Indirect ELISA for serosurveillance of Japanese encephalitis in pigs


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ABSTRACT

For monitoring the activity of Japanese encephalitis (JE) in pigs, no effective assay is available in Indian context. Being its endemicity in India and role of pigs as amplifiers, there was need to develop rapid and sensitive assay for serosurveillance of JE in pigs. An Indirect ELISA (I-ELISA) was standardized using ultracentrifuge sucrose density gradient purified JE antigen from standard virus strain. Cross reaction studies were done with West Nile antigen and comparative efficacy of I-ELISA assessed with Virus Neutralization Test (VNT). Optimum results recorded for detection of IgG and IgM, respectively, at 50 ng/well antigen and 1:200 and 1:300 serum dilutions. The positive - to - negative ratio of $\geq 2$ considered positive for JE antibodies. The overall prevalence of JE in pigs recorded to be 28.89 %. The concordance of the indirect ELISA (IgG) vis-à-vis VNT revealed good agreement ($kappa = 0.66$) and moderate agreement ($kappa = 0.56$) of standardized I-ELISA towards detection of IgG and IgM, respectively. The assay standardized in the present study could be useful for JE serosurveillance in pig population from endemic and non endemic areas in India.

Key words: Indirect ELISA, Japanese encephalitis, Pigs, Seroprevalence, Standardization.

INTRODUCTION

Japanese encephalitis (JE) is one of the important zoonotic diseases accounts for more than 10,000 deaths and 35,000 cases each year globally (Tsai, 2000). It is mosquito borne viral infection cause by Japanese encephalitis virus (JEV) of genus flavivirus, Flaviviridae family. Culex tritaeniorhynchus mosquitoes being principal vector for disease transmission. JE situation in Southeast Asia and Indian subcontinent is endemic to hyperendemic. Wide numbers of strains have been reported globally and phylogenetic studies revealed that JEV is continuously evolving in nature. Pig- mosquito-pig and bird-mosquito-bird are two basic transmission cycles in nature. Swine plays important role as an amplifying host in disease transmission, although clinically it is often remain under reported in pig population. It is enzootic in India and most commonly associated with rice growing fields in the rural areas where water lodging and irrigation system provides healthy environment for mosquito breeding. The epidemiological pattern and virus activity is greatly influenced by climate, geography and immune status of the host population (Gubler, 2002). Presence of swine population in peri-domestic area greatly influences the risk to human population.

Serological assays are the method of choice in JE diagnosis since virus isolation is very poor due to rapid development of neutralizing antibodies and low circulating viral numbers. Secondly, JE virus can only be isolated from cerebrospinal fluid (CSF) and brain (Solomon et al., 1998). Amongst various serological assays employed for serodiagnosis of JE, HI is most widely used. ELISAs are recognized as an important and promising tool for detection of antigen and antibodies in serum and CSF in recent past. IgM capture ELISA is the most accepted standard for diagnosis of JE in human. However, reports on the development of rapid and sensitive ELISA for serodiagnosis in animals is found to be scanty. More recently an indirect ELISA (Yang et al., 2006) and a quantitative ELISA (Xinglin et al., 2005) were developed to detect antibodies against JEV in swine. JE is endemic in India accounting large number of cases and death in the world. In India, serodiagnosis of JE in pigs is relied on HI only and no ELISA formats are available for monitoring JE infection in pig population. Therefore, present study was undertaken to standardize I-ELISA for serosurveillance of JE in pig population.

MATERIALS AND METHODS

A known standard strain of JE (753101) and West Nile (P 23085) viruses procured from the National Institute
of Virology (NIV), Pune were revived in suckling mice and C6 36 Aedes albopictus mosquito cell line according to the methods described (Gould and Clegg, 1991., OIE, 2004). The virus titer was estimated as plaque forming unit (PFU) per ml using agarose overlay and neutral red stain. Viral antigens were prepared as per the standard methods described with suitable modifications (OIE, 2004; Gould and Clegg, 1991 and Yang et al., 2006). The JE virus particles were first concentrated from infected cell culture fluid by ultracentrifugation at 50000 g (26000 rpm) for 2 h at 4°C in Sovall Ultra Pro 80 ultracentrifuge (Kendro Lab, USA) using fixed angle rotor (A 621) and the virus precipitate resuspended in 1/100th volume of GTNE buffer (200 mM Glycine, 50 mM Tris, 100 mM NaCl, 1mM EDTA, pH adjusted to 7.5 with HCl). This crude concentrate was further overlaid on 20-50 % (w/v) discontinuous sucrose (Sigma) gradient and again ultracentrifuged at 100000 g (27500 rpm) for 3 h at 4°C using swinging bucket rotor (AH 650). The antigen was recovered from the interface between the two sucrose layers. The purified antigen stored at -20°C used further for standardization of I-ELISA. The WN virus antigen was prepared by sucrose acetone extraction method as described by OIE (2004) from suckling mice brain (SMB). The protein content of the extracted antigen was determined by ND 1000 nanodrop spectrophotometer.

A total of 488 pig sera samples were collected from different endemic regions of JE infection. By VNT, virus neutralization titer was expressed as the reciprocal of the final serum dilution that prevented cytopathic effect (CPE) in 50% of the cell culture. A titer of >4 considered positive for JEV antibodies as described by Yang et al. (2006).

Indirect ELISA was standardized using known positive and negative sera confirmed by HI and VNT by checker board titration. Briefly, ELISA plates were coated with 50 µl /well of coating buffer containing JEV antigen in various concentrations of 0.5, 1.0, 2.0 and 5.0 µg/ml. The plates were incubated at 4°C overnight, washed four times with PBS-T. The unsaturated sites were blocked by 400 µl blocking buffer (PBS-T with 5% SMP) and incubated at 37°C for 2 h in humid conditions. Again washing was done and 50 µl of various dilutions of known positive and negative serum samples ranging from 1:50 to 1:1000 were added in duplicate to separate wells. The plates were further incubated at 37°C for 2 h and again washed as above and 50 µl HRPO conjugate of various dilutions (1:5000 to 1:15000) were added to each well. Plates were again incubated at 37°C for 2 h, washed and 100 µl OPD-substrate was added to each well, incubated for 5-10 min for color development. The reaction was stopped with 50 µl 4N H2SO4 after color formation and OD was read at 492 nm.

The positive to negative threshold for the standardized I-ELISA was determined (Dey et al. 2004). The ELISA as describe above was perform in triplicates and mean of OD values obtained were calculated. Then one standard deviation unit was added in mean OD of respective dilutions. By checkerboard titration, optimal antigen concentration and serum dilution was determined and analyzed. Samples showing positive to negative (P/N) ratio ≥2 were considered positive. The results obtained were statistically analyzed and various values viz., sensitivity, specificity, predictive values for positive test, predictive values for negative test and kappa values were calculated (Thrusfield, 2005).

RESULTS AND DISCUSSION

Standardization of I-ELISA: The antigen concentrations recorded were 1.01 mg/mL and 1.52 mg/ml, respectively, for JE and WN viral antigens. The optimal concentration of JE antigen and serum dilution for standardized I-ELISA (IgG) which showed maximum difference between positive and negative sera was determined to be 50 ng/well antigen and 1:200 serum and 1:10000 conjugate dilutions (Fig.1&2). Pig sera with OD ≥1.003 was taken positive for detection of IgG antibodies to JE, whereas, for detection of IgM antibodies in the swine sera assay optimized at 1:300 serum dilution with 1 µg /ml (50 ng/well) of antigen and 1:15000 goat anti pig IgM HRP conjugate (Fig.3&4). OD ≥0.883 was considered positive for IgM antibodies to JEV in pigs. For detection of WN antibodies using SMB antigen prepared from WNV (Strain No. P 23085) revealed optimum results at 5µg/mL (250ng/well) antigen concentration, 1:200 serum and 1:10000 conjugate dilutions.

Most of the researchers reported utility of IgM Capture ELISA for detection of antibodies to JE, DEN and WN viral infections in human (Solomon et al., 1998; Burke et al., 1982; Cardosa et al., 1993 and Ravi et al., 2006). However, reports on development and use of indirect ELISA for detection of IgG antibodies against these diseases in

FIG 1: Optimization of antigen dilution for I-ELISA (IgG) in Plgs at 1:200 serum quution and varying antigen concentrations.
TABLE 1: Prevalence of Japanese encephalitis in pigs

<table>
<thead>
<tr>
<th>Area/Region</th>
<th>Total Sera screened</th>
<th>I-ELISA (IgG)</th>
<th>I-ELISA (IgM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Positive</td>
<td>% Prevalence</td>
<td>No. Positive</td>
</tr>
<tr>
<td>Bareilly</td>
<td>48</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Deonar slaughter house</td>
<td>139</td>
<td>36</td>
<td>40</td>
</tr>
<tr>
<td>IVRI Pig farm</td>
<td>55</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>North-East region</td>
<td>88</td>
<td>31</td>
<td>20</td>
</tr>
<tr>
<td>Chandigarh</td>
<td>58</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>Goa</td>
<td>100</td>
<td>29</td>
<td>39</td>
</tr>
<tr>
<td>Overall Prevalence</td>
<td>488</td>
<td>135</td>
<td>141</td>
</tr>
</tbody>
</table>

Animals found to be scanty. Although, IgM antibodies predominantly described acute infection but IgG is a major antibody in humoral immunity and JEV specific IgG in serum needs to be explored (Xinglin et al., 2005). The findings of the present study could be comparable with some of the available literatures (Konishi and Yamaoka, 1982; Konishi and Yamaoka, 1983; Chang et al., 1884; Xinglin et al., 2005 and Yang et al., 2006). Chang et al. (1984) developed biotin labeled antigen sandwich ELISA for detection of JE antibodies in human and animal sera revealed that their assay could detect the antibody with lower titer which were read negative in HI test. In present findings, similar results were observed and indirect ELISA could detect the low antibody titre, as few of the samples read negative in HI were positive by standardized ELISA and VNT. In the present study, an attempt made to evaluate standardized I-ELISA revealed significant results in detection of JE in swine sera.

Comparative efficacy of I-ELISA: The sensitivity, specificity, accuracy, predictive value for positive test and predictive value for negative test for I-ELISA (IgG) with VNT, respectively, recorded were 82.85 %, 78.94 %, 80.82 %, 78.37 % and 83.33 %. Similarly, values for I-ELISA (IgM) with VNT recorded to be, sensitivity (62.65 %), specificity (81.57 %), accuracy (72.60 %), predictive value for positive test (78.87 %) and predictive value for negative test (83.33 %) (table 2). The concordance of the I-ELISA vis-à-vis VNT revealed good (kappa=0.62) and moderate (kappa = 0.46) agreement towards detection of IgG and IgM antibodies against JE in pigs, respectively.

Good agreement of I-ELISA (IgG) observed in the present study indicates the evidence of validity of standardized test for sero-survey of JE in pigs. Standardized ELISA assay showed higher predictive values for negative test which indicates correctness of detection of true negative samples. Comparatively low sensitivity and specificity of indirect ELISA could probably be due to smaller sample size of VNT. Variations in the correlations of standardized ELISA occur, if the sample size is small as revealed by Ravi et al. 2006. Another possible reason may be detection of only IgG
antibodies by former test, whereas VNT shows positivity for both neutralizing antibodies IgG and IgM (Ting et al., 2001). Relatively low sensitivity may also be attributed due to indirect ELISA format. IgM Capture ELISA is highly sensitive and specific because of the IgM capture format that eliminates potential background caused by extraneous antibody, resulting in less frequent nonspecific reactions and removing false positive reactions caused by rheumatoid factor as stated by Feinstein et al. (1985). Another advantage of IgM capture ELISA is elimination of competition between IgG and IgM for antigen binding thereby reducing the occurrence of false positive results (Feinstein et al., 1985 and Ravi et al., 2006).

One of the earliest ELISA developed for screening of JE in swine sera was by Konishi and Yamaoka (1982). The test was later on modified by shortening the ELISA reaction time and use of whole blood for serum for large scale surveys (Konishi and Yamaoka, 1983). Their assay showed high correlation with HI and very good agreement (96.1%). A sandwiched ELISA employing biotin labeled antigen was developed for detection and quantitation of JE antibodies from different species of animals by Chang et al., 1984. Burke et al. (1985) also used ELISA to study of IgM in pig sera, however, these assays were never used widely as epidemiological tool for JE surveys and HI remained one of the preferred and widely used test for serosurveillance of JE in pig. More recently, indirect ELISA (Yang et al., 2006) and a quantitative ELISA (Xinglin et al., 2005) were developed to detect antibodies against JEV in swine. The quantitative ELISA of Xinglin et al. (2005) showed 100% specificity and 91.2% sensitivity, whereas, indirect ELISA of Yang et al. (2006) showed comparable sensitivity with VNT and HI but specificity was recorded low.

**Cross reactivity:** Flaviviruses show cross reaction with closely relates viruses viz WN and dengue in various serological assays (OIE, 2004). The cross-reactivity can be eliminated to some extent by use of purified JE antigen or recombinant antigen based ELISA as recorded in the studies of Xinglin et al., (2005) and Yang et al., (2006). The results indicated weak positivity with WN antigen and all sera samples which showed positive signals for WN antibodies were strong positive for JE. These observations suggest that positivity to WN may be because of antigenic closeness of both viruses with each other. IgG antibodies have high degree of cross reactivity than IgM to homologous and heterologous flaviviruses and these antibodies persist longer than IgM antibodies in the sera (Holmes et al., 2005). Similar results have also been reported by Hogrefe et al. (2004), wherein performance study of IgG and IgM immunoglobulin ELISA using recombinant WNV antigen revealed cross-reactivity with other related flaviviruses viz., Saint Louis Encephalitis, Dengue, JE and Yellow Fever. The cross reactivity was more for IgG immunoglobulin. Ravi et al. (2006) also observed some degree of cross reactivity of conventional IgM Capture ELISA among closely related flaviviruses. Cross-reaction in IgM ELISA was attributed due to presence of rheumatoid factor in sera which gave false positive reaction. Cross reaction is very commonly noted with flaviviruses. It may be highest within a serocomplex and also occur between flaviviruses of different serocomplex as recorded by Cardosa et al. (2002); Martin et al. (2002) and Yoshii et al. (2003). In several other reports cross reactivity between various Flaviviruses has been reported using conventional tests (Yamada et al., 2003) as well as tests developed using recombinant antigens (Cardosa et al., 2002).

**Sero-prevalence:** Overall prevalence of JE recorded by standardized ELISA was 28.89 %. Higher prevalence was observed in North East region of India (35.22 %), followed by Chandigarh (31.03 %), Goa (29.00 %), Deonar slaughter house (25.89 %), Bareilly (20.83 %) and IVRI Pig farm (20.00 %) for IgG antibodies. Prevalence of JE IgM antibodies recorded higher with sera from Goa (39.00 %) followed by Chandigarh (34.48 %), Deonar slaughter house (28.97 %), Bareilly (22.91 %), North East (22.72 %) and IVRI Pig farm (20.00 %) (table 1). To assess cross reaction with WN, a total of 179 random sera were screened with SMB WN antigen and 10.05 % samples revealed weak positivity. However, all sera samples showing weak positivity for WN exhibited strong positive signals for JE, with OD difference of average 0.250. Pre coated ELISA plates with JE antigen gives clear difference between positive and negative samples up to 6 months.

Higher seroprevalence of JE in swine population was observed from all areas of collection. Such results are obvious as swine plays important role in JEV amplification

**TABLE 2:** Comparative efficacy of I-ELISA for diagnosis of Japanese encephalitis in swine

<table>
<thead>
<tr>
<th>Details</th>
<th>I-ELISA (IgG) vs VNT (n = 73)</th>
<th>I-ELISA (IgM) vs VNT (n=73)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of true positive samples (a)</td>
<td>29</td>
<td>22</td>
</tr>
<tr>
<td>No. of false positive samples (b)</td>
<td>08</td>
<td>07</td>
</tr>
<tr>
<td>No. of true negative samples (d)</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td>No. of false negative samples (c)</td>
<td>06</td>
<td>13</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>82.85</td>
<td>62.85</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>78.94</td>
<td>81.57</td>
</tr>
<tr>
<td>Efficiency / accuracy (%)</td>
<td>80.82</td>
<td>72.60</td>
</tr>
<tr>
<td>Predictive value for positive test (%)</td>
<td>78.37</td>
<td>75.86</td>
</tr>
<tr>
<td>Predictive value for negative test (%)</td>
<td>83.33</td>
<td>70.45</td>
</tr>
</tbody>
</table>
and harboring infection for longer period of time (Geeverghese et al., 1991 and Solomon et al., 2003). The regions from which the sera samples collected are well known endemic regions for JEV persistence. Sero-positivity in swine from Deonar slaughter house was also recorded to be high which is attributed to the fact that pigs brought for slaughter there are mostly from Goa, Karnataka, Andhra Pradesh states of India which are JE endemic regions (Geeverghese et al., 1991, Geeverghese et al., 1994 and Tiroumourougane et al., 2002). Varying degree of JE prevalence revealed in earlier studies from India in pigs viz. 40% from Bareilly and adjoining districts (Mall et al., 1995); Haryana (18% ) and Karnataka (47.22%) (Geeverghese et al., 1987); Tamil Nadu-26.4% (Kumanan et al., 2002) and Chandigarh- 30.03% (Ratho et al., 1999). These researchers studied JE prevalence by HI or VNT. This is probably the first Indian report on standardization of I-ELISA for detection of IgG and IgM antibodies against JE infection in pigs. The findings of the present study revealed higher positivity to IgM antibodies as compared to IgG from all regions except North East and IVRI Pig farm which is indicative of recent exposure of swine population to JEV infection. Further stated that all sera samples were collected during the months of June to October, which is the period of JE transmission or occurrence in India. These findings are in compliance with some of the previous findings, wherein boost in HI titer during epidemic months, i.e. October to December in pigs has been reported (Geeverghese et al., 1994). In another study from Nepal, lower prevalence of IgM antibodies than IgG antibodies was recorded in pigs by competitive ELISA because of the reason that none of the samples were collected during JE endemic months (Pant et al., 2006).

Since JE situation is endemic to hyper endemic in almost 21 states of India and still its detection in pigs is being done by HI or VNT only. No ELISA formats are available nor standardized for pigs in Indian context. Therefore, present task was undertaken to standardize indirect ELISA and to access its applicability for monitoring JE infection in pigs. Although, sensitivity and specificity recorded lower with present standardized assay as compared to other researchers, kappa values revealed good agreement which is suggestive of its utility for serosurveillance of IgG against JE in Indian pig population. Further modification of indirect format to IgM capture format is possible which shall improve the sensitivity and specificity of standardized assay.

CONCLUSION

ELISA appears to be most appropriate test for studies on JE virus when large number of samples to be studied in limited period of time. As facilities for detection of JE infection is limited to very few laboratories in India, and assays viz HI and VNT requires expertise because of live virus handling, indirect format of ELISA could be a promising tool to strengthen monitoring and surveillance activities of JE in pigs and to explore the role of other domestic animals in overwintering this infectious agent.

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REFERENCES


