
Epidemiological concordance of Japanese encephalitis virus infection among mosquito vectors, amplifying hosts and humans in India

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Received 10 July 2011; Final revision 6 January 2012; Accepted 1 February 2012;
first published online 24 February 2012

SUMMARY

A temporal relationship of Japanese encephalitis virus (JEV) transmission in pigs, mosquitoes and humans revealed that sentinel pig seroconversions were significantly associated with human cases 4 weeks before ($P=0\cdot04$) their occurrence, highly correlated during the same time and 2 weeks before case occurrence ($P<0\cdot001$), and remained significantly correlated up to 2 weeks after human case occurrence ($P<0\cdot01$). JEV was detected in the same month in pigs and mosquitoes, and peaks of pig seroconversion were preceded by 1–2 months of peaks of infection in vectors. Kaplan–Meier analysis indicated that detection of JEV-positive mosquitoes was significantly associated with the median time to occurrence of seroconversion in pigs ($P<0\cdot05$). This study will not only help in predicting JEV activity but also accelerate timely vector control measures and vaccination programmes for pigs and humans to reduce the Japanese encephalitis risk in endemic areas.

Key words: Arboviruses, epidemiology, Japanese encephalitis.

INTRODUCTION

Japanese encephalitis virus (JEV) is a member of the family Flaviviridae, genus *Flavivirus*. JEV is maintained in nature by a cycle that involves pigs and ardeid birds, which act as amplifying hosts [1]. Mosquitoes belonging to the *Culex vishnui* subgroup (*Culex vishnui* Theobald *sensu stricto*, *Culex pseudo-vishnui* Colless, *Culex tritaeniorhynchus* Giles) are the most important vector species in India [2]. An outbreak of JEV infection was first observed in Vellore district in Tamil Nadu, India in 1955 [3]. Since then the virus has been active in almost all parts of India

[4]. The incidence of Japanese encephalitis in recent times has shown an increasing trend in India and has become a major public health problem [5]. JEV has been endemic in Assam since 1976 [6] and in recent years clinical cases of JE have increased markedly [7]. Furthermore, the primary mosquito vectors and both the effective amplifying hosts for JEV, i.e. pigs and ardeid birds, can be found in this region [8–10]. The temporal pattern of JEV transmission, especially within its host and vectors, and the possible source of viral amplification within its host system remains indistinguishable in this region. It is generally accepted that the JE seroconversion rates in sentinel pigs reflect the prevalence of JEV in the area [11]. Sentinel pigs have the advantages of monitoring enzootic transmission events at a specific location [12] and being more cost-effective than other arboviral surveillance indicators, especially ardeid birds.

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Fig. 1. Map showing the location of Assam in India and Barbaruah and Lahoal in Dibrugarh district of Assam.

In Assam, pigs are likely to be the major amplifying host as there is a high prevalence of JEV antibody in swine sera [9]. The high occurrence of JE cases in Assam may be related to the increasing number of pigs in recent years. Pigs are currently not vaccinated against JEV in India, including Assam. Reliable prediction of epidemiological concordance of JEV infection in swine, mosquito and human populations during any specified period in a specific endemic locality is important for practical reasons that include initiation of mosquito control and vaccination programmes in pigs and humans and to analyse the mechanism of seasonal spread of infection from one host to another. So far, no systematic study of this nature has been conducted in India, the outcome of which is very important in order to develop a strategic plan for JE prevention and control and to assess the success of these programmes in endemic areas. Therefore, the present study aims at identifying the temporal transmission pattern of JEV infection by tracking the sentinel pigs, mosquito vectors and incidence of human JE cases in Assam. This information will provide baseline data to design and implement JE

prevention programmes not only in the state of Assam but also in other JE endemic areas of the country.

METHODS

Study area

The study was conducted during 2009 and 2010 in two areas, Barbaruah and Lahoal in Dibrugarh district, Assam, India (Fig. 1). These two areas were selected due to JEV endemicity, abundance of breeding places for JE vector mosquitoes and extensive pig rearing by the villagers [9]. Table 1 provides details of the increasing pig population in the study areas. Rice is the major crop cultivated between May and August and rice fields are generally located within a 2 km radius of human habitation.

Sentinel pig seroconversion

Twenty locally procured, post-weaned pigs (aged 3–6 months) of both sexes and JEV antibody-negative by

Table 1. Increasing trend of pig population in Lahoal and Barbaruah primary health centre, 2006–2009

Study area	Years			
	2006	2007	2008	2009
Lahoal	4936	5765	9897	13 796
Barbaruah	5674	7973	12 463	18 775

Source: Directorate of Animal Husbandry and Veterinary Department, Government of Assam.

haemagglutination inhibition (HI) test were distributed to locally identified households for rearing in each area in January 2009. A similar process was followed in the next year after allowing the beneficiaries to dispose of the first set of pigs in December 2009. Blood samples were collected every 2 weeks from the sentinel pigs. JEV antibody seroconversion in pigs was determined by HI test [13]. Sera were serially diluted twofold from 1:10 to 1:20480. Sera with an HI titre of $\geq 1:20$ were considered as positive [14].

JEV antigen preparation

Formalin inactivated antigen was prepared from cell culture propagated virus using the BHK-21 cell line [15] at the Regional Medical Research Centre, ICMR, Dibrugarh, Assam, India. The maximum haemagglutination titre of the prepared antigen usually reached 1:10240. This antigen was used to test pig sera by HI [13].

Mosquitoes

Adult mosquitoes were collected bi-weekly around sentinel pig sheds and in human habitats during dusk hours by using suction tubes (aspirators). Species identification was done by using the mosquito identification keys [16–18]. Mosquitoes were stored, in species order, in Eppendorf vials (2 ml) in pools of up to 50 mosquitoes in each vial. Species-wise per man hour density (PMHD) of mosquitoes was calculated according to the formula:

$$\text{PMHD} = \frac{N \times 60}{T \times P}$$

where, N = number of mosquitoes collected; T = time spent (in minutes); P = number of persons involved in collection.

Each pool was triturated mechanically in a sterile mortar and pestle in 3–5 ml of 2% fetal calf serum in minimum essential medium containing 50 μg gentamicin, 50 IU penicillin, 50 μg streptomycin and 50 μg amphotericin B per ml and centrifuged at 1100 g for 15 min at 4 °C. The supernatant was filtered through a 0.2 μm filter, aliquoted and tested for the presence of JEV antigen using an antigen-capture ELISA [19]. The supernatant was subjected to RNA extraction by using a QIAmp viral RNA mini kit (Qiagen, Germany). Purified RNA was used as template for cDNA synthesis using First Strand cDNA Synthesis kit (Fermentas, USA). Reverse transcriptase–polymerase chain reaction (RT–PCR) was performed to amplify the 348 bp *C-prM* gene region [20] of JEV by using cDNA (2 μl) as template.

Minimum infection rate (MIR)

The virus infection rate in mosquitoes was expressed as MIR/1000 mosquitoes of a particular species tested [21].

$$\text{MIR}/1000 = \frac{\text{no. of mosquito pools positive}}{\text{total no. of mosquitoes tested}} \times 1000.$$

Human JE cases

Collection of samples from the study area (Barbaruah and Lahoal) was limited to those that matched the World Health Organization clinical case definition of Acute Encephalitis Syndrome [22] and which were laboratory-confirmed, typically by detecting IgM antibodies in sera or cerebrospinal fluid (MAC ELISA kit; NIV, India) as well as through a fourfold rise of antibody titre in paired sera. A second sample was collected from patients with JEV infection 14–21 days after the first sample had been collected. The fourfold rise in neutralizing antibody titre in paired sera was determined by a microseroneutralization assay [23]. Written informed consent was obtained from the patient or guardian before collection of the sample. Ethical clearance for the present study was obtained from the institutional ethics committee of the Regional Medical Research Centre, ICMR, NE Region, Dibrugarh, Assam, India.

Data analysis

JEV infection in mosquitoes, i.e. MIR/1000 mosquitoes, was calculated for each month. Cross-

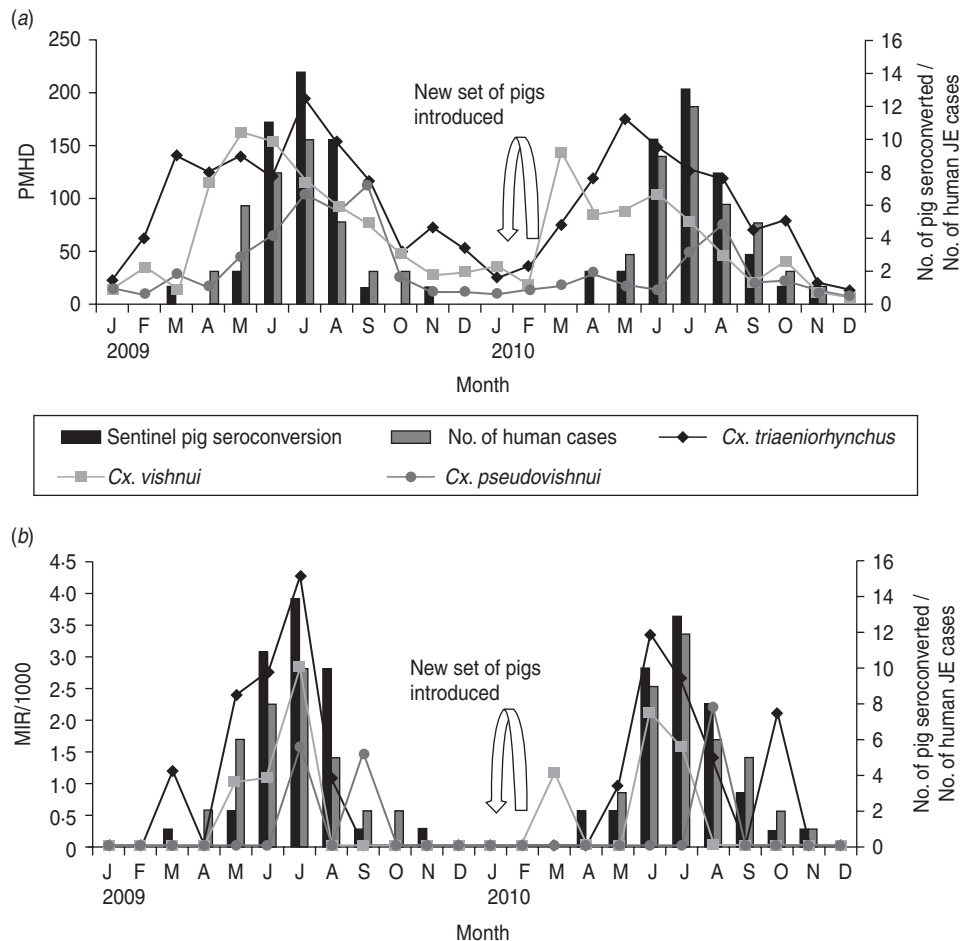


Fig. 2. Relationship between pig seroconversion, (a) abundance and (b) JEV infection in *Cx. tritaeniorhynchus*, *Cx. vishnui* and *Cx. pseudovishnui* vector mosquitoes. PMHD, Per man hour density; MIR, minimum infection rate.

correlation analysis was applied to detect the lag period between pig seroconversion and occurrence of human JE cases. A survival analysis was performed on the pig serology results stratified by the presence or absence of JEV-positive mosquito pools and a Kaplan–Meier survival curve was plotted. Data were electronically analysed using statistical software (SPSS version 13, USA).

RESULTS

JEV seroconversion was seen in all 40 sentinel pigs during 2009 and 2010, starting from March and April of the first and second year, respectively. Most of the sentinel pigs became positive within 3 months, i.e. June–August, with a peak in July in both years (Fig. 2).

A total of 28503 mosquitoes, i.e. *Cx. tritaeniorhynchus*, *Cx. vishnui* and *Cx. pseudovishnui* collected and stored in 586 pools were screened for JEV

over 2 years. Nineteen of 281 *Cx. tritaeniorhynchus* pools, 7/198 *Cx. vishnui* pools and 3/107 *Cx. pseudovishnui* pools collected during March to September were found positive by antigen-capture ELISA and RT–PCR. The results of this study clearly indicated the seasonal variation in occurrence of JEV infection in *Cx. tritaeniorhynchus*, *Cx. vishnui* and *Cx. pseudovishnui*, the principal JE vectors of India, in both PMHD and MIR peaks (Fig. 2). The MIR of *Cx. tritaeniorhynchus* was found to be highest with an average of 0.9 (range 0.0–4.3) during the 24-month study period followed by *Cx. vishnui* (average 0.4, range 0–2.9) and *Cx. pseudovishnui* (average 0.2, range 0–2.1).

The monthly number of pig seroconversions during 2009 and 2010 compared to the monthly abundance of the *Cx. vishnui* subgroup of mosquitoes (PMHD) and JEV infection (MIR/1000) is shown in Figure 2. In 2009, the peak in pig seroconversions coincided with the increase in abundance of all three *Cx. vishnui*

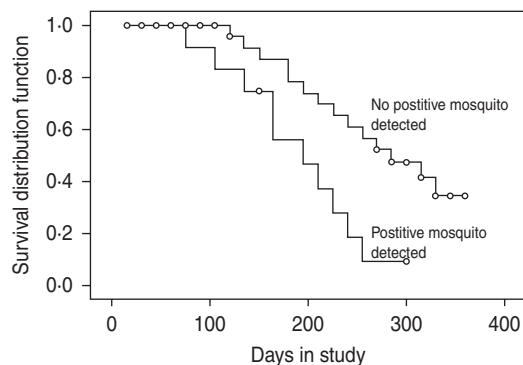


Fig. 3. Kaplan–Meier analysis to pig seroconversion stratified by mosquito positivity ($P < 0.01$).

subgroup mosquitoes. In 2010, the pig seroconversion peak occurred after the increase in abundance of *Cx. tritaeniorhynchus* and *Cx. vishnui* (Fig. 2). In 2009, the first pig seroconversion and JEV-positive mosquito pools occurred in the same month, March, but in 2010, the first sentinel pig seroconversion in April preceded the detection of positive mosquitoes in March. The highest MIR peaks of *Cx. tritaeniorhynchus* and *Cx. vishnui* and the pig seroconversion peak occurred in July 2009, whereas in 2010, the highest MIR peak in June was followed by the peak seroconversion in July. The last seroconversion peak of both years occurred in November which was preceded by a peak in MIR in September and October of the first and second year, respectively. Thus, from this observation, JEV infection was detected in the same month in pigs as well as mosquitoes and pig seroconversions were also preceded by 1–2 months of peaks of occurrence of JEV infection in the vectors. Kaplan–Meier analysis revealed that the detection of positive mosquitoes is significantly associated with the median time to occurrence of seroconversion of pigs ($P < 0.01$) (Fig. 3).

A graph based on plotting the onset dates of human JE cases and the seroconversion dates in sentinel pigs revealed a temporal concordance (Fig. 4). The same was evaluated by cross-correlation analysis by holding the number of human case counts constant by date of onset and shifting the number of pig seroconversions progressively earlier or later in time. Because of the seasonal trend present in both datasets, the time period was restricted from May to September. Cross-correlation coefficients with 95% confidence intervals revealed that sentinel pig seroconversions were significantly associated with human cases 4 weeks prior ($r = 0.48$, $P = 0.04$) to their occurrence and highly correlated during the same time period

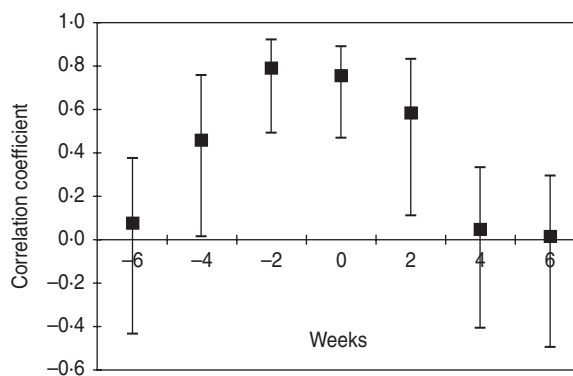


Fig. 4. Relationship between human case counts and lagged sentinel pig seroconversions (lag is represented on the x-axis) depicted by correlation coefficients and 95% confidence intervals.

($r = 0.76$, $P < 0.001$) as well as 2 weeks prior to case occurrence ($r = 0.79$, $P < 0.001$) and remained significantly correlated up to 2 weeks after cessation of human cases ($r = 0.60$, $P < 0.01$). These findings in the present study have revealed that initiation of porcine seroconversion predicted the incidence of JE cases in humans.

DISCUSSION

The present study demonstrated that JE seronegative sentinel pigs were sensitive indicators for detecting JEV transmission in a JE endemic area. Although various cross-sectional studies reported that sentinel pigs used for surveillance in various countries did not reflect JEV transmission to humans [24–27], in the Indian subcontinent porcine infections provided a means of detecting JEV activity in humans [19, 28, 29]. The present study further clearly illustrates how pig infections can be sensitive indicators for tracking tangential transmission of JEV in vector mosquitoes and subsequently to humans in an endemic area over a definite time period. The pattern of occurrence of JEV infection in mosquitoes, pigs and humans observed in this study area was striking in its synchrony.

In the present study, sentinel pig seroconversion which had been used to track JEV transmission subsequently in humans in endemic areas was significantly associated with human cases 4 weeks before their occurrence and up to 2 weeks after human case detection. This was also found to be highly correlated during the same time period and 2 weeks before human case occurrence. However, the concordance of sentinel pig seroconversion and human cases could be

better assessed by shortening the time interval of blood sampling from 2 weeks to 1 week [30].

Our study revealed that both MHD and MIR in the *Cx. vishnui* subgroup of mosquitoes, the principal JE vector in India [31] showed seasonal variation [8, 32, 33]. There were three distinct peaks of MIR in June, July and August in both years. It is noteworthy that JEV infection was detected in the same month in pigs and mosquitoes, and peaks of pig seroconversion preceded by 1–2 months the peaks of JEV infection in vectors. The present study revealed that the median time to seroconversion in pigs is significantly associated with the MIR of vector mosquitoes. More particularly, the MIR of vector mosquitoes can be used as a tool to predict the appearance of JEV infection in pigs [24, 34].

Our study gives a clear understanding of the temporal transmission patterns of JE in mosquitoes, pigs and humans. This information will be extremely helpful in developing a strategic plan for JE prevention and control and to assess the success of the programme. There is no drug treatment for JE. Vaccination of pigs represents an alternative strategy to control JE [35, 36]. An alternative approach of moving domestic pigs away from human habitation to encompass the flight range of the local *Culex* vectors has been suggested as a potential method of reducing JEV transmission to humans [37, 38].

The marked increase of unorganized pig farming and trading may also play a major role in the increase of JE incidence [35]. Intensive and extensive IEC (information, education, communication) activities among the people in JE endemic areas should be undertaken to increase understanding of the necessity for implementation of JE control programmes and proper maintenance of pig populations in these areas [9]. Assessment of the success of such a programme can be measured by strict surveillance of JE incidence in humans, animals and its vector.

In conclusion, our study has established the temporal relationship of transmission of JE in an Indian setting which may depict identical scenarios in other pig-rearing areas of South East Asia with a similar ecological niche and potential mosquito vectors. This temporal relationship will not only help in predicting JEV activity in a particular JE endemic area but also accelerate the initiation of timely vector control measures and animal vaccination programmes to reduce the risk of JEV infection in both pigs and humans. This will also alert the local health authority and the

administrator for management of any future outbreak of JE in areas under their jurisdiction.

ACKNOWLEDGEMENTS

J. Borah acknowledges the Indian Council of Medical Research, Government of India, for providing a senior research fellowship (SRF) to carry out the study. We thank the Directorate of Animal Husbandry and Veterinary Department, Government of Assam, India for providing pig population data. Technical assistance received from N. K. Baruah, P. K. Doloi, R. Doloi, L. J. Borah, P. Chowdhury and R. Topno in laboratory and field work is gratefully acknowledged.

DECLARATION OF INTEREST

None.

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