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ETIOLOGICAL SPECTRUM OF CO-CIRCULATING JAPANESE ENCEPHALITIS VIRUS GENOTYPE I AND III IN CLINICALLY DIAGNOSED AES CASES FROM WEST BENGAL, INDIA: AN INDICATION OF PUBLIC HEALTH THREAT IN NEAR FUTURE

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ABSTRACT

Japanese encephalitis virus, a mosquito-borne zoonotic pathogen, is the sole etiologic agent of Japanese Encephalitis; a neurotrophic killer disease is counted for one of the major causes of viral encephalitis worldwide as well as in our country including West Bengal and is also considered as a prime issue on public health concern. JE was first recorded in the state of West Bengal, India in 1973. Since then it is being reported every year from different districts of this state, though the vaccination has already being done. Moreover, the reports of JE incidences in this state indicate that there might be either partial coverage of the vaccine or the emergence of mutated/new strain of JEV, considering this fact, in order to identify the patterns of genetic change of JEV isolates circulating in West Bengal, the complete envelope (E) gene has been sequenced for 4 isolates (Malda, Nadia, Burdwan, Midnapore) from patients clinically diagnosed as Acute Encephalitis Syndrome (AES) cases, we performed insilico analyses on the sequences of envelop protein of those isolates were very demanding for understanding their molecular phylogeny, natural amino acid substitutions and their possible role in cellular function as well as sequence properties alteration in relation to protein conformation with respect to disease severity. This study demonstrated that homology modelling revealed that at least 6 out of 21 of the identified mutations fall into functionally significant domain of the viral E protein and are predicted to affect protein structure in response to disease severity in respective patients. In addition, it was assumed that the 15 amino acid substitutions on the stem and the transmembrane region of that protein of the Malda and Nadia isolates were responsible for neurological complications. Regarding molecular phylogeny, we observed that both genotype I and III co-circulates in this state; in near future, there might be a chance of JEV outbreak in this region/state.

INTRODUCTION

The flaviviruses persist as causative agents for a wide range of human related infectious diseases worldwide. However, the most common causes of AES is associated with the Japanese encephalitis group viruses which include Japanese encephalitis virus (JEV), Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV) and West Nile virus (WNV) within the *Flavivirus* genus under the family Flaviviridae[1,2]. The mosquito-born JEV is the sole etiologic agent of Japanese Encephalitis (JE), a neurotrophic killer disease and one of the causes of acute encephalopathy, affecting children and adolescents[3]. 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 young children (aged 2-15 years),survives with persistent neurological deficit and /or psychological sequelae[4-6].

In a zoonotic cycle, JEV is transmitted by mosquito (*Culex* sp.) vectors[7] between wild/domestic birds acting as reservoir host[8] and pigs acting as amplifying host[9]. Pig-mosquito-pig and bird-mosquito-bird cycle is responsible for the maintenance of the virus in nature. Man is the 'dead end' host[10, 11].

Like other flaviviruses, JEV, a positive stranded RNA (~ 11kb in length) virus contains single open reading frame (ORF) encoding a polyprotein that is processed into three structural (C, M, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins, flanked by 5'- and 3'-non-translated regions (NTRs)[12-14]. Among the three structural proteins, the envelope (E) protein is considered as the most antigenic part of the viral genome and is found to be involved in the majority of the biological properties of the virus, such as binding to the cell receptor, inducing immunological responses (neutralization, passive protection and antibody dependent enhancement), virion assembly and fusion activity at low pH[15, 16].

Since the isolation of this virus in Japan in 1935, it has spread all over the world including India[17] and has become a major public health problem.

JEV has been subdivided in five genotypes,[18-21] among them, genotype III is mostly circulated in the Southeast Asian countries[1]. However, it was recently documented that genotype III is replaced by genotype I in South Korea, Thailand and China[22].

In India, the existence of JEV was first reported serologically in 1954[23]. However, the disease was first recognized in India at Vellore in 1955[24]. Since then, epidemics of JE in different states have been recorded[25, 26]. It was mentioned that genotype III is predominant in India[1, 21,

[27], but recently genotype I has been introduced in this country[28]. 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[29-31]. Since then many out breaks have been reported[32-34]. In spite of vaccination programme undertaken against JE in some districts of West Bengal, every year sporadic cases are continuously being reported, which indicates the endemicity of JE in this state[3]. Moreover, the reports of JE incidences in this state indicate that there might be either partial coverage of the vaccine or the emergence of mutated/new strain of JEV.

In the present study, we aimed to detect the JEV infections amongst the patients admitted in the hospitals with AES and also to isolate its etiologic agent. Attempts were also made to work out an exhaustive phylogenetic analysis for the determination of the genotype of our isolates, followed by molecular characterization with detailed structural analysis of the isolates using E gene sequence based information from a wide range of geographically and temporally diverse, best representatives of JEV strains and also to analyze the impact of amino acid substitutions on envelope protein structure in relation to disease severity.

MATERIALS AND METHODS

Patients and clinical specimens

During the study period of 2005-2010, a total of 648 blood/CSF samples were referred and/or collected from the clinically suspected patients with AES. These patients had the history of high grade fever (≥39°c) for 2-15 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 etc. All these cases were admitted in different district hospitals, two medical colleges and ID & BG hospital in Kolkata, West Bengal. Standard ethical guidelines were followed during the blood/CSF collection. Details of the clinical event, investigations, treatment given and the prognosis of the patients during the 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 cases had moderately high sugar level (45-65mg/dL), slightly higher protein level varied from 50-70 mg/dL, and the WBC count was ≥ $6x10^6$/L. Cerebral malaria as well as bacteriological etiology was ruled out by the concerned hospitals. All these Samples were transported to the ICMR (Indian Council of Medical Research) Virus Unit, Kolkata, maintaining the cold chain. Sera were separated from the clotted blood samples and both the serum and CSF were stored at -80°c till tested.

In this study, out of 648 samples, only 4 blood/CSF samples had the history of fever for ≤ 3 days and developed typical JE like illness, were referred/collected from the 4 different district hospitals of Burdwan, Malda, Midnapore and Nadia in West Bengal (see Table 1). Of which one patient died on the 15th day of illness, two patients had been suffering from neurological sequelae and only one patient completely recovered (see Table 1). Therefore, considering these different clinical complications, these 4 samples were emphasized more and critically analyzed for the detection of mutational changes, if any or the emergence of new genotype, followed by analyzing the impact of amino acid substitutions (if any) on envelope protein structure in relation to disease severity.

Serology

For the detection of IgM antibody to JE, ELISA test was performed with the kit[35], purchased from National Institute of Virology (NIV), Pune; following the prescribed protocol. O.D was measured at 450 nm using an ELISA reader (Titertek Multiskan Plus, Lab systems Finland, Type- 314).

Virus strain

JEV P20778 strain (GenBank Accession No. AF080251) was obtained from National Institute of Virology (NIV), Pune. The lyophilized vial of the virus was reconstituted with sterile DNase-RNase free MiliQ water and was kept in aliquots at -80°c till the isolation of RNA and used as a positive control throughout the study.

Isolation of virus from samples

Attempts were made to isolate the virus from the samples, 200µl of 4 selected serum/CSF samples with a history of ≤3 days (acute phase) fever, were spread over the monolayer of the *Aedes albopictus* C6/36 mosquito cell lines (obtained from NCCS, Pune) and allowed to adsorb for 120 minutes in a 6 well tissue culture plate (Nunc, Roskilde, Denmark) with the incubation at 28°C under 5% CO_2 concentration. After adsorption, the excess sample materials were discarded and the wells were washed with 1X phosphate buffer saline (PBS; GIBCO BRL-Invitrogen, Grand Island, NY, US), followed by addition of 2ml Eagle's minimum essential media (MEM; GIBCO BRL-Invitrogen, Grand Island, NY, US) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL-Invitrogen, Grand Island, NY, US) and Penicillin Streptomycin antibiotic solution (PenStrep; GIBCO BRL-Invitrogen, Grand Island, NY, US) and were incubated again in the same condition as before, for growth of the virus, if any. It was observed regularly for the appearance of cytopathic effect (CPE)

up to 7-8 days. After the appearance of CPE up to the fifth successive passage, the tissue culture fluids were collected, followed by centrifugation at 1000×g for 5 minutes and the supernatants were kept in aliquots at -80°c till isolation/extraction of RNA, followed by RT-PCR test. Non-infected C6/36 cell culture was used as a negative control.

RNA extraction, RT-PCR and Nucleotide sequencing

RNA was extracted from infected tissue culture fluid and from JEV P20778 strain, reconstituted with sterile DNase-RNase free MiliQ water by using QIAamp RNA viral kit (Qiagen, GmbH, Hilden**,** Germany), following the manufacturer's protocol.

For the identification of the isolates as JEV, RT-PCR was carried out with the extracted RNA from the infected tissue culture fluid of 4 screened samples along with positive control strain. Qiagen one step RT-PCR kit (Qiagen, GmbH, Hilden**,** Germany) was used according to the manufacture's protocol, using RNA (50 pg to 1µg) and 0.6 µM of primer pairs (3); forward primer: JEnvF (w) 942-ACCATCCTCCTGCTGTTGGTCGCT-965 and reverse primer: JEnvR (w) 2506-CTTGTGATGTCAATGGCACATCCAGTGTCA-2477 which specifically anneal to the conserved region of structural envelop protein (E) of Japanese Encephalitis virus. 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 manufacturing protocol. Approximately 30-60 ng of purified cDNA templates were used in direct cycle sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Products were purified by ethanol precipitation, according to the manufacturer's instructions, and analyzed using an automated DNA sequencer, 3130XL Genetic Analyzer (PE Applied Biosystems, Foster city, CA, USA). The freely available Finch TV software (http://www.geospiza.com) was used to edit and correct the sequences.

Data set and FASTA file

Nucleotide sequence of complete envelope gene (1500 nucleotides) of our four isolates was converted into amino acid sequences using Transeq (www.ebi.ac.uk/Tools/emboss/transeq/). The searches of the SwissProt database (www.expasy.org/sprot/) were carried out with the program BLASTP 2.2.17[36] using one of the amino acid sequences of JEV isolates. Output file thus produced, contained 641 candidate sequences of JEV which fell in 40 different BLASTP 2.2.17 score classes. From these score classes, 19 SwissProt IDs (JEV-19, Table 2), having

high amino acid sequence score and structural annotations that cover all five genotypes from different geographical locations and sources and across a number of years, were carefully chosen for the preparation of a raw FASTA file (A1-file, data not shown) including Isolate sequences (ISO-4, Table 2). A1-file was then used to construct a Block FASTA file (data not shown). To obtain reliable aligned region for 500 amino acids homologous positions of JEVs E protein, multiple sequence alignment (MSA) was performed at EBI (http://www.ebi.ac.uk/) using ClustalW2 programme (www.ebi.ac.uk/Tools/msa/clustalw2/) and Output file thus obtained, was manually processed to remove all INDEL positions for the preparation of a new Block FASTA file (B1-file, data not shown) which was used for hydropathy profile and E Protein MSA evaluation (see below).

Moreover, the nucleotide sequences of 4 isolates (ISO-4, Table 2), 17 JEVs nucleotide sequences (JEV-11+ JEV-6, Table 2) out of 19 pre-selected JEVs (JEV-19, Table 2) for amino acid alignment and a nucleotide sequence of out group (OG-1, Table 2) were chosen for the preparation of a raw FASTA file (C1-file, data not shown). This C1-file was then used to construct a block FASTA file (data not shown), followed by MSA that was performed at EBI (http://www.ebi.ac.uk/) using ClustalW2 programme (www.ebi.ac.uk/Tools/msa/clustalw2/) and Output file thus obtained, was manually processed to remove all INDEL positions for the preparation of a new Block FASTA file (D1-file, data not shown) which retained only 1500 nucleotides homologous positions for E gene and was used for phylogenetic tree construction.

Multiple sequence alignment for JEV sequence block

A short listed form of B1-file was used for the preparation of MSA (see supplemental material) that contained only 13 out of 19 sequences of JEVs (JEV-11+ JEV-2, Table 2) along with 4 isolate sequences (ISO-4, Table 2) and this MSA was carried out using ClustalW programme of MEGA version 4.0 software[37]. This MSA was used to assess amino acids substitution positions of our 4 isolates with respect to 13 JEV strains. Relevant structural and sequence annotation was incorporated in the MSA (see supplemental material), based on crystallographic structural analysis of E protein of JEV (3P54. pdb) and WNV[38].

Hydropathy profile JEV isolates

B1-file was used for the evaluation of hydropathy profile where each JEV sequence was determined separately in web based STRAP programme (http://www.bioinformatics.org/strap/) using Kite and Doolittle[39] scale of amino acids with a

window size of 11 and the average hydropathy profile for 19 JEV sequences was computed using MS Excel programme. This average hydropathy was compared with isolates individual profiles. Only the differential regions were plotted using sigma plot software (Systat Software, Inc., San Jose, CA, USA) and the final graph was generated by applying a mild smoothening function. See Figure 3 (A), (B), (C).

Phylogenetic analysis

The phylogenetic tree was constructed by Neighbor-Joining (NJ) method[40], tested with Kimura 2-parameter model and other default parameters, using D1-file as input in MEGA version 4.0 software[37]. Bootstrap probability value for each node was calculated using 1000 pseudo replicates and a value greater than or equal to 50% was shown above the corresponding nodes.

RESULTS

Serology, Tissue culture and RT-PCR amplification

All the 4 screened acute samples were non reactive to JE IgM antibody by ELISA method (Table 1) and produced well defined CPE in the C6/36 cell line, characterized by cell rounding followed by early detachment of cells in comparisons with Non-infected C6/36 cell culture which was used as a negative control. All the 4 tissue culture isolates from 4 screened acute samples (Table 1) considered as JE positive, produced prominent band at 1.5 Kb in 1% Agarose gel and stained with ethidium bromide (Figure 1).

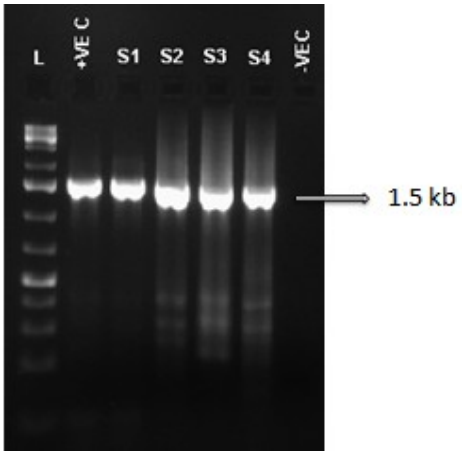

Figure1. Results of JEV specific RT-PCR showing band at 1.5kb (arrow head) in 1% agarose gel electrophoresis, obtained after the amplification of E gene of JEV from the prominent CPE producing tissue culture fluid of 4 isolates from blood/CSF samples. Lane S1-S4: JEV specific band of tissue culture fluid of 4 isolates, Lane +VE C: Positive control Lane –VE C: Negative control Lane, L: 1 kb plus DNA ladder

Table1. Result of IgM RT-PCR and virus isolation to detect the evidence of JEV etiology in 4 different clinical samples from AES cases of 4 different patients of 4 different districts of West Bengal with short case history and their present conditions. Here, (Y) and (D) are abbreviated for year and day, respectively.

Isolate	Age (Y)	Sex	Date of onset	Clinical symptoms of AES cases	Samples collected on (D)	Sample type	Results of ELISA (IgM) & RT-PCR	Isolation	Present Condition of the patient
IND/08/WB/ Malda	12	Male	24/10/08	High fever, unconsciousness	Second	Blood	- VE +VE	Yes	Suffering from neurological sequelae
IND/09/WB/Nadia	13	Male	15/08/09	High fever, convulsion, Neck rigidity, altered sensorium	Third	Blood CSF	-VE -VE -VE +VE	Yes	Towards recovery, with presently loss of griping in both hands and generalized loss of memory
IND/09/WB/Burdwan	29	Male	18/09/09	High fever, vomiting, Neck rigidity, altered-sensorium, convulsion	Third	Blood CSF	-VE -VE -VE +VE	Yes	Recovered
IND/10/WB/JEV28	4	Female	19/11/10	High fever, Neck rigidity, altered sensorium, convulsion, Unconsciousness, muscle stiffness, frothing	Third	Blood CSF	-VE -VE -VE +VE	Yes	Expired on 04/12/10

Amino acid Sequence analysis of isolates with respect to representative JEV strains

Domain specific Amino acid substitutions patterns of our 4 isolates (Nadia: IND/09/WB/Nadia: GenBank Accession No. JN189782, Burdwan: IND/09/WB/Burdwan: GenBank Accession No. JN189784, Malda: IND/08/WB/Malda: JN189783 and Midnapore: IND/10/WB/JEV28: JN703381, Table 2) were worked out from multiple sequence alignment (see supplemental material) and presented in Table 3, along with the presentation of E protein crystal structure information as in Figure 2 (A) and (B).

Table2. Information of JEV strains, Isolates and MVEV (as out group) used in the present study. Strains were grouped based on the study items (see text). Genotype information of JEVs were taken from others work.

Group		Strain	Country & Year	Host/ Source	Genotype	Swiss Prot Accession no.	GenBank Accession no.
JEV-19	JEV-2	GZ04-36	China, 2004	Mosquito	III	A1XJG1 (E)	DQ404112 (E)
		WTP-70-22	Malaysia, 1970	Mosquito	II	P90207 (E)	U70421 (E)
	JEV-11	SA14	China, 1954	Mosquito	III	P27395	M55506
		SA14 -14 -2	China, IU	Vaccine	III	Q99DQ9	AF315119
		SC04-17	China, 2004	Mosquito	I	A1XJE2(E)	DQ404093 (E)
		Nakayama	Japan, 1935	Human brain	III	A5JUL9	EF571853
		Ishikawa	Japan, 1994	pig	I	Q9DT72	AB051292
		FU	Australia, 1995	Human serum	II	Q9IJX6	AF217620
		JKT9092	Indonesia, 1981	Mosquito	IV	P88884(E)	U70409(E)
		GP78	India, 1978	Human brain	III	O90297	AF075723
		P20778	India, 1958	Human	III	Q76SI0 (E)	U70415 (E)
		733913	India, 1973	Human	III	Q82930(E)	Z34095 (E)
		Muar	Malaysia, 1952	Human	V	F1B5H1	HM596272
	JEV- 6	DH20	Nepal, 1985	Clone	III	Q82862	U03690
		K96A07	Korea, 1996	Mosquito	I	E1U802(E)	FJ938219 (E)
		JKT5441	Indonesia, 1981	Mosquito	II	P90206 (E)	U70406 (E)
		JKT7003	Indonesia, 1981	Mosquito	IV	P88883 (E)	U70408 (E)
		XZ0934	China, 2009	Mosquito	V	-	JF915894
		691004	SriLanka,1969	Human blood	III	Q82932 (E)	Z34097 (E)
ISO-4		IND/08/WB/Malda	India, 2008	Human blood	III	-	JN189783 (E)
		IND/09/WB/Burdwan	India, 2009	Human CSF	III	-	JN189784 (E)
		IND/09/WB/Nadia	India, 2009	Human CSF	III	-	JN189782 (E)
		IND/10/WB/JEV28	India, 2010	Human CSF	I	-	JN703381 (E)
OG-1		MVEV MVE-1-51	Australia, 1951	Mosquito	OG	P05769	AF161266

Table 3. Missense mutations of amino acids in E Protein sequence of different isolates. The directions and types of substitutions of isolates were worked out from the MSA block of 13 JEVs' (JEV-2+JEV-11, Table 2) E protein consensus sequences generated by MEGA version 4.0 software. Different positions were categorized into domains based on the crystallographic structure of E Protein of JEV (3P54.pdb) and WNV (2HG0.pdb).

Isolates (Gene Bank Accession Number)	Domain region, sequence position																							Total substitutions
	DI		DII		DIII		ST				TM													
	2	158	103	222	261	263	410	411	413	416	437	463	476	477	493	494	495	497	498	499	500		
Malda (JN189783)	N→H	Q→P	-		G→S	-	-	T→S	L→W	G→V	R→K	-	-	-	-	-	-	-	-	-	-		**7**
Nadia (JN189782)	N→H	Q→P	-	-	G→S	-	-	-	-	-	-	I→R	L→R	R→L	D→Y	F→S	L→S	A→D	N→M	V→S	H→M	A→L	**14**
Burdwan (JN189784)	N→H	-	-	-	G→S	H→P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**3**
Midnapore (JN189785)	N→H	-	N→K	A→S	G→S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**4**

Here, DI, DII, DIII, ST and TM designated as Domain I, Domain II, Domain III, Stem and Transmembrane region of E protein sequence, respectively.

Figure2.

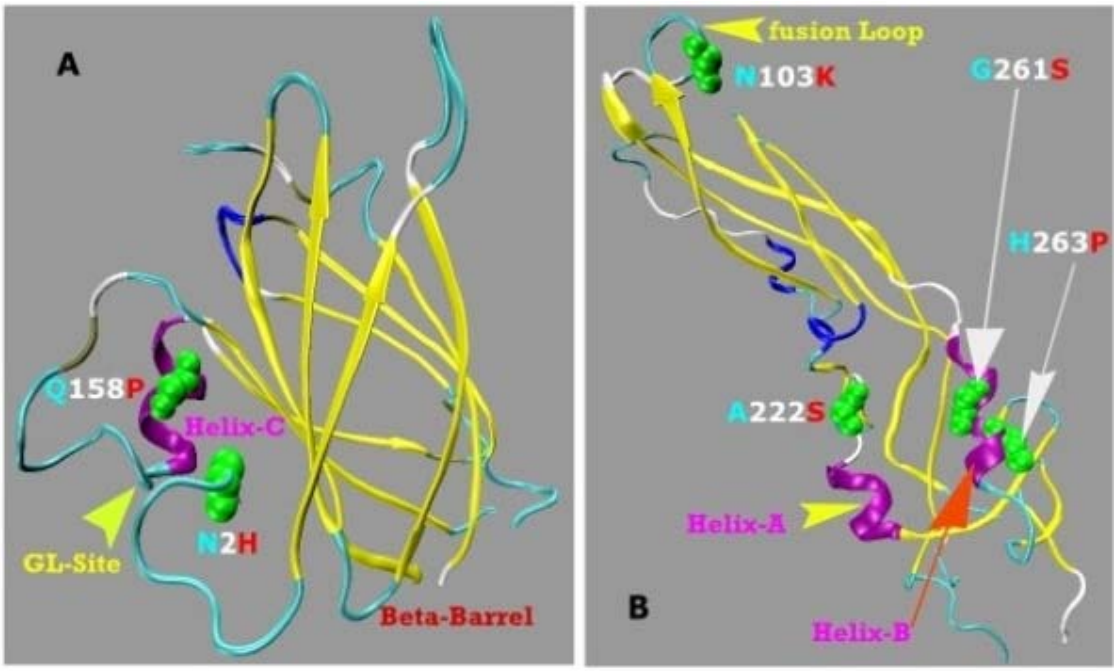

Figure2. Amino acid substitutions on Domain I (A) and Domain II (B) region of the E protein crystal structure derived/generated from JEV 3P54.pdb. Single letter code with Cyan color indicates wild type amino acid residue, red color indicates mutated/substituted amino acid residue. Critical regions such as helix C, glycosylation site, β- barrel were seen in Domain I and fusion loop; helix A and helix B were seen in Domain II.

Two kinds of amino acid substitutions were observed from the Table 3. One was common type and the other was isolate specific. These mutations were observed in the E protein structural domain I, II, stem anchor (ST) and transmembrane (TM) regions. No mutations were seen in the structural domain III. Common types of substitutions (N2H and G261S) were seen at the positions of E2 and E261 in domain I and II respectively. Isolate specific amino acid substitutions were found to vary in types, position as well as structural domain regions. A total of 11 amino acid substitutions were seen in case of Nadia isolate in both stem anchor and transmembrane regions whereas only stem anchor region specific mutations (total 4) were observed in case of Malda isolate (Table 3). Q158P substitution in domain II was seen in both of these isolates. Two unique substitutions, i.e., N103K in the fusion loop and A222S in domain II of the protein were seen in Midnapore isolate. But in case of Burdwan isolate, only one substitution was observed in domain II of the protein as H263P.

Additionally, in domain I, a total of two and in domain II, a total of four substitutions were observed for all isolates. Most of these substitutions were located in the helical or loop region of domains of the protein (Figure 2 A and B). None of the substitutions were observed in the domain III which is believed to be the most antigenic in nature in response to host cell[41]. On the Other hand, a total of five and ten substitutions occurred in the stem anchor and transmembrane region respectively, only in case of Malda and Nadia isolates (Table 3).

Effect of amino acid substitutions on hydropathy profile of isolates

Each isolate contained amino acids substitutions with respect to best representative JEVs that were used in this study. How did these point mutations change the code of the protein? These were assessed by comparing individual hydropathy of isolates with average hydropathy of other JEVs and the differential regions were shown in the Figure 3 (A), (B), (C). Other regions of the profiles were either identical or almost similar (data not shown) and hence not included in the Figure 3 (A), (B), (C).

Figure 3.

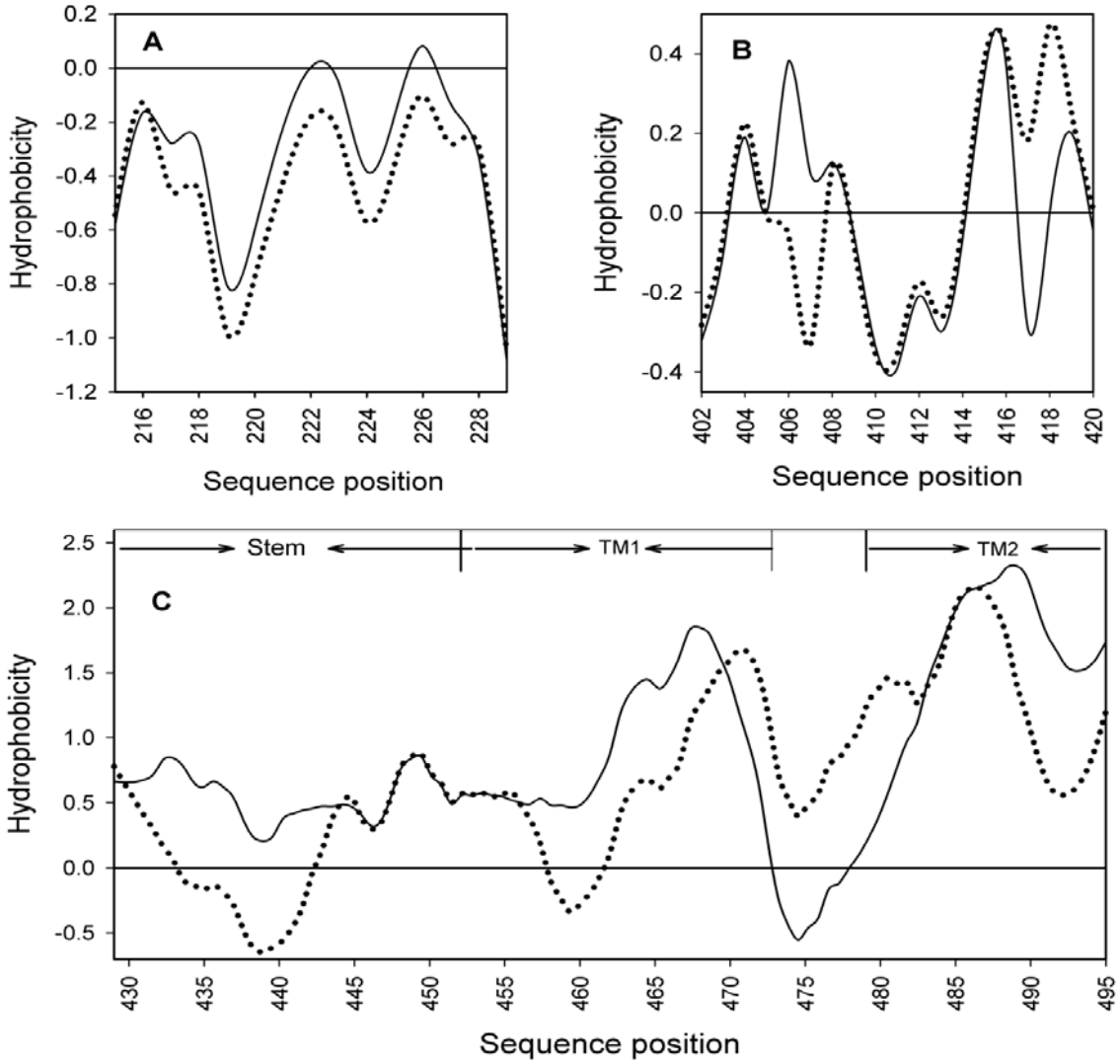

Figure3. Hydropathy profile for specific regions of E protein for Midnapore (A), Malda (B) and Nadia (C) isolates. Solid line indicates an average hydropathy of 19 JEV strains (JEV-19, Table 2) and the dotted line indicates the hydropathy of individual isolates.

In case of Midnapore isolate (GenBank Accession No. JN703381), a distinct change was observed in sequence hydropathy for the region of E215-E230 where it became more hydrophilic than the average hydropathy of the representative of other JEV strains (Figure 3 A). Similar sequence hydropathy property changes were observed in case of Malda (GenBank Accession No. JN189783) and Nadia isolate (GenBank Accession No. JN189782) for the region of E402-E420 (Figure 3 B) and E430-E495 (Figure 3 C) respectively. Malda

isolate has shown remarkable alteration of sequence hydropathy property for the region of E402-E420, wherein hydrophobic region has become hydrophilic and hydrophilic has become hydrophobic. In case of Nadia isolate, sequence hydropathy property has been changed for a wide range of sequence including stem anchor (ST) and transmembrane (TM) regions. Here, hydrophobic property has decreased drastically for ST and TM1 and TM2 regions and that for inter membrane region has increased.

In case of Burdwan isolate (GenBank Accession No. JN189784), no notable difference in hydropathy profile was observed with respect to average value of hydropathy profile of representative JEV strains (data not shown).

Molecular phylogeny of isolates

The phylogenetic tree for the E gene sequences of 4 JEV isolates with a group of 19 previously published geographically and temporally diverse JEV strain (including 3 from India and 16 from worldwide) was shown in Figure 4. The tree was rooted within the Japanese encephalitis serogroup by using Murray Valley virus strain MVE-1-5 (GenBank Accession No. AF161266) and contains 5 distinct clades corresponding to genotypes I-V. These Five distinct genotypes were seen as representative JEV strains that have been used in earlier works[18-21] along with the phylogenetic relationships of our 4 JEV isolates. According to dendrogram, following points were noteworthy to mention that-Firstly, a comparison of E gene nucleotide sequences of 4 isolates (Nadia: IND/09/WB/Nadia: GenBank Accession No. JN189782, Burdwan: IND/09/WB/Burdwan: GenBank Accession No. JN189784, Malda: IND/08/WB/Malda: JN189783, Midnapore: IND/10/WB/JEV28: JN703381) with other 19 JEV strains showed that 3 E gene sequences (GenBank Accession No. JN189782, JN189784, JN189783) belonged to GIII and single E gene sequence (GenBank Accession No. JN703381) belonged to GI. Secondly, these 3 E gene sequences were most similar (98%-99%) with the first Indian JEV strain P20778 (GenBank Accession No. U70415), followed by (96%-97%) similarity with Japanese Nakayama strain (GenBank Accession No. EF571853). On the other hand, the single GI E gene sequence showed 94%, 95% and 96% nucleotide similarity with 3 prototype JEV strains, namely, the Chinese strain SC04-17 (GenBank Accession No. DQ404093), Korean strain K96A07 (GenBank Accession No. FJ938219) and Japanese strain Ishikawa (GenBank Accession No. AB051292) respectively.

Figure 4.

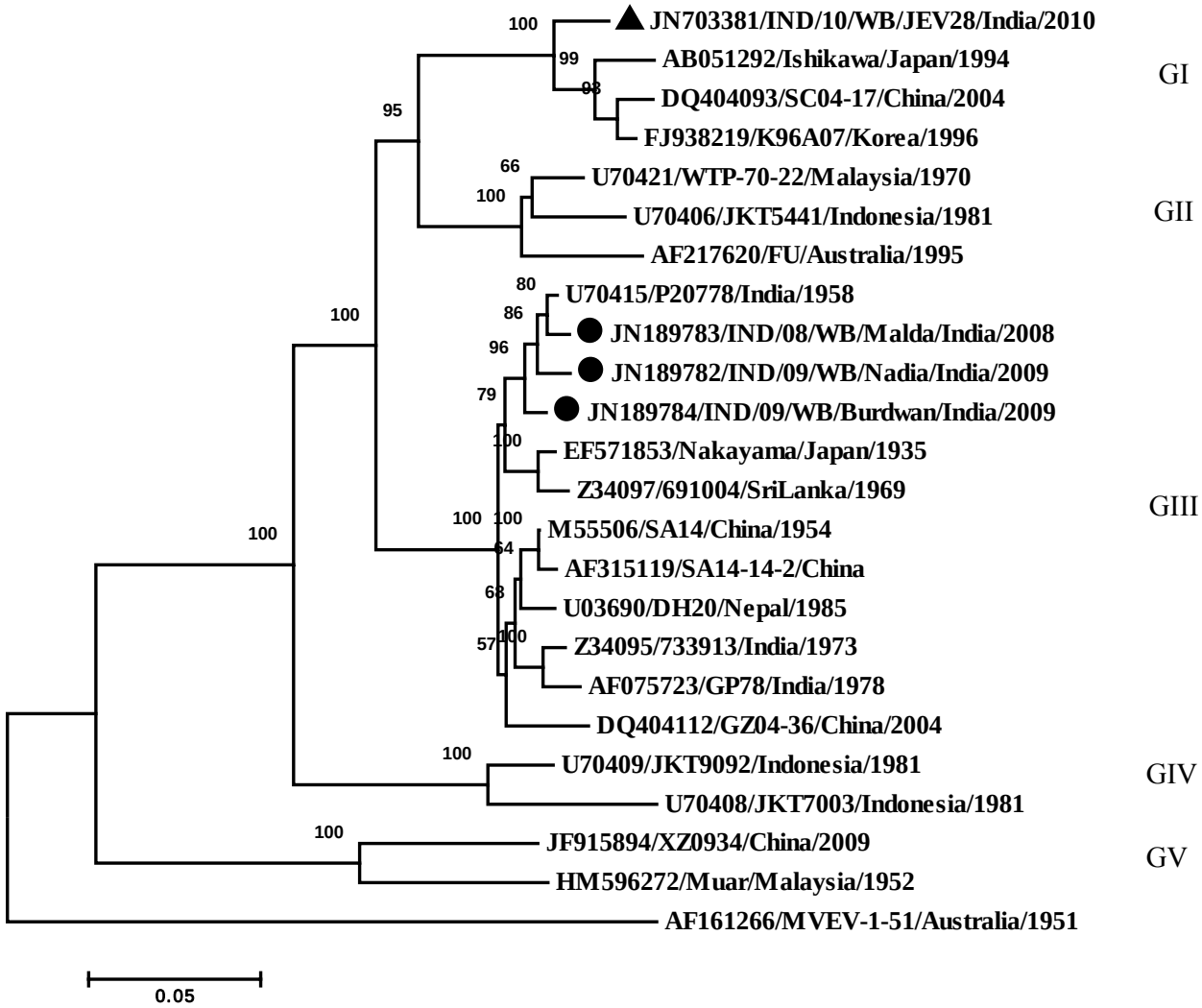

Figure4. Phylogenetic analysis based on complete E gene nucleotide sequences of 4 JEV isolates (ISO-4, Table 2) from 4 AES cases of 4 districts in West Bengal, with reference to other 19 JEV (JEV-19, Table 2) strains from worldwide. The Murray Valley Virus strain (MVE-1-51) was used as out group (OG-1, Table 2) for generating the rooted tree. The tree was generated by Neighbor-Joining (NJ) method, tested with Kimura 2-parameter model. The robustness of phylogram was evaluated by 1000 bootstrap pseudo replicates. Bootstrap values (≥50% of replicates) were shown in corresponding nodes. 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.

DISCUSSION

In West Bengal, JE is really a public health concern. Most of the JE cases remain undiagnosed as most of the infections are subclinical. It is a rural disease and the people who suffer from this disease cannot avail the costly diagnostic facility due to their low economic status and the poor communication facility between the hospitals/diagnostic labs and their residence. Therefore, the patients only avail the immediate treatment from the local doctors, with no diagnostic facility and are not referred to for further services.

In the present study, out of 4 samples tested, all of these samples were non-reactive against IgM antibody to JE and had the history of illness for ≤3 days, indicates non-immune response at this stage of illness[42, 43]. Moreover, this observation amply proves that infection occurs very recently and JEV, the etiologic agent multiplies in the patient's body, considered as viremic stage[44]. Those samples were subjected for the isolation of the virus using tissue culture system, followed by RT-PCR test, for the confirmation of the isolation/identification of virus as JEV. All of the 4 screened samples produced prominent CPE, characterized by cell rounding followed by early detachment of cells and were considered positive. To confirm the isolates as JEV, tissue culture infected fluids were used to extract RNA, followed by RT-PCR test where all of these 4 isolates were considered as positive against JEV specific E gene primers. 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 performed a good result.

Enveloped glycoprotein of JEV is the major structural protein that involves in host cell surface receptor interaction, fusion of cell membranes and viral assembly processes including highly immunogenic property which elicits major antibody response in host cell[15, 16]. Antigenic determinant/epitope regions of E protein have been assessed by engineered substitutions either individually or in combination for the critical region of the protein[45]. This amino acid substitutions in the envelop protein of JEV have also been possible to relate pathogenicity and neuroinvasiveness of the virus with these mutations[45]. Thus, bioinformatics' studies/Insilico analysis on the sequences of envelop protein of each of our isolates were very demanding for understanding their molecular phylogeny, natural amino acid substitutions and their possible role in cellular function as well as sequence properties alteration in relation to protein conformation with respect to disease severity.

In the present study, natural amino acid substitutions were identified in the enveloped glycoprotein of each isolates with respect to the existing best representative strains of JEV (see Table 3 and supplemental material) and also evaluated their possible effect on E protein sequence property (Figure 3 A, B and C), followed by structural properties (Figure 2) along with viral infectivity in relation to disease severity (Table 1).

The envelop protein of JEV which contains 500 amino acids is highly conserved glycine rich protein. An analyses of sequence block constituted by JEV-19 and ISO-4 (Table 2) was 374 (74.8%) invariant, 20 (4%) hydrophobically substituted and 25 (5%) hydrophilically substituted positions. Rest 81 (16.2%) positions were non conservative substitutions. Very few substitutions were observed in our isolates (Table 3) indicating that these emerged recently. None of these substitutions occurred in the domain III (Table 3) which is the source of important epitopes that elicit hosts antibody responses[41]. However, some crucial substitutions that might affect the structural and functional role of the protein include DI, DII, stem anchor and transmembrane regions. Although individual role of each amino acid substitutions in each isolates were difficult to assess but their collective effect was reflected in their symptomatic effect on patients (Table 1, Table 3). In addition, unique mutations in isolates might have relation with their severity in infection. In this aspect, Midnapore isolate (GenBank Accession No. JN703381) appeared most severe amongst all of our isolates which contained two crucial mutation (out of four), i.e., One at E103 in fusion loop and the other at E222 position of DII (Table 3, Figure 2). Fusion loop in domain II of E protein played important role in viral infectivity and membrane fusion[46]. This fusion loop is 13 residues long (98 to 110 residues) that adopts finger like structure and is highly conserved among all flaviviruses. In a structural study, it was predicted that substitution of the hydrophobic residue at the position of 107, do not affect the native conformation of the fusion loop[47]. A number of interactions such as salt bridge (between Asp98 and Lys110), disulfide bond (between Cysteine 105 and Cysteine 74) and hydrogen bond make the loop structurally stable[48]. What could be the possible effect of N103K mutation seen in our Midnapore isolate? The fact is that lysine (K) residue possesses lengthy, amphoteric and charged side chain in comparison to asparagine (N), an effect on infectivity and host membrane interaction of the Midnapore isolate was expected. Due to longer side chain, lysine might establish various weak non-covalent interactions which were otherwise difficult for wild-type residue (i.e. asparagine). It is also expected that lysine in comparison to asparagine stabilize the helix of

fusion loop (from residue 100 to 104) as it is helix former[49]. Again lengthy side chain of lysine might cause spatial proximity and thus extra interactions with other residues which are otherwise not possible for asparagine. It was suspected that the above physicochemical advantages along with the positive charge on side chain of lysine might contribute to host infectivity. This assumption is supported by two lines of evidences. Firstly, in an experiment, it was shown that host membrane interaction is severely impaired at low pH for introduction of negative charged residue (i.e. Asp) at position 107 (i.e. L107D) of fusion loop. Secondly, the phenotypic effect of patient was infected by Midnapore isolate shows higher level of severity (Table 1). Another mutational position at E222 in case of Midnapore isolate, present in the upstream of helix A (Figure 3 B), may be assumed as red zone as mutation at 226 regions in envelope protein of Chikungunya virus caused epidemic[50]. It is noteworthy that the single mutation i.e. A222S in case of Midnapore cause change of hydropathic properties for wide range of sequences from 215 to 230 (Figure 3 A). The fact was that A226V substitution enhanced infectivity in Chikungunya virus[50] and the mutation A222S in Midnapore isolate has been occurred in the same region and it was expected that this substitution contributed to the severity in infectivity which was supported by the observation that patient infected with Midnapore isolate showed high degree of severity and had died (Table 1). Other substitutions in domain II were G261S (in all isolates, in helix B Figure 2 B) and H263P (in Burdwan isolate: GenBank Accession No. JN189784, in helix B Figure 2 B). All these residues were on the exposed surface near the hinge region and were exposed to the solvent. Isolate of Nadia (GenBank Accession No. JN189782), followed by Malda (GenBank Accession No. JN189783) were the next in terms of severity point of view which have shown mutations in the DI, DII, stem anchor and transmembrane region (Table 3. The substitution Q158P in domain I of Malda and Nadia isolates was located in the E_0-F_0 glycosylation loop[51]. The loop contains a helix in case of JEV and WNV and that is absent in dengue and tick-Born encephalitis virus. Thus, the conformation of the loop varied in these two cases[51]. It is known that proline is a helix breaker and thus the above substitution has been occurred in the helix region of the loop would destroy the helical structure of the region and thus it was apparently thought that the conformational shift of the loop from JEV type to dengue type. Moreover, analysis of the accessibility of the residue, glutamine in crystal structure of JEV (3P54.pdb), showed that it is highly buried into the protein core. Now upon substitution with the proline which is hydrophobic in nature would certainly affect the exposure of the residue as well as

associated region. The fact was that extent of surface exposure of residues and side chain orientation of the glycan of the loop which act as the determinant for receptor specificity and virulence properties[51, 52] might get affected to a great extent due to the above substitution in case of those isolates. Comparable severity was observed with Malda isolate where mutations were in stem region. Stem anchor region (400 to 457) of the E protein shows four substitutions in case of Malda in the $H1^{pred\ 53}$ region and one substitution in case of Nadia isolate (Table 3) in the $H2^{pred\ 53}$ region.

A huge conformational rearrangement was apparent for the stem region (region 401 to 420) for Malda isolate as position specific sequence properties was seen to alter (Figure 3B) in that hydrophobic region has become hydrophilic and vice versa. Two non-conservative mutations (namely L411W and G413V) were responsible for the above changes. This region of sequence is responsible for establishing trimeric contact[54] and thus an altered interaction for trimer formation was expected in the present case. The fact that $H2^{pred}$ was involved in PrM-E protein interactions stabilization and the observation that single non-conservative substitution (I437R) has occurred in this region in case of Nadia isolate which was the only reason for increasing hydrophilicity of this segment of sequence (Figure 3 C), modulation of PrM-E interactions was expected.

However, the correlation of these interactions with the virulence property of this isolate was needed to be worked out. H263P mutation in case of Burdwan strain was another important substitution in domain II. $Histidine^{E263}$ was present in helix B (Figure 2 B) in hinge region of domain II that maintain pH dependent oligomerisation state of the protein was substituted by proline indicating disruption of such function. Again, introduction of proline in this position also would destroy the helix structure as proline is helix breaker. The isolate of Burdwan remain almost identical symptomatic effect as other existing JEVs.

Two common amino acids substitutions, namely N2H in domain I and G261S in domain II were seen in all of our isolates. The N-terminal Asparagine is structurally present in the hinge region between the domain I and Domain II. Thus, the conversion of Histidine for Asparagine would contribute to the pH mediated structural transition. The substitution G261S occurs in the helix B region (Figure 2 B). Although it contributes to the strength of the helix, it destroys a conserved glycine residue. Such conversion is known to have devastating effect on protein structure and function[55].

Though Cumulative positional changes in nucleotides has been assessed in molecular phylogeny (Figure 4), hydropathy plot for selected regions (Figure 3 A, B, C) has shown the changes of sequence properties which might affect on local chain conformation and topology, degree of exposure of these peptide segments of envelop protein into solvent which played important role in the modulation of interactions with host cell and viral infectivity. Stem anchor and transmembrane regions were very crucial for targeting and establishing interaction of envelop protein was seem to be modulated to a great extent in case of Nadia isolate as it has been shown 11 mutations in these regions (Table 3, Figure 3 C).

Recent studies have shown that the viral envelope (E) protein has become established phylogenetic markers for JEV[56-59], because of this region being free from selective pressure that corroborates obscure long-term evolutionary relationship[60]. In the present study, phylogenetic analysis based on E gene sequences of 4 isolates, along with 19 JEV strains, confirmed that 3 sequences (GenBank Accession No. JN189782, JN189784, JN189783) of 3 isolates (Nadia: IND/09/WB/Nadia, Burdwan: IND/09/WB/Burdwan, Malda: IND/08/WB/Malda) belonged to JEV GIII and the single sequence (GenBank Accession No. JN703381) of one isolate (Midnapore: IND/10/WB/JEV28) belonged to JEV GI (Figure 4). Moreover, this study represents the first report on JEV GI and GIII were found co-circulating in the state of West Bengal.

According to our study, the isolate belonging to genotype I of JEV from the costal district Midnapore (22.25°N 87.65°E and 23 meters above sea-level) which is situated on the banks of the Kangsabati River and adjacent to Orissa and Jharkhand border, might be facilitate the transmission of new genotype by travellers who had spent and returned from JE endemic country, with possible JEV infection[61].

On the other hand, this district has got so many Lakes, swamps, forest and rice fields which are considered to provide a wintering and staging ground for several migratory waterfowl. Such areas are also very suitable for breeding and survival of mosquitoes[28]. In view of these conditions, genotype I of JEV might have been introduced into West Bengal through migratory birds or cyclonic wind-blown mosquitoes from newer geographic region[22, 62]. However, as to the exact mode of introduction of genotype I of JEV into West Bengal it is still unknown. It is needless to say that further studies to determine the role of travellers, migratory birds and wind-blown mosquitoes in JEV transmission are a must.

The State Health Department of Govt. of West Bengal undertook the vaccination programme against JE in Midnapore in 2008[3] using live attenuated JE vaccine derived from genotype III strain SA-14-14-2. In this connection, the efficacy of the vaccine to protect against genotype I of JEV needs careful evaluation. In near future, there might be a chance of JEV outbreak in this region/state, if this vaccine fails to protect sufficiently against genotype I of JEV.

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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.

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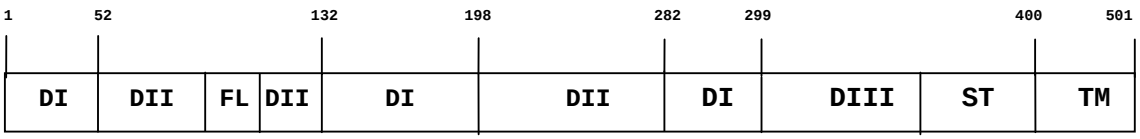
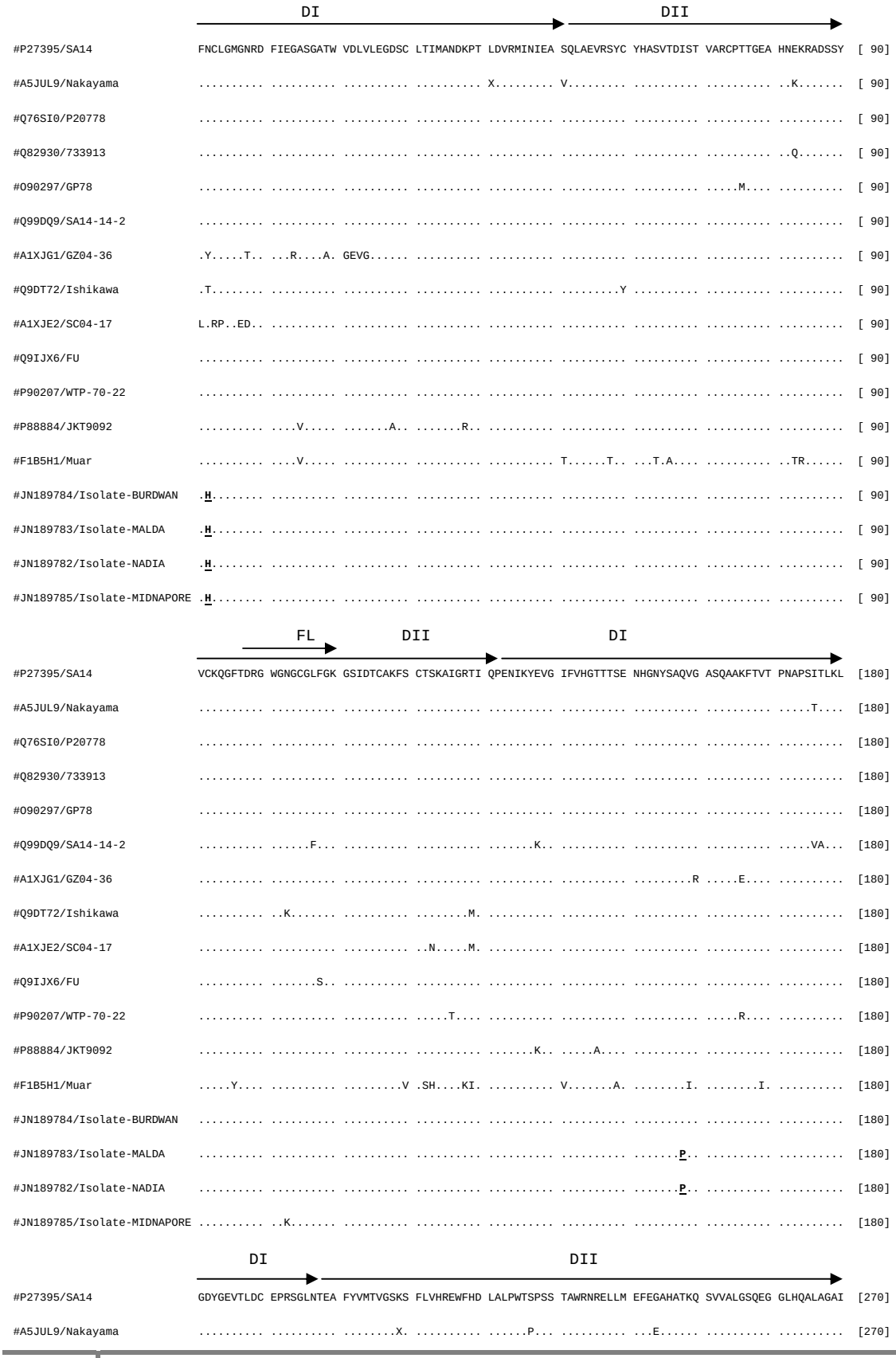
ABBREVIATIONS

AES- Acute Encephalitis Syndrome, JEV- Japanese encephalitis virus, MVEV- Murray Valley encephalitis virus, SLEV- St. Louis encephalitis virus, WNV- West Nile virus, MSA- Multiple sequence alignment

SUPPLEMENTAL MATERIAL

Schematic representation of structural as well as sequence annotation of E protein of WNV. Here, DI, DII (FL), DIII, ST and TM designated as Domain I, Domain II (Fusion loop), Domain III, Stem and Transmembrane region of E gene sequence of WNV, respectively.




DI
DII
#P27395/SA14 FNCLGMGNRD FIEGASGATW VDLVLEGDSC LTIMANDKPT LDVRMINIEA SQLAEVRSYC YHASVTDIST VARCPTTGEA HNEKRADSSY [90]
#A5JUL9/Nakayama .... X..... V..... ....K..... [90]
#Q76SI0/P20778 .... [90]
#Q82930/733913 ....Q..... [90]
#O90297/GP78 ....M.... ..... [90]
#Q99DQ9/SA14-14-2 .... [90]
#A1XJG1/GZ04-36 .Y.....T.. ...R....A. GEVG..... .... [90]
#Q9DT72/Ishikawa .T..... ....Y .... [90]
#A1XJE2/SC04-17 L.RP..ED.. .... [90]
#Q9IJX6/FU .... [90]
#P90207/WTP-70-22 .... [90]
#P88884/JKT9092 ....V..... .....A.. .....R.. .... [90]
#F1B5H1/Muar ....V..... .... T.....T.. ...T.A.... ....TR..... [90]
#JN189784/Isolate-BURDWAN .H..... .... [90]
#JN189783/Isolate-MALDA .H..... .... [90]
#JN189782/Isolate-NADIA .H..... .... [90]
#JN189785/Isolate-MIDNAPORE .H..... .... [90]
FL
DII
DI
#P27395/SA14 VCKQGFTDRG WGNGCGLFGK GSIDTCAKFS CTSKAIGRTI QPENIKYEVG IFVHGTTTSE NHGNYSAQVG ASQAAKFTVT PNAPSITLKL [180]
#A5JUL9/Nakayama ....T.... [180]
#Q76SI0/P20778 .... [180]
#Q82930/733913 .... [180]
#O90297/GP78 .... [180]
#Q99DQ9/SA14-14-2 ....F... ....K.. ....VA... [180]
#A1XJG1/GZ04-36 ....R .....E.... ..... [180]
#Q9DT72/Ishikawa ....K..... ....M. .... [180]
#A1XJE2/SC04-17 ....N.....M. .... [180]
#Q9IJX6/FU ....S.. .... [180]
#P90207/WTP-70-22 ....T.... ....R.... ..... [180]
#P88884/JKT9092 ....K.. .....A.... .... [180]
#F1B5H1/Muar .....Y.... ....V .SH....KI. ..... V.....A. .....I. .....I. ..... [180]
#JN189784/Isolate-BURDWAN .... [180]
#JN189783/Isolate-MALDA ....P.. .... [180]
#JN189782/Isolate-NADIA ....P.. .... [180]
#JN189785/Isolate-MIDNAPORE ....K..... .... [180]
DI
DII
#P27395/SA14 GDYGEVTLDC EPRSGLNTEA FYVMTVGSKS FLVHREWFHD LALPWTSPSS TAWRNRELLM EFEGAHATKQ SVVALGSQEG GLHQALAGAI [270]
#A5JUL9/Nakayama ....X. ....P... ....E..... .... [270]

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#Q76SI0/P20778              .......... .......... .......... .......... .......... .......... ...E...... .......... ..........  [270]
#Q82930/733913              .......... .......... .......... Y.......R. ......P... .......... ...E...... .......... ..........  [270]
#O90297/GP78                .......... .......... .......... .......... .......... .......... ...E...... .......... ..........  [270]
#Q99DQ9/SA14-14-2           .......... .......... .......... .......... .......... .......... .......... .......... ...H......  [270]

                                   DI                                   DII
#A1XJG1/GZ04-36             .......... .......... .....G.... .......... ......P... .......... ...E...... .......... ..........  [270]
#Q9DT72/Ishikawa            ..F....... ..K....... .......... .......... .S........ .......... ...E...... .......... ..........  [270]
#A1XJE2/SC04-17             .......... .......... .......... .......... .S........ .......... ...E...... .......... ..........  [270]
#Q9IJX6/FU                  .......... .......... .......P.. .......... .S........ .......... ...E...... .......... ..........  [270]
#P90207/WTP-70-22           .......... .......... .......... .......... .S........ .......... ...E...... .......... ..........  [270]
#P88884/JKT9092             .......... .......... .......... .......... ......P... .......... .S.E...... .......... A.........  [270]
#F1B5H1/Muar                .......M.. .....F.... ...L...T.. ........N. .....L.... .N.....I.L ...E...... .......... A.........  [270]
#JN189784/Isolate-BURDWAN   .......... .......... .......... .......... .......... .......... ...E...... .......... S.P.......  [270]
#JN189783/Isolate-MALDA     .......... .......... .......... .......... .......... .......... ...E...... .......... S.........  [270]
#JN189782/Isolate-NADIA     .......... .......... .......... .......... .......... .......... ...E...... .......... S.........  [270]
#JN189785/Isolate-MIDNAPORE .......... .......... .......... .......... .S........ .......... ...E...... .......... S.........  [270]

                               DII        DI                          DIII
#P27395/SA14                VVEYSSSVKL TSGHLKCRLK MDKLALKGTT YGMCTEKFSF AKNPVDTGHG TVVIELSYSG SDGPCKIPIV SVASLNDMTP VGRLVTVNPF  [360]
#A5JUL9/Nakayama            .....N.... .........R .......... .......... ....A..... .......... .......... .......... ..........  [360]
#Q76SI0/P20778              .......... .......... .......... .......... ....A..... .......... .......... .......... ..........  [360]
#Q82930/733913              .......... .......... .......... .......... ....A..... .......... .......... .......... ..........  [360]
#O90297/GP78                .......... .......... .......... .......... ....A..... .......... .......... .......... ..........  [360]
#Q99DQ9/SA14-14-2           ........M. .......... .......... .......... .......... .......... .......... .......... ..........  [360]
#A1XJG1/GZ04-36             .......... .......... .......... .......... .R..A..... .......... .......... .......... ..........  [360]
#Q9DT72/Ishikawa            .......... .......... .......... .......... ....A..... ......T... .......... .......... ..........  [360]
#A1XJE2/SC04-17             .......... .......... .......... .......... ....A..... ......T... .......... .......... ..........  [360]
#Q9IJX6/FU                  .......... .......... .......... ......NS.. R...A..... ......T... .......... .......... ..........  [360]
#P90207/WTP-70-22           .......... .......... .......... .......... ....A..... ......T... .......... .......... ..........  [360]
#P88884/JKT9092             .......... .......... ....T..... .......... ....A..... ......L... .....N.... .......... ..........  [360]

                                DII      DI                        DIII
#F1B5H1/Muar                .......... .......... .......... .......... S...A..... ......Q.T. T........S .......L.. ..........  [360]
#JN189784/Isolate-BURDWAN   .......... .......... .......... .......... ....A..... .......... .......... .......... ..........  [360]
#JN189783/Isolate-MALDA     .......... .......... .......... .......... ....A..... .......... .......... .......... ..........  [360]
#JN189782/Isolate-NADIA     .......... .......... .......... .......... ....A..... .......... .......... .......... ..........  [360]
#JN189785/Isolate-MIDNAPORE .......... .......... .......... .......... ....A..... ......T... .......... .......... ..........  [360]

                                           DIII                                STEM/TM
#P27395/SA14                VATSSANSKV LVEMEPPFGD SYIVVGRGDK QINHHWHKAG STLGKAFSTT LKGAQRLAAL GDTAWDFGSI GGVFNSIGRA VHQVFGGAFR  [450]
#A5JUL9/Nakayama            .......... .......... .......... .......... .......... .......... .......... ........K. ..........  [450]
#Q76SI0/P20778              .......... .......... .......... .......... .......... .......... .......... ........K. ..........  [450]
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#Q82930/733913              .......... .......... .......... .......... .......... .......... .......... ........K. ..........  [450]
#O90297/GP78                ..A....... .......... .......... .......R.. .......... .......... .......... ........K. ..........  [450]
#Q99DQ9/SA14-14-2           .......... .......... .......... .......... .......... .......... .......... .......... ......D...  [450]
#A1XJG1/GZ04-36             .......... .......... ......TR.. .......... .......... .......... .......... ........K. I.........  [450]
#Q9DT72/Ishikawa            .....S.... .L.I...... .......K.. .....R.... .......... .......... .......... .R......K. ..........  [450]
#A1XJE2/SC04-17             .....S.... .......... .......... .......... .......... .......... .......... ........K. ..........  [450]
#Q9IJX6/FU                  .......... .......... .......... .......... .......... .......... .......... ........K. ..........  [450]
#P90207/WTP-70-22           .....S.... .......... .......... .......... .......... .......... .......... ........K. .L........  [450]
#P88884/JKT9092             .....S.... .......... .......... ........P. .......... .......... .......... ........K. ..........  [450]
#F1B5H1/Muar                ....T..... ...L...... .F........ .......... .S.....T.. .......... .......... ........K. ..........  [450]
#JN189784/Isolate-BURDWAN   .......... .......... .......... .......... .......... .......... .......... ........K. ..........  [450]
#JN189783/Isolate-MALDA     .......... .......... .......... .......... .........S W.V..K.... .......... ........K. ..........  [450]
#JN189782/Isolate-NADIA     .......... .......... .......... .......... .......... .......... .......... ......R.K. ..........  [450]
#JN189785/Isolate-MIDNAPORE .......... .......... .......... .......... .......... .......... .......... ........K. ..........  [450]

                                                STEM/TM
                                                ──────────►
#P27395/SA14                TLFGGMSWIT QGLMGALLLW MGVNARDRSI ALAFLATGGV LVFLATNVHA  [500]
#A5JUL9/Nakayama            .......... .......... .......... .......... ..........  [500]
#Q76SI0/P20778              .......... .......... .......... .......... ..........  [500]
#Q82930/733913              .......... .......... .......... .......... ..........  [500]
#O90297/GP78                .......... .......... .......... .......... ..........  [500]
#Q99DQ9/SA14-14-2           .......... .......... .......... .......... ..........  [500]
#A1XJG1/GZ04-36             .......... .......... .......... ......I... ..........  [500]
#Q9DT72/Ishikawa            .......... .......... .......... .......... ..........  [500]
#A1XJE2/SC04-17             .......... .......... .......... .......... ..........  [500]
#Q9IJX6/FU                  .......... .......... .......... .......... ..........  [500]
#P90207/WTP-70-22           .......... .......... .......... .......... ..........  [500]
#P88884/JKT9092             .......... .......... ..I....... .M...V...T ..........  [500]
#F1B5H1/Muar                .......... .......... ..I....... .......... .L........  [500]
#JN189784/Isolate-BURDWAN   .......... .......... .......... .......... ..........  [500]
#JN189783/Isolate-MALDA     .......... .......... .......... .......... ..........  [500]
#JN189782/Isolate-NADIA     .......... ..R....... .....LY... .......... ..SSD.MSML  [500]
#JN189785/Isolate-MIDNAPORE .......... .......... .......... .......... ..........  [500]
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Alignment of the E protein amino acid sequences of JEV GI-V. Dots indicate consensus region in the sequences. The letter X represents amino acids that are unidentified in Nakayama strain. Bold faces, underlined amino acids represent position specific unique amino acid substitutions in E protein amino acid sequences of isolates. Relevant structural and as well as sequence annotation was incorporated, based on crystallographic structural analysis of E protein of WNV and JEV.