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## Molecular characterization and pathological studies of Japanese encephalitis virus in pigs of Assam

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### Abstract

Japanese encephalitis (JE) is the most important cause of epidemic encephalitis worldwide. JE exists in a zoonotic transmission cycle between Animals/water birds and human by *Culex* mosquitoes. Assam is one of the highly endemic states for JE sharing approximately 46.95% of total Japanese encephalitis (JE) positive cases reported from the country in 2014. The present study was carried out with the objective of molecular characterization and pathological alterations of JE infection in pig population of Assam. 247 sera samples of pig were screened from Kamrup, Jorhat, Lakhimpur and Goal para and 11.33% of these sera samples showed positive antibody against JEV. The envelope protein gene of JE virus was detected by RT-PCR in 19 blood samples and 5 tissue samples of pigs. The phylogenetic analysis of the JE positive samples belonged to the genotype GIII and was found to be closely related to the human isolates of JEV from Malda (West Bengal), Nadia (West Bengal) and Vellore, India. Hence, suggesting a possible mode of JEV transmission to the mosquito vector and humans inhabiting in the pig rearing areas of Assam.

**Keywords:** Japanese encephalitis virus, molecular characterization, pathology, pig, Assam

### 1. Introduction

Japanese encephalitis (JE) is the most important cause of epidemic encephalitis worldwide. It is a mosquito-borne, zoonotic viral disease caused by the JE virus (JEV) belonging to the genus *Flavivirus* and family *Flaviviridae*. JEV exists in a zoonotic transmission cycle between animals/water birds and human by *Culex* mosquitoes and humans are a dead-end host. It is prevalent in eastern and southern Asia such as China, India and Japan, and has spread to Indonesia, northern Australia, Papua New Guinea and Pakistan [5]. Factors such as global warming, changing agricultural practices and increase in vector populations have enhanced the rise in JE incidence among humans in India during the last decade [5]. Assam is one of the highly endemic states for JE and shares approximately 46.95% of total Japanese encephalitis (JE) positive cases reported all over the country in 2014 [10].

JEV is an enveloped virus with single-stranded positive sense RNA molecule approximately 11 kb in length and contains a single long open reading frame (ORF) that encodes a polyprotein flanked by 5' and 3' non translated regions (NTRs). The polyprotein is of approximately 3,400 amino acids and is subsequently cleaved into three structural proteins, designated as capsid protein (C), membrane (M, a mature form of its precursor protein prM), and envelope protein (E), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The prM and E proteins play critical roles in several biological activities, such as hemagglutination, neutralization, viral binding to cellular receptors and membrane fusion. The E protein has a major role in determining the neurovirulence or neuroinvasiveness. Phylogenetic analyses mainly focused on partial sequences derived from either the C/ prM or E gene and JEV can be categorised into five genotypes (GI – GV) based on the nucleotide sequence of E gene [1].

Pigs act as amplifying hosts, and ardeid birds such as pond herons and egrets are the maintenance reservoirs for the virus. JEV infection causes reproductive failures in pigs characterized by abortion, stillbirths and mummified fetuses [2]. There has been extensive study reported on mosquitoes in JE endemic areas in India. So far there are reports on seroprevalence of JEV infection in pigs in different parts of the country [8, 9], JEV isolation

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from sentinel pig in India <sup>[5]</sup>, genotypic characterization of a JEV isolate associated with reproductive failure <sup>[2]</sup> and complete genome sequence of genotype III JEV isolated from stillborn piglet <sup>[3]</sup>. Considering the endemicity of JEV infection in pigs, the present study was carried out with the objective of molecular characterization and pathological alterations of JE infection in pig population of Assam.

## 2. Materials and Method

### 2.1 Collection of Samples

Serum samples from pigs were collected from 4 districts (Kamrup, Jorhat, Lakhimpur and Goalpara) of Assam state (India) between 2017-18. None of the pigs had been vaccinated against JEV. Serum was separated by centrifugation at 1000 x g for 5 min and stored at -20°C until further use. Blood samples from jugular vein were collected and transported to the laboratory at 4°C. Tissue samples were collected from stillborn foetuses and transported to the laboratory at 4°C.

### 2.2 Antibody detection by ELISA

Green Spring Enzyme Immunoassay for Qualitative Determination (Shenzhen Lvshiyuan Biotechnology Co., Ltd.) was used for detection of antibodies against JEV according to the manufacturer's instructions.

### 2.3 Pathological examination

Post mortem examination was conducted in six stillborn foetuses from two sows and one gilt. The gross pathological alterations in the foetuses were recorded.

### 2.4 Viral RNA Isolation

Viral RNA was extracted from the blood and tissue samples using QIAamp RNA Blood Mini Kit according to manufacturer's instructions. Quality check and quantification of the extracted RNAs was done by gel electrophoresis (1.5% formaldehyde Agarose gel) and eppendorf Bio Photometer plus at optical density (OD) 260nm/280nm respectively.

### 2.5 RT-PCR

RT-PCR was carried out using a Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (#K1621, #K1622) according to the manufacturer's instructions. 10 microlitres of viral RNA solution, prepared as described above, were reverse-transcribed with 1 µl 100 µM Oligo (dT)18 Primer were mixed. The mixture was incubated at 60°C for 10 minutes and chilled on ice. The remaining reagents, which consisted of 4 µl of 5X Reaction buffer, 20 units of Ribo Lock RNase Inhibitor (20U/ µl), 2 µl of 10mM (deoxyribonucleotide triphosphate) dNTP mix and 200 units of Revert Aid M-MuLV Reverse Transcriptase in a final volume of 50 µl were added. The mixture was incubated at 42°C for 60 minutes and the reaction was terminated by heating at 70 °C for 10 minutes. The cDNA was either stored at -20 °C or amplified immediately. A pair of specific published primer for JEV was used to amplify the cDNA. The sequence for forward and reverse primer used was 5'-TTACTCAGCGCAAGTAGGAGCGTCTCAAG – 3' (JF1) and 5'- ATGCCGTGCTTGAGGGGGACG – 3' (JF2) respectively (Yeh *et al.*, 2010). The RT-PCR was carried out as 50 µl volume reaction and in each reaction mixture 6 µl of cDNA as template was added to 5 µl of 10X Dream Taq PCR buffer, 1 µl 10mM dNTPs, 1 µl of each forward and reverse primer (10pmole each), 5 units of Dream Taq DNA

polymerase and final volume was made to 50 µl using nuclease free water. For amplification the thermal cycler was programmed so as to have an initial denaturation at 94 °C for 15 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds and a final extension at 72 °C for 1 minute. The PCR amplicon was checked for the presence of 241bp product specific for JEV by electrophoresing in 1.5% agarose gel. 100bp ladder was used as marker and Ethidium bromide as tracing dye under UV illuminator. Positive and negative controls were used for the validation of the RT-PCR.

### 2.6 Sequencing and Phylogenetic analysis

RT-PCR positive amplicons were purified using Min. Elute Gel Extraction Kit (Qiagen, Germany) and were sequenced from both ends (Sci. Genome, Chennai). Phylogenetic analysis of JEV positive samples was performed for the envelope protein gene. Nucleotide sequences of 3 sequenced JEV positive samples along with 50 reference sequences retrieved from the NCBI Gen Bank nucleotide database were analyzed to establish their genetic relationship. Phylogenetic and molecular evolutionary analyses were conducted with MEGA 6 software using neighbour joining method. The robustness of the groupings in the neighbour-joining analysis was assessed with 1000 bootstrap replicates.

## 3. Results and Discussion

### 3.1 Antibody detection by ELISA

A total of 247 sera samples of pig were screened for the detection of antibodies against JEV. 12.5% of the sera samples showed antibody against JEV from Kamrup district, 10.52% from Jorhat district, 11.66% from Lakhimpur district and 9.52% from Goal para district. In total, 11.33% of samples showed antibody against JEV from the four districts of Assam (Table 1).

### 3.2 Pathological examination

The stillborn foetuses showed skeletal abnormality along with the following pathological lesions in the brain. The head appeared more rounded and enlarged. The stillborn foetuses showed skeletal deformity in both the fore and hind legs (Fig. 1). Upon opening the skull, the foetuses revealed hydrocephalus and the entire cranial cavity was filled with fluid (Fig. 2). Hydranencephaly was observed in the stillborn foetuses with only remnants of brain tissue (Fig. 3).

### 3.3 Molecular detection by RT-PCR

The envelope protein gene of JE virus was detected by RT-PCR. In total, out of 90 blood samples and 51 tissue samples tested by RT-PCR, 19 blood samples and 5 tissue samples were found positive for JEV (Fig 4).

### 3.4 Phylogenetic analysis

The genetic relationship of the JEV positive samples was established by constructing phylogenetic trees based on the JEV envelope protein gene sequences. Three JEV positive sequences of the present study were considered for the phylogenetic analysis, which showed maximum identity of 100% with Japanese encephalitis virus isolate IND/08/WB/Malda envelope protein gene (isolated from human from West Bengal, India), 98% with Japanese encephalitis virus isolate Vellore P20778-T (isolated from human from Vellore, India) and 99% with Japanese encephalitis virus isolate IND/09/WB/Nadia envelope protein

gene (isolated from human from West Bengal, India). These 3 JEV positive samples have been referred to as JEV 1/SW/NRCP/2018, JEV 2/SW/NRCP/2018 and JEV 3/SW/NRCP/2018 in Fig. 5. Four distinct phylogenetic groupings were identified in the phylogenetic tree that corresponded to the four genotypes (GI, GII, GIII and GV) of the envelope (E) protein gene. The three JEV positive samples reported in the present study belonged to the genotype GIII. A classical swine fever virus isolate L168 (AY283666.1) was used as an outgroup and the phylogenetic tree was rooted by the sequence of classical swine fever virus isolate L168, confirming the pigs were infected by the circulating genotype GIII of Japanese encephalitis virus envelope protein gene.

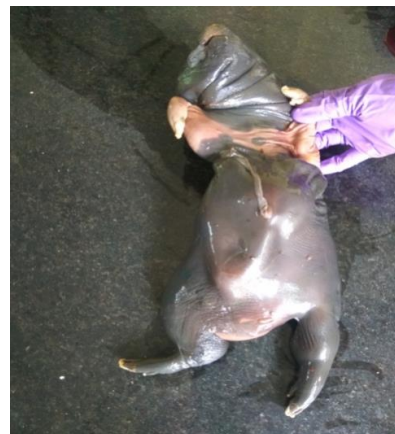
In the present study, a total of 247 nos. sera samples have been screened for JE antibody by ELISA. Of these, 11.33% (28 samples) have been found to possess antibodies against JEV. JE is endemic disease in India, particularly in states where extensive rice cultivation, irrigation facilities and pig rearing are practiced. It is also worth mentioning that human JEV is endemic in north-eastern states of India and Assam is mostly vulnerable for Japanese encephalitis (JE) infection as compared to other states in India. It shares approximately 46.95% of total Japanese encephalitis (JE) positive cases reported all over the country in 2014 ([www.nvbdc.gov.in](http://www.nvbdc.gov.in)). In Assam, pigs are likely to be the major amplifying host as there is a high prevalence of JEV antibody in swine sera as reported earlier [4]. JE causes reproductive failure in pigs and therefore the possibility of abnormalities in six stillborn foetuses from two sows and one gilt were examined. The stillborn foetuses showed lesions in the brain that has been similar to the findings of [2]. The detection of JEV by RT-PCR in the blood samples, in the present study indicated the presence of a high and a prolonged JEV viremia in the pigs. Although these pigs were naturally infected with JEV, this also gives an indication towards the likelihood of mosquito vector infection and direct pig-to-pig transmission. Among the tissue samples, JEV was detected mostly from cerebrospinal fluid and remnants of the brain tissue. The phylogenetic analysis based on the envelope protein gene indicated that the JE positive samples from the fields of Assam belonged to the genotype GIII and was found to be closely related to the isolates of Malda (West Bengal), Nadia (West Bengal) and Vellore. There are findings of GIII genotype reported in India by [11, 12] from human patients, from pigs [2] and from equines [7]. Therefore, our findings indicate that genotype GIII still continues to be a predominant genotype in India.

#### 4. Conclusion

In conclusion, the present study demonstrates the presence of JEV infection in pig population of Assam by finding both serological evidence in the pig sera and PCR positive blood and tissue samples for the JE virus. The severe pathological lesions observed in the brain of the stillborn foetuses were caused by JEV associated reproductive failure. The phylogenetic analysis based on the envelope protein gene indicated that the JE positive samples from the fields of Assam belonged to the genotype GIII and was found to be closely related to the JEV isolates reported from human. Hence, indicating a possible mode of JEV transmission to the mosquito vector and humans inhabiting in the pig rearing areas Assam.

**Table 1:** Seroprevalence of JEV from four districts of Assam

Sl. No.	District	No. of Samples	No. of Positive samples	Percentage of Positive samples
1	Kamrup	88	11	12.5%
2	Jorhat	57	6	10.52%
3	Lakhimpur	60	7	11.66%
4	Goalpara	42	4	9.52%
Total		247	28	



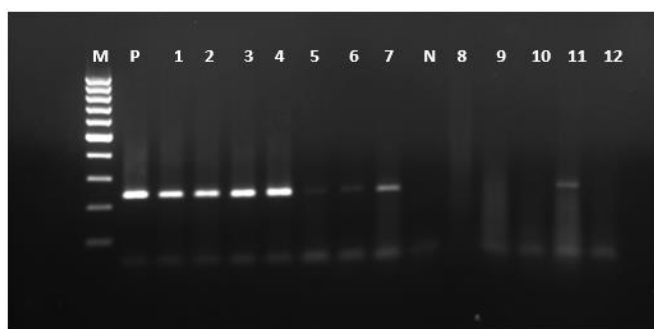
**Fig 1:** Stillborn foetus with skeletal deformity



**Fig 2:** Stillborn foetus showing hydrocephalus in the brain



**Fig 3:** Stillborn foetus showing hydranencephaly



**Fig 4:** Detection of JEV in porcine samples by RT-PCR M: Molecular marker 100 bp DNA ladder; P: JEV positive control; N: negative control; Lane 1-7, 11: JEV positive samples; Lane 8-10, 12: JEV negative samples



**Fig 5:** Phylogenetic tree of Japanese encephalitis virus from field samples

The bootstrap analysis by neighbor joining method was performed using MEGA 6 program. Classical swine fever virus was considered as an outgroup in the analysis

## 5. Acknowledgement

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