Sero-positivity of Japanese Encephalitis Virus in Swine Using Virus Neutralization Test

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ABSTRACT

Japanese encephalitis (JE) is a re-emerging mosquito borne zoonotic flaviviral disease and is the most common cause of encephalitis in children of Asia leading to several deaths every year in our country. The present investigation was undertaken to assess the sero-positivity of Japanese encephalitis virus in swine population using virus neutralization test. A total of 181 swine serum samples were collected from various regions of Uttar Pradesh, Chandigarh, Kerala and Goa. The study revealed overall sero-positivity of 59.11%. The sero-positivity in different regions was found to be 78.94% for Goa, 60.97% for Kerala, 56.14% for Uttar Pradesh and 43.47% for Chandigarh. The present study revealed the high sero-positivity of JEV in swine population of various Indian states and highlights the need for more extensive surveillance from different parts of country to confirm the exact status of JE for formulating the necessary preventive measures.

Keywords: Japanese encephalitis, sero-positivity, swine, virus neutralization test

Introduction

Japanese encephalitis (JE) is a re-emerging mosquito borne zoonotic flavivirus disease, reported from large parts of Asia, western Pacific countries, and northern Australia (Solomon, 2006; Wang and Liang, 2015). The reported annual worldwide incidence in humans is 67,900 (Campbell et al., 2011). The case fatality rate of 20-30 per cent and the presence of debilitating neuropsychiatric sequelae in 30-50 per cent of the survivors, adds further to the gravity of the disease. The huge outbreaks of Japanese encephalitis in years 2005, 2006 and 2009 in Gorakhpur, India, highlights the continued burden of this disease in developing countries (Parida et al., 2006; Tiwari et al., 2012).

JEV is maintained naturally in a cycle where ardeid water birds act as reservoir host and role of vector is played by culicine mosquitoes primarily Culex tritaeniorhynchus (Bhattacharya and Basu, 2014). Pigs are the amplifier host of JEV since they allow manifold virus multiplication and maintain comparatively prolonged viraemia (Wiliams et al., 2001). The correlation between the seasonal sero-prevalence of JE in pigs and periods of outbreaks of JE in humans and the fact that sero-conversion in pigs occurs 2-3 weeks before infection in human indicates the importance of sero-surveillance targeting pig population in endemic areas as an epidemiological tool for forecasting of JE outbreaks in humans (Flohic et al., 2013). In India domestic pig rearing is an important risk factor for the transmission of JE to humans. There are many unorganized piggeries and the rural populations who rear pigs live in close proximity with these animals. Vaccination of pigs is not feasible logistically as a prevention measure due to the cost factor, the high turnover of piglets and the presence of maternal antibodies in piglets. The surveillance of pig population is the best measure that can be adopted.

It was against the above backdrop that the present study was undertaken to estimate the sero-positivity of JEV in swine population in different regions of India using virus neutralization test (VNT), the gold standard test for flavivirus detection.

Material and Methods

Collection of samples

A total of 181 blood samples from swine were collected from Uttar Pradesh, Chandigarh, Kerala and Goa during the period from August 2015 to January 2016. The collections were made in sterile vials and
transported to laboratory under chilled conditions. Serum was separated by centrifugation at 1,000 × g for 5 min and stored at -20°C until further use.

**Virus and cells**

GP-78 strain of Japanese encephalitis virus procured from Sanjay Gandhi Postgraduate Institute of Medical Science and maintained in the Division of VPH, ICAR-IVRI was used in this study. Vero cells obtained from NRC, Equine, Hisar was employed.

**Growth and maintenance of cell line**

The Vero cells were grown into monolayer under aseptic conditions in 25 cm² and 75 cm² tissue culture flasks (Nunc). Filter sterilized Dulbecco’s minimum essential medium (DMEM, Hyclone) with L-glutamine, glucose, sodium pyruvate and without sodium bicarbonate was used for growth and maintenance of the cells. Sodium bicarbonate at the rate of 3.7 g/L of medium was added to maintain the pH and antibiotic-antimycotic containing 10,000 µg/ml streptomycin, 10,000 units/ml penicillin and 25 µg/ml amphotericin (Gibco) was also added. The cells were grown in DMEM containing 10% fetal bovine serum (FBS, Cellclone), whereas DMEM with 2% FBS was used as maintenance medium for cells.

**Virus propagation**

The JE virus was propagated in Vero cells and maintained further as described below. The virus stock (250 µl) was mixed with 750 µl of DMEM. The mixture was spread onto a Vero cell monolayer having 80 per cent confluence in 25 cm² cell culture flask. The flask was incubated for 1 h at 37°C in 5% CO₂ atmosphere with intermittent shaking. Later the mixture was discarded from the flask and 5 ml DMEM with 2 per cent FBS was added. The flask was incubated at 37°C in 5% CO₂ atmosphere with intermittent observations till the development of cytopathic effect (CPE). The flask showing CPE was freeze thawed for three times and the supernatant was used for subsequent passages in fresh Vero cell culture flasks.

**RT-PCR**

The presence of virus in cell culture was confirmed using RT-PCR previously standardized in the Division of Veterinary Public Health, IVRI, Izatnagar (Dhanze et al., 2015). After sufficient production of virus, the aliquots of respective passages were stored in -80°C.

**Virus neutralization test**

**Propagation of cells in 96 well micro titre plate to form monolayer**

Vero cells propagated in tissue culture flasks were trypsinized and added to 96 well micro titre plate in different dilutions viz. 5000, 10,000 and 20,000 cells per well to find out the seeding rate of cells required for good monolayer formation.

**Calculation of TCID₅₀**

TCID₅₀ was calculated using the Reed and Muench method. The virus was titrated in 10 fold series in maintenance medium and 100 µl was added per well in a single 96-well cell culture plate. Further, Vero cell suspension in growth medium with 10% fetal bovine serum was added to each well of the plate. The plate was covered with lid and kept at 37°C under 5% CO₂ tension for 3-4 days. After incubation the presence or absence of CPE in each well was noted. Viral titre was calculated by Reed and Muench method.

**Protocol followed for VNT**

VNT was performed in 96-well tissue culture plates using Vero cells. Serum samples were heat inactivated at 56°C for 30 min and diluted to 1:8 dilution in a 50 µl volume. It was mixed with an equal volume of 300 TCID₅₀ of the JEV. After incubating the virus-serum mixture at 37°C for 1 h, Vero cell suspension was added to each well. The plates were incubated at 37°C in 5% CO₂ for 5 days and CPE in each well was recorded. Absence of CPE indicates neutralization and the sample was graded as positive for JE antibodies.

**Results and Discussion**

Japanese encephalitis has become a major public health problem in India with every year cases in children reported from different parts of the country. Swine plays an important role in epidemiology of JE and serve as a suitable sentinel for predicing the outbreaks in humans. In the present study, a total of 181 swine serum samples were collected from various regions of India. The samples were screened using VNT as it is described as the gold standard for diagnosis of JE virus (OIE, 2010).

**Maintenance and propagation of JEV**

JEV was successfully maintained and propagated in Vero cell line. The virus showed characteristic cytopathic effect (CPE) like multinucleation, cytoplasmic oozing and detachment of cells after 3-4 days of infection (Fig. 1). The presence of virus in cell culture was confirmed using RT-PCR. The amplified product was revealed at 220 bp in agarose gel electrophoresis (Fig. 2).

**Propagation of cells in 96 well micro titre plate to form monolayer**

It was found that addition of 20,000 cells per well in the micro titre plate provided a good monolayer formation after an incubation period of 48 h.
Calculation of TCID$_{50}$

The TCID$_{50}$ was calculated using Reed and Muench method and it was found to be $10^{4.5}$ ml.

Sero-positivity of JEV

Out of 181 swine serum samples screened using VNT, 107 samples were found to be positive for JEV antibodies. Thus, the overall sero-positivity in swine population was 59.11 per cent (Table 1).

Table 1. Region wise consolidated results of swine serum samples tested

<table>
<thead>
<tr>
<th>Area/ region</th>
<th>No. of samples</th>
<th>No. of positive samples</th>
<th>Seropositivity (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U.P.</td>
<td>57</td>
<td>32</td>
<td>56.14</td>
</tr>
<tr>
<td>Kerala</td>
<td>82</td>
<td>50</td>
<td>60.97</td>
</tr>
<tr>
<td>Chandigarh</td>
<td>23</td>
<td>10</td>
<td>43.47</td>
</tr>
<tr>
<td>Goa</td>
<td>19</td>
<td>15</td>
<td>78.94</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>181</strong></td>
<td><strong>107</strong></td>
<td><strong>59.11</strong></td>
</tr>
</tbody>
</table>

Earlier studies have reported seroprevalence in the range of 12–44 per cent among swine population from different parts of the country (Tiwari et al., 2012). The reason for high positivity in the present study could be the time of sample collection. The samples were collected from the month of August 2015 to January 2016. According to the previous study conducted in the division of VPH, IVRI, Izatnagar, the seasonal seroprevalence of JE was found to be maximum in the post monsoon period (July to October), which coincides with high vector activity and rice cultivation period in the endemic areas (Dhanze et al., 2014). Further, observations of other research workers have concluded that maximum human outbreaks of JE in endemic areas occur during this period (Solomon, 1997; Kumari and Joshi, 2012).

The sero-positivity in Uttar Pradesh was found to be 56.14 per cent while in previous studies it was recorded as 27.88 per cent (Rawat, 2013) and 29.33 per cent (Dhanze et al., 2014). However, it has been reported that in the study areas having endemic status for JE, the sero-positivity in swine population can go up to 100 per cent (Acha and Szyfrez, 2003).

The highest sero-positivity of 78.94 per cent was found in Goa which might be due to the low sample size (15/19). Further sampling is required to know the exact status of JE in Goa. Previously, a sero-positivity of 29 per cent was reported for Goa (Kohle, 2008). In Chandigarh, a sero-positivity of 43.47 per cent was recorded in the present study while previous studies reported sero-positivity between 30.3 to 31.03 per cent (Acha and Szyfrez, 2003).

The present study revealed the high endemic status of JEV in various Indian states and highlights the need of extensive surveillance in different parts of India to confirm the status of JE for formulation of preventive measures accordingly.
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References


