Evidence of JEV in *Culex tritaeniorhynchus* and pigs from high altitude regions of Tibet, China

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**ABSTRACT**

**Background & objectives:** *Culex tritaeniorhynchus* is the primary vector of Japanese encephalitis virus (JEV) which is a major threat to animals and humans health. This virus has been reported earlier from low altitude regions of Tibet, in mosquitoes, Tibetan pigs and local Tibetans, but no reports are available for the probable propagation of JEV to high altitude regions (3100 m) of Tibet. This study aimed to find the evidence of JEV in *Cx. tritaeniorhynchus* and pigs from high altitude regions of Tibet, China.

**Methods:** In total, 102 porcine serum samples and eight pools of *Cx. tritaeniorhynchus* were subjected to real-time PCR (RT-PCR) for the amplification of a fragment (~ 420 bp) of the NS1 gene. The resultant amplicons of the genes were subsequently analyzed and sequenced.

**Results:** Overall seroprevalence of JE in Tibetan pigs was 6.86%, while five pools of *Cx. tritaeniorhynchus* were found positive for JEV. Results showed genotype III as the most prevalent (100%) among JEV positive isolates. Furthermore, phylogenetic analysis of the JEV positive strains showed 98.8–99% similarity to four reference strains from China (JEV-Hubei, Whe, HYZ and CQ11–66).

**Interpretation & conclusion:** The present study revealed that JEV has extended its geographic range to high altitude regions of Tibet. The factors like increased tourism and transportation might play key role in the transmission of JE that comprises a potential health risk for humans and animals.

**Key words** *Culex tritaeniorhynchus*; Japanese encephalitis; phylogenetic analysis; Tibet; Tibetan pigs

**INTRODUCTION**

Japanese encephalitis (JE) is one of the most important public health issues and a potential threat to human lives across different countries of the world. Japanese encephalitis virus (JEV) is a single-stranded RNA virus belonging to the genus *Flavivirus*, family Flaviviridae that is the only etiological agent of Japanese encephalitis (JE)¹. It is a mosquito-borne viral disease and causes encephalitis in humans and abortion in pigs². This pathogen has been responsible for many human deaths and >30000 cases are reported annually all over the world³. JE is transmitted humans through the bite of infected *Culex* species mosquitoes, particularly *Culex tritaeniorhynchus* and *Cx. pipiens* mosquitoes that are reported to act as mean reservoirs of the virus in north and south of the China, respectively⁴. The pigs are one of the most important natural hosts of JE and this disease is mainly responsible for stillbirth, abortion and reproductive disturbance (in them) thereby causing serious economic losses to the pig industry in China⁵. JEV is transmitted *via* mosquitoes to pigs, which then act as main source of transmitting the virus to the humans². However, with the development of JE vaccine, it is quite possible to avoid a serious loss to human lives and lower the impact of JE on society and pig industry.

In recent years, public awareness for JE prevention has been increased through campaigns and various other strategies by the stockholders in China, but still numerous cases have been reported sporadically in several provinces of China²,⁴,⁵ and different parts of the world including India, Nepal, Korea, Myanmar, and Vietnam²,⁶,⁸. Altogether, these findings suggest that JEV is still present in most parts of the Asia and China.

Tibet has been recognized as non-endemic region of JE because of its unique geographical and environmental conditions that acts as a natural barrier between mosquito and reservoirs transmission; therefore, no vaccination has been practiced for JE in this region⁹,¹⁰. Recently, JEV has been reported in low altitude areas of Nyingchi prefec-
ture (Motuo and Chayu) of Tibet\textsuperscript{10-11}. This might be due to population expansion, frequent travel and transportation from nearby endemic regions, tourism attraction, ambiance change, pig rearing, and the lack of vaccination surveillance. Tibetan pigs are relatively an ancient original indigenous breed, a rare plateau type pig in the world and are the only high altitude pasture pig breed in China. It is mainly distributed in the Yarlung Zangbo River Valley and eastern Tibet region\textsuperscript{12}. To determine the status of JEV in high altitude regions of Tibet, this study aimed to describe JE in Tibetan pigs and mosquitoes at an altitude of 3100 m which might act as a reference to avoid the risk of JEV transmission to humans and other animals by the bites of mosquitoes or through amplifying host.

**MATERIAL & METHODS**

**Study site**

The present study was carried out in Qinghai Tibetan Plateau that has an average height of 3100 m, the largest continuous high elevation ecosystem (Fig. 1). Geographically the study area is close to Nepal, India, Myanmar and influenced by the Indian Ocean and Pacific, with a humid tropical and semi-humid climate. The average temperature during the study period was 11.3–22.4°C with large difference between day and night temperatures.

Sichuan-Tibet highway is one of the highest roads in the world and the most precipitous road that connects Sichuan province to Tibet, while Yunnan-Tibet highway is another important and highest road that connects Yunnan province to Tibet and also attracts many tourists every year.

**Samples collection and tests**

In the study, a total of 102 serum samples from 1–6 month old pigs were collected randomly from local slaughter houses in between June and September 2015. Sera were analyzed for IgM antibodies against JEV using a commercial ELISA kit (Bio-Swamp, China) according to the instructions of manufacturer. All the JEV positive samples were used to extract RNA by using the TIANamp virus DNA/RNA kit (TianGen, China). A total of eight pools (33 mosquitoes per pool) of *Cx. tritaeniorhynchus* mosquitoes were also collected near pig sty’s in Tibet during the study period, and JEV was extracted from these mosquitoes using the TIANamp virus DNA/RNA kit (TianGen, China) as per manufacturer’s instructions.

Real-time (RT-PCR) amplification approach was used to amplify a fragment (~ 420 bp) of the *NSI* gene (non-structural protein at 3’ conservative region of ORF) by using PCR primers; forward: ATCACAAGAAAAAGATGAGATGT and reverse TTAGTGCTCATCAGGCATTCC. However, reverse transcription was done by Quant One Step RT-PCR kit (TianGen, China) with the following cycling parameters: 35 cycles at 55°C for 30 sec, and 72°C for 1 min and annealing at 55°C for 40 sec; and final extension at 72°C for 10 min. The PCR products were separated on agarose gel (1.5 %) along with ethidium bromide (at the rate of 0.5 μg/ml) following electrophoresis in 0.5×TBE buffer at 5 V/cm for 60 min (Fig. 2). The products were purified using a TaKaRa MiniBEST agarose gel DNA extraction kit version 4.0 (Takara Biotechnology, Dalian, China) according to manufacturer’s instructions. The obtained positive products were sequenced by a commercial company (Quintara Biosciences, Wuhan, China).

![Fig. 1: The possible spread routes of JEV in southwest of China in 2015.](image1)

![Fig. 2: Results of RT-PCR amplification for the *NSI* gene of JEV. (M—Marker; and 1, 2 and 3—Positive samples.)](image2)
Phylogenetic and sequence analysis

The nucleotide sequences of the NS1 gene of JEV were compared with previously reported JEV sequences available at NCBI database (Table 1). Multiple alignments and phylogenetic analysis techniques were conducted using DNASTAR (Laser gene v7.1) and molecular evolutionary genetic analysis (MEGA 6.0) software for windows. Phylogenetic trees were constructed using the neighbour-joining method. The evolutionary distances were estimated using the Kimura two-parameter method and the reliability of the trees was estimated using 1000 bootstrap replications.

RESULTS

Out of 102 tested sera, 7 (6.86%) were positive for the presence of IgM antibodies against JEV, whereas the rest were negative. RNA from the seven positive specimens was isolated by using RT-PCR and named as Tibet-pig-JEV1, Tibet-pig-JEV2, Tibet-pig-JEV3, Tibet-pig-JEV4, Tibet-pig-JEV5, Tibet-pig-JEV6 and Tibet-pig-JEV7. Furthermore, five pools (5/8) of Cx. tritaeniorhynchus mosquitoes were found positive, namely Tibet-Culex-JEV1, Tibet-Culex-JEV2, Tibet-Culex-JEV3, Tibet-Culex-JEV4 and Tibet-Culex-JEV5.

The product of all 12 positive specimens were sequenced and analyzed with other related sequences from NCBI database. The result revealed that the nucleotide sequence of the Tibetan specimens were 98.8–99% identical to that of the JEV-Hubei, Whe, HYZ and CQ11–66 strains (Accession number: KR265316, EF107523, JN381853 and KC183732), and 96.6–96.9% identical to that of the JaOAr72, IND-WB-JE1, Anyang 300, SC04-17, XJ69, JKT6468 and XZ0934 strains (Accession number: AB569990.2, JX050179.1, KF711994.1, GU187972, EU880214, AY184212 and JF915894. While, the sequence identities of other strains were less than 89.4%.

To determine the evolutionary relationships between the isolated strains and representative JEV strains, a phylogenetic analysis was performed based on the NS1 gene nucleotide sequence (Fig. 3). The results of the phylogenetic analysis showed that all the twelve Tibetan positive isolates belonged to genotype III and were most closely related to the representative strains of JEV-Hubei, Whe, HYZ and CQ11–66 strains (Accession number: KR265316, EF107523, JN381853 and KC183732).

The nucleotide sequences of isolated strains shared 97.3–100% sequence identity with reference strains (Fig. 4). Among them, the sequence homology between isolated strains and some reference strains reported from China (JEV-hubei, Whe, HYZ and CQ11–66) illustrated a close homology (Fig. 3).

![Fig. 3: Phylogenetic tree constructed by the neighbour-joining method in DNASTAR (v 7.1), using nucleotide sequences of the NS1 gene (▲—Cx. tritaeniorhynchus samples; ■—Tibetan pigs samples. Numericals I, II, III, IV and V—Different genotypes of the Japanese encephalitis).](image)

<table>
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<th>GenBank Accession No.</th>
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DISCUSSION

Japanese encephalitis virus is mainly transmitted by *Culex* mosquitoes, and leading cause of morbidity and mortality in humans and animals. The prevalence of JEV is determined by various factors, such as geographical environment (altitude and mountains), climatic variations (temperature, humidity and rainfall), vectors (abundance, activity and poisoned rate), and public health system (immunization coverage, effective antibody levels, pigs and exposure to mosquito bite)\textsuperscript{11, 13}. Moreover, factors like increased tourism, transportation, and increased domestic pigs population might be the possible risk factors for the presence of JEV in high elevation regions of Tibet.

In this study, JEV positive isolates were found closely related to Hubei, Sichuan, Chongqing and Yunnan regions of China suggesting their transmission through increased transportation between these provinces as JE has been reported mainly from the southwest parts of China (Sichuan, Chongqing and Yunnan) which are recognized endemic regions of JE in China\textsuperscript{5}. Along side Yunnan, Sichuan, Chongqing and Tibet are also one of the major scenic spots in China, while Sichuan-Tibet highway and Yunnan-Tibet highway are the only traffic routes that link these three regions, that can contribute to the spread of JEV in Tibet.

Moreover, typical weather change in the Tibet and immediate unpredictable climate changes might play a key role for the spread of JEV from low to high elevated regions of Tibet. Other possible reasons of transmission might be increased mean temperature, pig production (amplifying hosts for JEV), water birds and non-vaccinated humans to higher elevated regions of Tibet that can lead to a serious problem in future\textsuperscript{14-15}. On the other hand, Tibet’s unique mountains and rivers, forms a natural barrier structure to prevent or reduce the chances for the spread of JEV. Until now, researchers have not reported *Cx. tritaeniorhynchus* in high altitude areas of Tibet; however, JE has been continuously reported in the low altitude areas of Tibet. The isolation of JEV strains from *Cx. tritaeniorhynchus* in the high altitude regions of Tibet revealed that these areas are now exposed to *Cx. tritaeniorhynchus* bite and spread of JE virus. According to the data analysis, *Cx. tritaeniorhynchus* mosquitoes were mainly distributed in the ditches and found during their breeding months, i.e. July and August. Studies have reported that the distribution and abundance of *Cx. tritaeniorhynchus* are important risk factors for the infection of JEV, and the existence of the mosquito is connected with the season\textsuperscript{16}, hence the spread of JE between local Tibetans and Tibetan pigs has seasonal characteristics.

Pigs are the most important source and intermediate host of JE infection and play an important role in the spread of this disease. Studies have shown that the seroprevalence of JE in local pigs has close connection with the inhabitants. Previously, high seroprevalence of JE in pigs was reported in Yunnan (63.33%) and Sichuan (29.81%) provinces\textsuperscript{17-18}. In the present study, the seroprevalence of JE was 6.86% in Tibetan pigs which was lower than that in Yunnan and Sichuan.
CONCLUSION

This study provided the evidence that JEV is prevailing among high altitude (average 3100 m) regions of Tibet, as confirmed by phylogenetic analysis of JE strains. Hence, this study provides new and valuable knowledge for the epidemic regularity of JE in Tibetan region, and to understand the distribution of JEV in nearby regions. The local government should make strategies for the control and prevention of the emerging JE in this region, especially by paying attention to monitor JE along the Sichuan-Tibet highway and Yunnan-Tibet highway area. The enzootic and epizootic transmissions are source of infection and non-vaccinated children are at high risk of the infection from mosquito bites. Therefore, effective measures and increased surveillance of JEV should be ensured in high altitude regions of Tibet, China.

Conflict of interest

None of the authors have any conflict of interest.

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