DISCUSSION
6. DISCUSSION

6.1 Japanese Encephalitis Virus

JE has become a major public health problem in the state of West Bengal due to its complexity and lack of any specific treatment. In its first appearance it was a rural disease and appeared in the form of large epidemics at intervals. Now it has become endemic in many rural areas of West Bengal. 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 symptomatic treatment with no diagnostic facility and are not referred for further services.

In the present study, out of 828 samples tested, 291 samples (35.1%) were positive to JEV infection; of which 245 were positive by ELISA method and 46 were positive by RT-PCR method [table 1]. RT-PCR test was performed from the ELISA negative acute samples (upto < 3 days’ febrile illness) to ascertain the actual positive cases. That was the actual stage of viremia when viral titre was supposed to be high and viral RNA could be detected at that stage. In all the ELISA positive samples, patients had a history of illness for ≥10 days, indicative of active immune response at this stage of illness (Lewthwaite et al., 2010; Solomon et al., 1998).

Yearly distribution of JE cases from 2005-2011 showed that initially in the year 2005, JE cases were much higher, but suddenly it declined in the year 2006 [figure 3]. Again from the year 2007, it was again increasing. In comparison to the year 2010, although the percentage positivity of JE cases was lower in the year 2011, but the highest number of JE cases were detected in this year.
Discussion

Males were significantly (P<0.01) more affected than the females [figure 4, table 2]. These findings corroborates well with earlier studies (Swami et al., 2008). The reported JE cases represent the tip of iceberg in this state. The pediatric-adolescent age group (≤20yrs) were mainly affected by the JEV infection. The highest numbers of positive cases have been recorded in the age group of 11–20 years, followed by ≤10 years [table 2, Figure 5]. The main source of income in the rural area is mainly dependent on cultivation. People of the age group of 11-20 years usually take active part in crop field for the cultivation. The vector responsible for this disease usually breeds in the stagnant water in the paddy field and the majority from this age group gets exposed to the vector directly. The age group of ≤10 years were affected possibly due to the lack of immunity in them (Chatterjee et al., 2004). 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 the earlier vaccination, in them.

Effective vaccine against JEV is now available. Due to the widespread use of JE vaccine, JE cases have been declined in China, Korea, and Japan (Kabilan et al., 2004). 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 [figure 5]. In West Bengal, the vaccination programme was initiated in 2006 (PATH, 2009). Although in some districts of West Bengal like Burdwan, Birbhum, West Midnapore, 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 positive cases amongst the children and young adults (≤20 years) were found from those districts where the vaccination against G-III (SA14-14-2 strain) were done [table 3]. The recurrence of sporadic cases in every year even from the vaccine covered districts amply proves the silent activity of JEV in those districts.
Discussion

Moreover, in the year 2011 adults were almost equally affected by JEV infection in both vaccinated and non-vaccinated districts [table 3, figure 5]. The maximum numbers of positive cases were reported from the districts of Malda, Midnapore [East], Jalpaiguri, Nadia and Murshidabad, where no vaccination has yet been initiated [table 3, figure 6]. In 2011, JE cases were recorded from the district of Jalpaiguri and Coochbehar, which are located in the hilly and cold climatic regions [table 3]. These two districts are adjacent to the state of Assam, where an outbreak of JEV had been recorded in July 2011 (Report Times of India, July 29, 2012). Possibly, people of those two districts were infected by the vector mosquitoes that were migrated from the neighboring state.

During the study period, the interesting observation was made that, the majority of the positive cases, i.e., 163 out of 291(56%), had the history of no literacy and was found to be significantly higher (P < 0.05) than the 128 cases (44%), who belonged to the literate group [Table 4]. Moreover, within the literates, the lower educational level like primary, lower secondary and secondary constituted the maximum number (90.2%) of the positive cases while the higher educational level like higher secondary, graduate or post graduate professional group was least (9.8%) affected [figure 7, table 4].

This study also revealed that the majority of the positive cases, i.e., 189 out of 291 (65%) belonged to the low income group (income level ≤ 5000/ month) and was found to be significantly (P < 0.0001) higher than that of the high income group (income level > 5000/ month), consisting of 102 (35%) positive cases [Table 4]. Mostly this community of the rural population adopt and accept cultivation as the main source of income. To raise their economic status, they also built up piggery (Hurk et al., 2009) and minipoultry as an accelerated source of income in their own hut. The stagnant water of paddy field affords a very congenial home for breeding of Culex mosquitoes (Chakravarty et al., 1975) that act as a vector for JEV.
(Geevarghese et al., 2004). On the other hand, pigs, domestic birds like ducks, fouls which are known to be the favourable source for the maintenance of JEV in nature. Thus, this low income group become directly or indirectly exposed to JEV infection by the vector mosquitoes and became one of the important risk factors in relation to JE incidences. This observation tallied well with the earlier observation (Luo et al., 1995)

In this study, we observed that 183 JE positive cases (62.9%) were the habitat of the mud houses was significantly (P < 0.0001) higher than the 109 (37.1%) positive cases living in the brick houses [Table 4]. This observation suggests that the house type (i.e., made up of mud or brick) is another contextual risk factor in relation to the JE incidences. This factor very much depends on the economic status. The people living in brick houses belonged to high income group were less affected whereas the people belonged to the low income group, living in mud houses with unhygienic conditions like household crowding and lack of proper ventilation, were more susceptible to JEV infection. These seem to be the risk factor for acquiring JEV infection. This observation is in tune with earlier observations in other studies (Badari 1985).

It is well known that the *Culex* mosquitoes are the vectors that breed in rice field and birds are the reservoir for JEV transmission. The residence of the JE victim people being in the close proximity to the breeding site, *i.e.*, rice fields, lakes or ponds. They are more prone to the infection by the mosquitoes. Migratory birds, the reservoir of JEV, usually visit these places for food and the vector mosquitoes transmit this disease from birds to the human. It was observed that a total of 219 positive cases (75.3%) had their residences in the periphery of rice fields, lakes or ponds. These cases were significantly higher (P<0.0001) than those of the 72 positive cases (24.7%) who resides far away from the above mentioned places [Table 4].

-149-
In regard to the seasonal variations, monsoon and post-monsoon (autumn) period influence to accelerate JE incidences. Most of the JE positive cases, i.e., 257 out of 291 (88.3%) cases found in the monsoon and post-monsoon period, were significantly (P < 0.00001) higher than the cases, found in the pre-monsoon or other seasons (11.7%) [Table 4]. The monthly distribution of JE cases from 2005–2011 [Figure 8] reveals the highest number of JE cases was reported in the month of September, followed by October and November. These are at the end of the monsoon season with all the paddy fields covered with stagnant rain water for the need of the crops, which are preferred by the member of the *Culex* for breeding place (Chakravarty et al., 1975) and mosquito density begins to rise with extensive paddy plantation.

HAI study shows that the JEV is in circulation in the vaccinated districts of Birbhum, Burdwan and Hooghly, where only a negligible portion of the children population (≤10 years) had the JE antibody, while the rest of that age group had no antibodies to JEV [table 5, figure 9]. This population are highly susceptible to JEV infection and at any moment JE outbreak with high mortality can take place. This is supported by the evidence of JEV circulation in those areas as most of the age group contained JE antibody in them through sub-clinical infection. Although the vaccination programme against JEV took place in the district of Burdwan, Birbhum and Hooghly in the year 2006, 2007 and 2009 respectively, the detection of HAI antibody level in those districts are not up to the mark and were not giving abundantly to cover the total population against the JEV infection.

Genetic variation among JEV strains isolated from widely different time periods and geographical regions has been reported in several studies. Complete genome sequences (verified) of only five isolates from India are available in GenBank, of which one is from equine [table 6]. We exposed the full-genome sequences of two JEV isolates. The IND-WB-JE1 strain was isolated from the district of Malda, where no vaccination has yet been done, and
the other one, IND-WB-JE2 isolate was achieved in the year 2010 from a male patient of 17 years of age; reside at the district of Birbhum, where vaccination were done in the year 2007. The homology of the nucleotide and amino acid sequences were precisely compared with other JEV strains, particularly with other Indian isolates [table 7]. Phylogenetic analysis using 10915 bp full-length coding region nucleotide sequences of the two isolates from West Bengal i.e. IND-WB-JE1 (GenBank: JX050179) and IND-WB-JE2 (GenBank: JX072965) indicated almost close clustering of the West Bengal isolates in GIII [Figure 12]. Phylogenetically these isolates showed maximum homology (99% nucleotide similarity) with the first isolated strain of India i.e. Vellore P20778 (GenBank: AF080251), obtained from a patient from Vellore (south India), during 1958 and with H225 strain (GenBank: JX131374), isolated in the year 2009 from a horse in Haryana (North India). This result is quite surprising since the two West Bengal strains and H225 strain were isolated from geographically distant locations at a time gap of more than 50 years. The Vellore P20778 strain is also related to Beijing 1 strain (GenBank accession No. L48961), which was isolated from a human brain in Beijing, China in the year 1949. A detail evolutionary trend could not be studied due to insufficient recorded data on whole genome sequences from other states in India.

The predicted amino acid substitutions were compared between the two West Bengal isolates and other Indian isolates, vaccine strain SA-14-14-2 [table 7]. Some non-synonymous nucleotide changes were found in the protein coding region of the two West Bengal isolates in comparison with the Vellore P20778 strain. In IND-WB-JE2 isolate, a non-synonymous unique mutation G9645T was found in the NS5 gene, which corroborates A688S change in the polypeptide. Nonstructural protein NS5 is one of the major components of viral RNA replicase complex (Lin et al., 2006). NS5 contains sequences homologous to methyltransferase, involve
in methylation of the 5’ RNA cap structure and RNA-dependent RNA polymerase, which is the key enzyme for viral replication.

In West Bengal, JE is still remaining as a public health threat. JEV had been detected in paediatric-adolescent group people, resided in the vaccinated districts. This might be due to partial vaccination in those districts or the current circulating strain is ignoring the vaccine, with some crucial mutation in its viral genome or some new genotype is being introduced. The later possibilities could not be ignored, as from 2010 onwards, a good number of cases were also been recorded among adults and from non-vaccinated districts. In the year 2010, GI strain of JEV was isolated from Gorakhpur, which is a JE endemic region of the state Uttar Pradesh, India (Fulmali et al., 2011). Previously GIII was in circulation in that area. It is worthy to mention that, very recently in our laboratory we have also achieved only two isolates from a non-vaccinated district East Midnapore, which belonged to GI (Annexure II; Published paper 5). Although this vaccine conferred $\geq 70\%$ and $\geq 60\%$ protection following intraperitoneal (i.p.) and intracerebral (i.c.) injection to mice, but still some people could escape the protection (Liu et al., 2011).

We have genetically characterized the West Bengal JEV strains by analysis of its complete nucleotide and deduced amino acid sequences and comparison of the majority of the available fully sequenced JEV genomes. This study, therefore, constitutes the first report on complete gene based phylogenetic analysis of JEV isolates from AES cases of West Bengal.

From this study, it is evident that, JEV is still under circulation in almost all the districts of West Bengal. To combat the JE disease successfully and to reduce the mortality rate from the lower age group, vaccination in the total population is required. Continuous monitoring on the circulating strain is also required in the state of West Bengal. Otherwise, the changing trend of the circulating strain will create devastating public health problem in near future.
6.2. Chikungunya Virus

6.2.1 Monotypic infection of CHIKV

In India, the first epidemic due to CHIKV infection was detected and identified in the city of Kolkata in 1963, which persisted up to 1965. Since then, no cases of chikungunya were detected in Kolkata or West Bengal till the year 2006. In 1995, a serological survey conducted on the population of Kolkata revealed the presence of a negligible percentage of antibodies against CHIKV, predominantly in the age group ≥50 years, indicating infection in the remote past and the disappearance of CHIKV from the Kolkata population (Hati et al., 2009). In the year 2006, the first outbreak that occurred at Baduria (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 the districts of the South Bengal region were affected by CHIKV infections by the year 2008 [table 9]. In 2009, CHIKV-positive cases were recorded from North Bengal, i.e. from Malda and Dakshin Dinajpur [table 9]. In the year 2011, CHIKV infection was also recorded from the district of Jalpaiguri, located at the northern region of this state. This observation indicates that since the re-emergence of CHIKV in West Bengal in 2006, CHIKV-positive cases have significantly increased from 7.91% to 47.1% during the 6 years of the study [table 8].

Although all age groups were affected by CHIKV, slightly higher numbers of positive cases were observed in the age group of 31–40 years (28.1%) followed by 41–50 years (20.5%) [Table 10]. Similar studies in other parts of India also report the re-emergence of CHIKV and the most affected age group in these studies tallies well with our observations (Dwibedi et al., 2011; Dutta et al., 2011). Females were more affected than the males [figure 16] as they reside
in the house at daytime and may get exposed to the vector *Aedes* sp., which is domestic, and peridomestic in nature and a day-biter.

Cumulative reports for the 6 years indicate the highest number of CHIKV-positive cases in the month of November followed by October [Figure 17], which is the post-monsoon period of this state and the vector density is very high at this time. Similar observation was also reported from other parts of India (Dwibedi et al., 2011).

Phylogenetic analysis showed all the West Bengal isolates, achieved during the year 2006-2011 belonging to the ECSA genotype [Figure 20]. The IND-11-WBST1, IND-11-WBST2 and IND-11-WBST3 isolates, achieved in the year 2011, form a separate subcluster. These three isolates contained both E1-226A and E1-K211E mutations [table 12], which are highly conserved in the CHIKV strains, circulated in the Asian urban areas where *Aedes aegypti* were the natural vectors for transmission of this strain (Sumathy and Ella, 2012). In India, both of these mutation was first reported in late 2009 in the isolate AP0109 (GenBank: HM159390) from Hyderabad, South India and was observed in all the 2010 isolates from the neighboring state Tamil Nadu (Sumathy and Ella, 2012). The E1-K211E mutation was also observed in France in the year 2010, which was isolated from a person who travelled Rajasthan, India and received the infection there (Sumathy and Ella, 2012). The IND-11-WBST1-3 isolates were isolated in the year 2011 from Kolkata, which is one of the metropolitan cities of India. The remaining 13 strains, which contained A226V mutation, were isolated from rural/semi urban areas of West Bengal between the year 2006 and 2010, where *Aedes albopictus* might be the vector, as this vector can survive in both rural and urban environments (Pialoux et al., 2007).

In the E2 region, G60D and I211T mutation have important role on the infectivity of the CHIKV in a specific vector (Tsetsarkin et al., 2009). The E2-I211T mutation in CHIKV
can modify the effect of the E1-A226V mutation in *Aedes albopictus*, but cannot influence the virus infectivity for *Aedes aegypti*, whereas, the E2-G60D mutation is a determinant of CHIKV infectivity for both *Aedes albopictus* and *Aedes aegypti*, but only moderately regulate the effect of the E1-A226V mutation in *Aedes albopictus* (Sumathy and Ella, 2012). The E2-G60D and E2-I211T mutation together can increase the infectivity of the CHIKV with A226V mutation in *Aedes albopictus*. Both of these mutations were found in all the isolates of West Bengal. The IND-11-WBST1, IND-11-WBST2 and IND-11-WBST3 isolates also contained alanine residue at position 264 of E2 gene. Both E1-K211V and E2-V264A variations were first reported from France in September 2010 (Grandadam et al., 2011). This was an autochthonous imported case from India. In India, the same was reported from Tamil Nadu, Andhra Pradesh and Delhi in 2010 (Shrinet et al., 2012).

In the E3 region of the IND-11-WBST1, IND-11-WBST2 and IND-11-WBST3 isolates contained E3-V303I and E3-P320S mutation, which were not reported earlier. The study revealed that two sub lineages of ECSA genotype of CHIKV is circulating in the state of West Bengal. It might be due to the presence of abundant population of both *Aedes albopictus* and *Aedes aegypti* in this state.

In West Bengal, due to the lack of any immunity against CHIKV, the virus has spread rapidly in this state and affected the population irrespective of age group (Taraphdar et al., 2012d). This spread has been supported by the presence of abundant *Aedes sp.* population, which are already responsible for dengue endemicity of this state (Sarkar et al., 2010; Taraphdar et al., 2010; published paper 9 and 10 at annexure II). Since its resurgence, the circulating mutated strain is maintaining the same genetic pattern in every year till 2010. But in 2011, it has been observed that another strain is circulating in this state, which also affected a large number of people in one year only. This might have been introduced in this state by the
Discussion

infected travellers, as the city of Kolkata has both domestic and international connections via
airport, seaport and rail. Although the presence of two sub lineages of ECSA strains have been
reported from south India, but this constitute a new report from this state as well as from the
Eastern part of India.

Continuous monitoring on the circulating strains with detail entomological study is
required to understand the epidemiology of the virus in this region and to draw any conclusion
on vector transmission of the disease.

6.2.2 Dual infection study

West Bengal is a well known endemic zone for DENV and many epidemics have been
documented from this state (Sarkar et al., 2010; Taraphdar et al., 2010). 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
after that CHIKV re-emerged at Baduria, North 24 Parganas in the year 2006 (Taraphdar et al.,
2012d). 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
24) (Taraphdar et al., 2012d; published paper 2 in annexure II). From the calculated P-values,
it can be concluded that during 2006–2011 the total number of chikungunya cases was
significantly higher than the total dengue cases [Table 14].

During the year 2010-2011, a total of 115 patients were affected by both CHIKV and
DENV infection (Table 15; published paper 1 in annexure II). Out of 115 dual-infected cases,
only 11 patients (9.57%) were <16 years of age [Table 16]. The highest number of co-infected
Discussion

cases was found in the age group of 31–40 years (31.3%) [Figure 22]. The female/male ratio was 1.8:1, which is significantly high (P = 0.03). Like the monotypic CHIKV infection, here also the females were much more affected than males [table 16] because they reside in the house at daytime and might have exposed to the vector *Aedes* sp., which is domestic in nature, a day biter and the vector for both DENV and CHIKV (Saxena et al., 2006; Kannan et al., 2009).

It has been observed that people in the urban/semi-urban areas were more affected by both monotypic and dual infection [table 16]. During the rainy seasons (June to September) stagnant fresh water favoured 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 [figure 23].

District wise distribution of dual infected cases showed that four districts of West Bengal i.e. Kolkata, Nadia, North 24 parganas and South 24 parganas were affected by both the viruses [figure 24]. These four districts were also predominantly affected by monotypic infection of CHIKV [figure 15].

In case of dual infection, ELISA result revealed that the OD value of the Chikungunya IgM antibody was at least four times higher than the OD value of the dengue IgM antibody. This result amply justifies that the CHIKV infection took place in the later phase of biphasic manifestation of fever, which was found in all the dual infected cases [table 17] (Taraphdar et al., 2012a; published paper 1 in annexure II). Swelling of joints and severe arthralgia were the common symptoms in the case of CHIKV infection, but was rare among the dual-infected patients. Most of the cases with only DENV infection were associated with abdominal pain, which was present in only three cases with dual infection by both CHIKV and DENV. Diarrhoea was reported only by the dual-infected patients (21.7%). All the dual infected
patients recovered quickly. The striking observation among the dual infected cases was that no hemorrhagic manifestation was observed among them.

Although both CHIKV and DENV individually affected a large number of people of this state, but after 1965, the dual infection caused by both viruses was recorded after 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 (Kumar et al., 2008). *Aedes aegypti* are predominated mainly in the urban areas, whereas *Ae. albopictus* can survive in both rural and urban environments (Pialoux et al., 2007). The vectors can carry both of the viruses, 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 areas and raised the vector densities.

### 6.2.3 CNS involvement in CHIKV infection

In India, the first large series of neurological complications like encephalitis, myopathy, neuropathy, myelopathy and myeloneuropathy due to CHIKV infection was reported from Maharashtra in 2006 (Chandak et al., 2010). In the same year, neurological complications like altered level of consciousness in the form of confusion, disorientation, drowsiness and delirium were observed amongst the hospitalized patients in Kota, India (Rampal et al., 2007), where 1/3rd of the Chikungunya affected patients had the complications at the CNS levels in the form of encephalitis, encephalomyelitis and optic neuritis and tolled some lives. Acute CNS infections in children with febrile illness (<2 weeks’ duration), altered mental status and seizures were observed at Bellary (Lewthwaite et al., 2009). In Andaman and Nicobar Island, four cases of acute flaccid paralysis due to CHIKV were reported (Singh et al., 2008).
In West Bengal, India, although the virus has become endemic in nature since its resurgence in the year 2006, but no Chikungunya cases has yet been reported with neurological complications till 2010. While studying the involvement of Japanese Encephalitis Virus (JEV) in the acute encephalitic syndrome (AES) cases, we made attempt to detect CHIKV infection in the JE negative AES cases. Only four cases had the CHIKV infection in them. All these cases were confirmed by ELISA and RTPCR method. In all four cases CSF report showed lymphocyte pleocytosis with moderately raised CSF protein (range 66-145 mg/dl) which indicated the cases of viral encephalitis [table 18]. Here the case--1 and case--2 had the acute encephalitis syndrome; case--3 had acute disseminated encephalomyelitis whereas the case—4 had the symptoms of meningo encephalopathy with bulbar involvement. In the case--3, both the blood and CSF sample was RTPCR negative [table 19]. It may be explained by the fact that the viral RNA can be detected within the viremia stage which persists in the blood and CSF from 2—10 days (Kam et al., 2009). But exclusively the sample of the third case was detected on 18th day of illness when the IgM antibody has already ushered in the blood and CSF and neutralized the viral particles. So it could not be detected by RTPCR method.

In all these four cases, CHIKV was detected as the etiologic agent for CNS manifestation from this state for the first time. The re-emerging CHIKV is of central-east African origin, replaced the previous Asian genotype from this country (Pialoux et al., 2007). It is remain unclear whether the severity of the disease is due to the lack of immunity in the population or due to the changing genotype. The rapid spread of CHIKV has become a problem from the public health point of view. These works only report referred cases and represent the tip of iceberg of the infected population. Total cases are much higher than those dealt with.