in a well equipped laboratory, as the virus disappears after that period. However, detection of IgM antibodies by ELISA method and detection of rise of antibody titres with the convalescent samples by Haemagglutination inhibition test are well accepted serological methods for the diagnosis of dengue infection.

This paper reports the detailed investigation of repeated outbreak of unknown fever in the year 2002, 2004 and 2007 in the district West Midnapore and establishes dengue activity in rural areas on the basis of laboratory investigations.

**Methods**

**Location and Meteorological information**

Midnapore was the biggest district in the state of West Bengal, having a sea port from the ancient time, with historical back grounds. Presently the district has been divided into East and West Midnapore. The district West Midnapore, our study area, is situated at an altitude of 23 meters above sea level on the bank of Bay of Bengal with an average rain fall of 1656 mm, mostly between the months of May and September. In the summer the temperature ranges from 42°C to 50°C with high humidity.

**Sample collections and investigations**

**Investigation in the year 2002**

Cases started occurring initially in the Guadaha village of Gorbeta block followed by Salboni block. These two villages were worst affected and are situated 30-40 km from the district head quarter, surrounded by paddy fields and agricultural lands. Source of drinking water was tube wells. Village Guadaha has a total population of 1541. It was a typical rural village with kachha houses and kachha road. The village has reported 311 cases since its onset, at the local primary health centre. During investigation, it was revealed that several people suffered from two episodes of fever, headache, nausea, abdominal pain and retro orbital pain at an interval of 4 days to 6 days. In most of the houses visited, stagnant and stored water were observed for regular uses, which facilitated the breeding of the mosquitoes. Mosquitoes were abundant at the village and day biting mosquitoes were also present. From 1st to 7th July, the numbers of daily attendants of fever cases in the OPD of the hospital were collected. During this visit a total of 288 blood samples were collected from the acute cases from two villages. Of which we could collect 63 blood samples from the OPD and from the indoor patients of the hospitals and rest of the 110 blood samples were collected by the local health workers and the medical technicians of the health centre on door to door visit.

The Salboni village has a total population of 5819. It is semi urban in settings. This village has reported 540 cases up to 14th July, 2002. In both the villages there were many cases, who suffered but did not report to any health facility. Only 115 blood samples could be collected from acute cases from the affected village.

**Clinical manifestations**

All the cases had the clinical features of continued fever, headache, pain all over the body, anorexia, epigastric pain, nausea and/or vomiting, extreme weakness, bleeding manifestations like, epistaxis, haematemesis, haematuria etc. only in one case, paethchial rash was observed.

Regarding seven death cases, one case died of paralytic ileus, three cases died of haematemesis, two cases died of haematuria and one case was brought dead to the hospital with a history of rapid up rise of fever followed by sudden collapse.

**Investigation in the year 2004**

This year, village Kesia, and Laxmanpur, of Garbeta block were affected. These two villages are about 90 km from the District Headquarter and adjacent to Salboni of West Midnapore. During investigation, it was revealed that the total populations of these two villages are 4059. Date of first case report was 16th May 2004, total number of fever cases up to 3rd June were 792. All the age groups were more or less affected. During this visit in the year 2004, 139 and 115 samples were collected from Kesia and Laxmanpur respectively, in the manner adopted in the year 2002. The two patients who died in these villages had suffered from high fever with headache and cough associated with severe respiratory distress. During the time of investigation the outbreak was in declining state. There was no evidence of sex differentiation among cases. The distribution of clinical presentation among the cases and the age wise distribution of fever cases were collected from the health centre.

**Clinical manifestations**

All the cases had continued high fever, rashes all over the body with cough and mild respiratory symptoms. Majority of them had headache, nausea / vomiting, malaise and drowsiness with duration of 3 days to 5 days. Joint pain and backache were the common features in all the cases.

A total of 63 convalescent paired sera were collected after 15 days by the local health authority with the help of medical Technicians and health workers.

**Investigations in the year 2007**

This year, again an outbreak of unknown fever took place in a mild form in all over the district. The clinical manifestation was almost same with the previous years. No death was recorded in this year. The health authority of the district West Midnapore took the initiative for collection of blood samples. A total of 239 blood samples from all over the district were collected by the doctors of Sadar Hospitals, block hospitals and the primary health centers. These samples were sent to us for necessary diagnosis.
A total of 58 convalescent paired sera were collected by the local health authority with the help of medical technicians and the health workers. All the sera collected during different years were transported on wet ice to the ICMR virus unit, Calcutta, where they were stored at -80°C until tested. The possibilities of bacterial and prokaryotic etiology in the collected sample were excluded through investigations at the local hospitals. All the sera were tested within 1 month to 2 months from the date of collection.

Laboratory investigation

Virus isolation

Attempts were made to isolate the virus from the acute samples, if any, by using C6/36 cell lines. Two hundred micro liters of selected samples (2 days fever cases with dengue like symptoms) were spread over the monolayer of C6/36 cell line and allowed to adsorb for 120 minutes in an incubator at 28°C under 5% CO₂ concentration. After adsorption, the excess sample materials were discarded and minimum essential media (MEM; GIBCO BRL, US) supplemented with 2% fetal bovine serum (FBS; GIBCO BRL, US) and Penicillin Streptomycin antibiotics (PenStrep; Gibco) were added in 24 well tissue culture plate (Tarsons) and were incubated again in the same condition as before. It was observed regularly for the appearance of cytopathic effect (CPE) up to 7-8 days.

Serology

The acute samples were tested for the presence of Dengue IgM antibody by IgM capture ELISA (MAC-ELISA) method, using a kit (Prepared by National Institute of virology, Pune, India), following the prescribed protocol. O.D was measured at 450 nm using an ELISA reader (Titertek Multiskan Plus, Lab systems Finland, Type- 314).

Haemagglutination inhibition test

A total of 195 convalescent samples could be collected from the affected areas and were tested along with the respective acute sera for any rise of HAI antibody in the convalescent state. The HAI test was carried out according to the method of Clarke and Casals [19]. For confirmation of the etiologic agent, the convalescent sera along with the acute samples were subjected to HAI test against Dengue and JE antigen. Samples were considered positive, if they had a fourfold rise of titer in the convalescent paired sera in comparison with the corresponding acute samples.

Results

Sample collections and investigations

The total number of fever cases attended the OPD of Guiadaha hospital from 1st July to 17th July has been presented (Fig 1).

There was a steady increase in number of patients. On 15th July, 2002 highest no. of Patients attended the OPD.

![Fever cases attended at OPD of Guiadaha hospital from 1st July to 17th July](image.png)
Virus isolation
CPE was not developed and no virus could be isolated from any of the acute sera collected from fever cases.

Serology
Out of 781 acute blood samples, collected during these three years, Dengue Virus specific IgM antibodies were observed only in 446 (57.10%) samples. Year wise collection of acute samples and detection of Dengue IgM antibody in them, are presented (table 1).

**TABLE 1:**

<table>
<thead>
<tr>
<th>Year</th>
<th>Total number of samples collected and tested</th>
<th>Total number of IgM positive to Dengue</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>288</td>
<td>142</td>
</tr>
<tr>
<td>2004</td>
<td>284</td>
<td>156</td>
</tr>
<tr>
<td>2007</td>
<td>239</td>
<td>148</td>
</tr>
<tr>
<td>TOTAL</td>
<td>781</td>
<td>446</td>
</tr>
</tbody>
</table>

Among the 781 patients' samples tested, 453 were male and 328 were female. Out of 453 male, 217 (47.90%) were positive. On the other hand, 185 (56.40%) of the 328 samples collected from the female cases, were positive to Dengue IgM antibody. The sex wise distribution of suspected cases of Dengue and Dengue IgM positive cases have been presented (table 2).

**TABLE 2:**

<table>
<thead>
<tr>
<th>Year &amp; Sex wise distribution of Dengue positive cases in the district of West Midnapore, during the study period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>2002</td>
</tr>
<tr>
<td>2004</td>
</tr>
<tr>
<td>2007</td>
</tr>
</tbody>
</table>

There was a decrease in the number of the samples collected, during the three outbreaks, as compared to the year 2002. The age wise distribution of Dengue suspected cases and Dengue IgM positive cases have been presented (table 3).

**TABLE 3:**

<table>
<thead>
<tr>
<th>Age group (in years)</th>
<th>Year</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>2002</td>
<td>81</td>
<td>36</td>
</tr>
<tr>
<td>11-20</td>
<td>2004</td>
<td>44</td>
<td>40</td>
</tr>
<tr>
<td>21-30</td>
<td>2007</td>
<td>44</td>
<td>66</td>
</tr>
<tr>
<td>31-40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41-50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>51+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>1292</td>
<td>564</td>
<td></td>
</tr>
</tbody>
</table>

In all the three years, the maximum IgM positive cases were observed in the age group of 0-10 (28.69%), as compared to the other age groups (Fig. 2).

**FIG 2:**

A total of 77 samples out of 195 convalescent sera revealed fourfold rise of antibody titre, in comparison with the acute sera, against Dengue antigen. Apart from that, only 27 sera had very low antibody titre against JE, both in acute and the convalescent stage. Rest of the convalescent samples did not produce any reaction either against dengue or against JE antigen. The results of the HAI test against Dengue and JE antigen have been given (table 4).

**TABLE 4:**

<table>
<thead>
<tr>
<th>Year</th>
<th>No. tested</th>
<th>No. positive</th>
<th>Rise of antibody to</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>74</td>
<td>9</td>
<td>22 (30%)</td>
</tr>
<tr>
<td>2004</td>
<td>63</td>
<td>29</td>
<td>12 (46%)</td>
</tr>
<tr>
<td>2007</td>
<td>58</td>
<td>7</td>
<td>7 (12%)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>129 (28.92%)</td>
<td>11 (5.58%)</td>
<td>49 (10.98%)</td>
</tr>
</tbody>
</table>

Discussion
The epidemiology of Dengue is of continuing health importance, as the incidence of DF and DSS is increasing worldwide and is appearing in areas where it was previously unreported. In India, Den-1 and Den-4 was first reported in 1964 [25-26] and Den-3 in 1968 [27]. DHF was first reported in Calcutta, West Bengal in 1963 and in 1964 [13-14] Ever since, several reports of DF and DHF outbreaks have come from different cities in India and these include reports from Ludhiana [28], Lucknow [29], Chennai [30], Mangalore [31], Assam [32]
and Vellore [33]. In Delhi, although frequent outbreaks of DF have been occurring since 1967, major outbreaks of DHF and DSS have been reported in the year 1999 and 2001 [34-35]. All these outbreaks of DF and DHF/DSS have been recorded from the rural areas and the urban areas. No such outbreak has yet been recorded from the rural area like the district of West Midnapore, West Bengal. In the year 2002, all most one fifth of the total populations of the two villages were affected. Out of seven deaths, five cases had the symptoms of dengue haemorrhagic fever and one had the history of shock syndrome. More over the history of two episodes of fever at an interval of 4 days to 6 days in the same locality indicates the possible circulations of two types of Dengue viruses. This has in turn facilitated the haemorrhagic manifestations. The detection of IgM antibody to dengue virus by ELISA test, in 446 samples out of 781 cases, amply proves that the febrile illness was due to the dengue virus infection in the recent past (Table-1).

According to the sex wise distribution of the suspected cases (Table-2), there was a significant higher incidence of dengue IgM Sero positivity in females and not in males. This may be explained by the fact that the female individuals usually resides at home at the day time and get exposure to the mosquitoes (Aedes aegypti), as it is a domestic and peridomestic in nature.

A significant higher incidence of dengue IgM Sero positivity were observed in the age group of 0-10 years followed by 11-20 years (Table 3), which is similar to the observations made by many workers at different times and from different parts of India. The results obtained in the convalescent sera by HAI test, revealed 4 fold rise of antibody titre in 77 samples, as compared with the acute sera. Only 37 convalescent sera produced group B flavivirus reaction and had a very low titre of antibody to JE in them, indicate their exposure to JE elsewhere in the remote past (Table 4). The activity of JE in this district has already been established [36]. So on the basis of the house hold condition, epidemiological information, detection of IgM antibody to Dengue virus in the acute sera and rise of dengue antibody titre in the convalescent sera confirms the etiologic agent of the febrile illness was dengue virus. It is worthy to mention that the selected acute sera/samples did not produce CPE in the tissue culture system and no virus could be isolated due to the fact that, the initiation of antibody (IgM) response mounted the activity of the virus during the course of infection at the late stage. On the other hand, samples might have lost the viability of the Dengue virus during transport and repeated freeze thawing conditions, as this may led to lose of viral titre or RNA, making it difficult to isolate the virus from the selected samples.

This scenario on the outbreak of dengue in three years at intervals, in the district of West Midnapore, happens to be the same as it has taken places in different cities in India. More over many of the ecological conditions in this area do not differ substantially from those where the disease has been prevalent. However, Dengue infections have been recorded mainly from the urban areas of the countries, all over the world. Due to the rapid and increased urbanization, Dengue has spread all over the world only in the urban area. But the reports of DF and DHF from the rural area either absent or scanty. This is an important observation that DF and DHF have first emerged in the rural area as the major public health problem in the district of West Midnapore, West Bengal, India; that needs to be taken notice of. Hence, it constitutes a new report from a rural and coastal district like West Midnapore, West Bengal; which needs continuous surveillance and molecular studies on the circulating serotypes and their genotypes for addressing the probabilities of DSS/DHF incidence in future.

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Competing interests:

The first two authors are Research Fellows at the ICMR Virus Unit and the communicating author is an Assistant Research Officer of ICMR Virus Unit. The authors have no financial and competing interests that are affected by the material in the manuscript.
References


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|                       | Amongst The Common Fever Cases In West Bengal. |
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