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Development of recombinant nonstructural 1 protein based indirect enzyme linked immunosorbent assay for sero-surveillance of Japanese encephalitis in swine

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ABSTRACT

Japanese encephalitis virus (JEV) causes severe neurological disease in humans, especially among children. The disease is endemic in several South Asian countries including India. Swine play a major role as amplifier host for JEV and act as a source of infection to humans through mosquito bite. Early detection of either virus or antibodies in swine will aid to undertake control measures to prevent virus spread to humans. Swine seldom show symptoms of JEV infection and the viraemic phase lasts for a short period of 3 to 4 days indicating the potential of detection of antibodies, which remain for relatively longer period, as a suitable alternative. Cost effective and sensitive assays for the detection of JEV antibodies in swine are not available indigenously. Hence, we have developed a recombinant nonstructural protein 1 (rNS1) based enzyme linked immunosorbent assay for the detection of IgG antibodies against JEV in swine. The test is robust, highly sensitive (91%), specific (97%), reproducible and affordable. Field validation of the assay was done by screening 3628 swine Serum samples collected from different parts of India. The overall sero-positivity was found to be 32.22%. The developed ELISA can be readily incorporated into surveillance programs for detection of Japanese encephalitis virus activity in swine population thereby aiding in prediction of outbreaks in humans.

1. Introduction

Japanese encephalitis (JE) is a re-emerging mosquito borne flaviviral zoonotic disease. Japanese encephalitis virus (JEV) is one of the major causes of encephalitis in children and it is estimated that annually JEV causes 50,000-67,900 cases leading to 10,000-20,000 deaths (Campbell et al., 2011). Actual number of cases and deaths are certainly much higher than reported due to lack of suitable diagnostics and surveillance systems especially in developing countries. The increasing global population, novel agricultural practices and climate change have facilitated the perpetuation of JE vector, *Culex* spp., thereby increasing the threat of JE cases in human population.

Swine, being an amplifier host of JEV plays an important role in the epidemiology of JE cases occurring in humans. JEV in swine undergoes two amplification cycles, during first cycle roughly 20% of swine become infected and develop antibodies within 10 days, followed by a second cycle 1-2 weeks later in which sero-conversion occurs in almost 100% swine population. The human clinical cases occur following extrinsic incubation period of 1-2 weeks in mosquitoes after second cycle of amplification in swine (van den Hurk et al., 2009). Detection of antibodies against JEV in swine population is therefore a feasible alternative to predict outbreak of JE and to initiate necessary control measures to prevent the spread of infection to humans. Availability of a reliable diagnostic test with high sensitivity and specificity can be an effective tool to conduct sero-surveillance of JE in swine population especially in Southeast Asian countries where JE is endemic in human population.

Virus neutralization test is considered as the gold standard for detection of JEV antibodies. However, requirement of skilled laboratory personnel, constant supply of JEV and round the year maintenance of

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cell lines makes the test labour intensive and expensive, consequently limiting it to referral laboratories. Different ELISAs using whole JEV antigen and envelope (domain III) protein have been tested with varying sensitivity and specificity in the recent past (Xinglin et al., 2005; Yang et al., 2006; Fan et al., 2010; Kolhe et al., 2015). Unfortunately these ELISAs suffer from one or more limitations. The whole JEV antigen based ELISA involves handling of live virus exposing the laboratory workers to occupational infection. Further, the whole JEV antigen may cross react with closely related *Flavivirus* namely, West Nile virus which compromises the specificity of the test. Further, the complete envelope protein possesses certain cross reacting epitopes (Chiou et al., 2008) and the use of only domain III of envelope protein reduces the test sensitivity to an unacceptable extent (80%) as revealed in our previous study (Rawat, 2013). The nonstructural protein 1 (NS1) of JEV, unlike envelope protein has been reported to overcome the problem of cross reactivity with other related flaviviruses (Shu et al., 2001; Kitai et al., 2007, 2011).

Considering the inherent drawbacks attached to the existing diagnostic tests, a recombinant NS1 based indirect IgG ELISA was standardized in the present study with the objective of developing a robust diagnostic assay for JE sero-surveillance in swine population. To the best of our knowledge, this is the first report on the development of indirect IgG ELISA using recombinant NS1 protein for sero-surveillance of JE in swine.

2. Materials and methods

2.1. Virus strain

The reference strain of Japanese encephalitis virus (JEV) GP78 procured from Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India and maintained in Vero cell lines was used for cloning and expression studies.

2.2. Cloning and expression of NS1 gene

The ssRNA was extracted from JEV infected vero cell culture supernatant using TRIzol-LS reagent (Ambion, Invitrogen) as per manufacturer instructions. The oligonucleotide primers (JENSF1 (sense): 5′-CGCGGATCCATGACTGGATGTGCCATTGACATC-3′; JENSR2 (antisense): 5′-GTGCTCGAGTTCACCATTGAAAGCATCAAC-3′) for amplification of full length NS1 gene were designed and restriction sites for the enzymes *Bam*HI and *Xho*I were incorporated. The NS1 gene was amplified using two step RT-PCR and purified PCR product was cloned into pET32a expression vector (Novagen) followed by transformation of recombinant plasmid into *E. coli* BL21 *pLys*S competent cells. The transformed colonies were screened for the insert in their plasmids using *Bam*HI and *Xho*I enzymes. A few of the transformed colonies, which showed insert in their plasmids, were cultured overnight in LB broth. About 1% of the overnight culture was transferred to 10 ml of LB broth containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) and incubated in shaker incubator at 37 °C for 2-3 h till the cells reached mid log growth phase having OD_{600} between 0.6-1.0. Subsequently, the culture was induced using 1 mM IPTG (Isopropylthio-β-galactoside) and incubated at 37 °C with vigorous shaking. One ml of culture was collected at hourly interval to study the expression kinetics of NS1 gene by SDS-PAGE.

2.3. Purification of recombinant protein

The poly-histidine (6x-his) tagged recombinant protein was purified under the denaturing conditions by nickel chelating affinity chromatography using imidazole gradient method as elaborated in prior art (Dhanze et al., 2018). In brief, the harvested cells containing protein in the form of inclusion bodies were solublized in lysis buffer containing urea (100 mM NaH_2PO_4, 10 mM Tris base, 8 M urea, pH 8) by vigorous pipetting and vortexing followed by incubation at room temperature for 1 h. The suspension was centrifuged at 10,000 rpm for 20 min at 4 °C and the supernatant containing crude protein lysate was passed through Ni-charged resin (Bio Rad) column. The column was washed using urea lysis buffer having pH adjusted to 6.3 and then by urea lysis buffer (pH 7.4) containing 10 mM of imidazole. The protein was eluted by passing urea lysis buffer (pH 7.4) containing imidazole in increasing concentration through the column.

Urea lysis buffer with 20 mM imidazole (6 ml) was passed through the column followed by subsequent passing of 6 ml each of buffer containing 40 mM, 80 mM, 100 mM, 200 mM and 500 mM imidazole. The purified recombinant protein was dialyzed sequentially in decreasing concentrations of urea in PBS. After dialysis the protein was in soluble form. The protein concentration was measured using Bradford reagent and stored at −80 °C for subsequent use.

2.4. Raising of hyperimmune Serum

Hyperimmune Serum against recombinant NS1 protein was raised in a one year old New Zealand white rabbit after taking due permission from the Institute Animal Ethics Committee. The rabbit was injected subcutaneously with purified rNS1 protein (250 µg) mixed with incomplete Freund's adjuvant in 1:1 ratio. The rabbit was subsequently injected with two boosters, first with 250 µg and second with 100 µg protein mixed with incomplete Freund's adjuvant at 21 days intervals. One week after the second booster, hyperimmune Serum was collected and used to confirm recombinant protein by dot blot and western blot (WB) analysis.

2.5. Virus neutralization test

Virus neutralization test (VNT) was used to screen 131 field swine Serum samples to identify a set of positive and negative reference sera panel for standardization of ELISA. All the samples were tested in duplicate in 96-well tissue culture plates using porcine stable (PS) kidney cells as described by Gulati et al. (2011). Serum samples were heat inactivated at 56 °C for 30 min in order to inactivate the components of the complement. Two-fold serial dilutions of the Serum in a 50 µl volume were prepared and an equal volume of 300 $TCID_{50}$ of the JEV was added. The virus-Serum mixture was incubated at 37 °C for 1 h followed by addition of 100 µl of PS kidney cell suspension (2×10^5 cells/ml) to each well. The plates were then incubated at 37 °C with 5 % CO_2 for 72-96 h and cytopathic effect (CPE) in each well was recorded. The absence of CPE indicates neutralization of the virus and the sample was inferred as positive for JEV antibodies. The reciprocal of the highest Serum dilution that completely inhibited CPE in the wells was expressed as the neutralization titer. Titers of 8 and above were considered positive for JEV antibodies.

2.6. Standardization of ELISA

Indirect IgG ELISA was standardized using the checker board titration method for optimization of antigen concentration, Serum dilution and conjugate dilution. For standardization of ELISA, set of JE positive (n = 58) and negative (n = 73) swine sera, previously confirmed by VNT as mentioned above, were used as reference positive and negative samples. The VNT titer of positive sera was between 1:8 and 1:256 and titer of negative sera was < 1:8. At first different concentrations of rNS1 protein from 0 to 10 µg/ ml in carbonate buffer (pH 9.6) were prepared and 50 µl of each concentration was added to 96 well polystyrene maxisorp plate (Nunc, Denmark). Plate was incubated overnight at 4 °C. The wells were washed three times with PBS-T (0.05% Tween 20) to remove excess unadsorbed antigen. The unbound sites were blocked with 300 µl of blocking buffer i.e. 5% skimmed milk powder (SMP) (Difco) in PBS-T for 2 h at 37 °C. Blocking with two more blocking buffers, plain PBS-T (0.05%) and 2% bovine Serum albumin

(BSA) in PBS-T was also tried. After incubation the blocking buffer was removed and plate was washed as described earlier. Two fold dilutions of positive and negative reference sera ranging from 1:100 to 1:800, diluted in blocking buffers were added to the plate in 50 µl volume. Plate was incubated at 37 °C for 1 h and washed three times with PBS-T. Two different dilutions (1:5000 and 1:10,000) of goat anti-pig IgG horse radish peroxidase conjugate (Bethyl) diluted in blocking buffer was added to each well (50 µl), followed by incubation at 37 °C for 1 h. The plate was washed four times with PBS-T and freshly prepared 100 µl of substrate solution (10 ml citrate buffer, 10 mg OPD, 8 µl H_2O_2, pH 4.5) was added to each well in dark. Color was allowed to develop for 5 min followed by stopping of reaction by addition of 4 N H_2SO_4 (50 µl). The absorbance was measured at 492 nm using microplate reader (Thermo Scientific, Multiskan Ex). Antigen control, Serum control and conjugate control were also used during the process of standardization. Amongst the different antigen concentration, blocking buffer, Serum dilution and conjugate dilution tried, the combination that gave highest OD_{492} ratio between reference positive and negative Serum (P/N value) were scored as optimal working conditions.

For the purpose of the interpretation of results of test Serum, comparison of S and N values was done, where S stands for optical density of test Serum and N stands for optical density of negative control. The samples showing S/N ratio of ≥2 were considered as positive for JE. Initially, the cut-off value was calculated by taking into account OD_{492} of 120 negative Serum samples using the equation: Cut-off = Average OD of negative samples + 3 (Standard deviation). However, to avoid inter-laboratory variation, S/N ratio of ≥2 was considered as criterion for interpretation of the test results and the negative control having OD_{492} value equivalent to half of the cut-off value was selected to perform the subsequent assays.

The shelf life of pre-coated and blocked ELISA plate stored at 4°C was checked at every 15 days interval by screening a set of reference positive and negative Serum samples.

2.7. Diagnostic efficacy of rNS1 based indirect IgG ELISA

The diagnostic efficacy of rNS1 protein based indirect IgG ELISA in terms of sensitivity and specificity was calculated in comparison with VNT using formulae described by Thrusfield (2005).

2.8. Validation of standardized rNS1 ELISA

Validation of the standardized ELISA was carried out as per the OIE guidelines (OIE, 2014). Five different laboratories within the ICAR-Indian Veterinary Research Institute (IVRI) participated in the inter-laboratory validation. The pre-coated ELISA plates along with the protocol, reagents and set of 10 positive and 10 negative coded Serum samples were supplied to the five laboratories. The inter-institutional validation was carried out at three institutes of Indian Council of Agricultural Research, one State Agriculture University and one institute under the Department of Animal Husbandry, Dairying and Fisheries, Govt. of India. The pre-coated ELISA plates along with the reagents and set of 20 positive and 20 negative coded Serum samples were supplied to the institutes. Kappa value was calculated to know the agreement between results of our laboratory and the other laboratories/institutes as per Thrusfield (2005).

2.9. Screening of field samples using standardized rNS1 ELISA and whole virus antigen ELISA

A total of 3628 swine Serum samples collected from all 6 zones of India (North, Central, West, East, Northeast and South) were screened using the rNS1 protein based indirect IgG ELISA. Out of 3628 samples, 500 samples were also screened with previously developed IgG ELISA employing whole JE virus antigen (Kolhe et al., 2015) for the purpose of selecting the best format for future sero-surveillance of JE in pigs.

3. Results

3.1. Production and purification of recombinant NS1 protein

The recombinant NS1 protein was expressed with thioredoxin (Trx) tag and 6 His tag at the N and C terminals, respectively. The expressed full length NS1 protein was of 59 kDa in size (Supplemental Fig. S1). The NS1 protein was totally expressed as insoluble inclusion bodies, with negligible protein expressed in the soluble fraction. Study of hourly kinetics of clones induced with 1 mM IPTG on SDS-PAGE revealed that protein expression started within 1 h of induction and was maximum around 5 h of induction, after which it was almost constant. The protein was purified by affinity chromatography. The SDS PAGE analysis revealed that protein started appearing with 40 mM imidazole elute and increased steadily thereafter with maximum concentration in 80 mM imidazole elute followed by gradual decline in subsequent elutes (Supplemental Fig. S2). The imidazole elutes with higher protein concentratrion were pooled rNS1 and protein concentration was found to be 350 µg/ml by Bradford method. The purity of recombinant protein was confirmed by single band in SDS PAGE (Supplemental Fig. S 2) and by dot blot and western blot analysis.

3.2. Optimal conditions for rNS1 based indirect IgG ELISA

The checkerboard titration revealed that an antigen concentration of 500 ng/well (10 µg/ml) and 1:200 dilution of the Serum was optimum for the ELISA (Supplemental Fig. S3). Amongst the blocking buffers used, 5% skimmed milk powder (SMP) in PBS-T (0.05%) was found to be the best and the same was used to dilute Serum and conjugate. The P/N ratio was higher when 5% skimmed milk powder in PBS-T (0.05%) was used as diluent buffer for Serum and conjugate in comparison to plain PBS-T (0.05%) as diluent buffer (Supplemental Table 1).

The optimum dilution of goat anti-pig IgG HRPO conjugate was 1:10,000. The shelf life of coated and blocked ELISA plate was found to be 6 months during storage at 4 °C.

3.3. Evaluation of diagnostic efficacy of rNS1 based indirect IgG ELISA

The result of samples screened by VNT and rNS1 ELISA are summarized in Table 1. The diagnostic sensitivity and diagnostic specificity of rNS1 ELISA was found to be 91% and 97%, respectively.

3.4. Validation of rNS1 IgG ELISA

The results of inter-laboratory and inter-institutional validation when compared with the results of our own laboratory revealed excellent reproducibility of rNS1 IgG ELISA. Results of inter-laboratory validation were found in concordance for all the labs and hence kappa value was 1 indicating the perfect agreement between all the laboratories (Supplemental table S2). The kappa value of inter-institutional validation results ranged between 0.823 to 1.0, which again indicate almost perfect agreement between all the institutes involved in validation (Supplemental table S3).

Table 1
Contingency table of samples screened by VNT and rNS1 ELISA.

rNS1 ELISA	VNT		Total
	Positive	Negative	
Positive	53 (True positive)	2 (False positive)	55
Negative	5 (False negative)	71 (True negative)	76
Total	58	73	131

Table 2
Results of sero-surveillance of swine population for JEV infection across India using rNS1 ELISA.

Sl. No.	Zones of India	Number of swine sera screened	Number of sera positive	Percent positivity of anti-JEV IgG
1	North	2099	572	27.25%
2	East	703	202	28.7%
3	West	488	181	37.1%
4	Central	73	46	63.01%
5	South	164	115	70.12%
6	Northeast	101	53	52.47%
	Total	**3628**	**1169**	**32.22%**

3.5. Sero-positivity in swine population

Out of 3628 swine sera samples screened, using rNS1 ELISA, 1169 samples were found positive for IgG against JE virus (Table 2). The overall sero-positivity was 32.22% in swine population of India.

3.6. Comparison of rNS1 protein based ELISA with whole JE virus antigen based ELISA

Out of 500 field swine Serum samples tested, 28% and 32.22% of the samples were found positive by rNS1 ELISA and whole JE virus antigen ELISA, respectively. The diagnostic sensitivity of rNS1 ELISA was found to be 91 per cent and specificity of rNS1 ELISA was found to be 97 per cent while diagnostic sensitivity and specificity of whole virus antigen ELISA was 82.85% and 78.94%, respectively.

4. Discussion

Recombinant NS1 protein was expressed and purified with the objective to develop a sensitive and specific ELISA for detection of IgG against Japanese encephalitis virus in swine Serum samples. In the previous studies, IgG ELISA using purified whole JE virus antigen has been standardized for swine Serum samples (Xinglin et al., 2005; Yang et al., 2006; Kolhe et al., 2015). The slow growing nature of JEV makes it difficult to produce it in bulk for use as antigen. Moreover, use of whole JEV as antigen increases chances of cross reactivity resulting in false positive samples as revealed by comparatively low specificity of whole virus antigen based ELISA(s) (Yang et al., 2006; Kolhe et al., 2015). The whole JEV antigen contain epitopes for envelope protein also which are proven to be cross reactive (Chiou et al., 2008). Due to these reasons, the possibility of using recombinant proteins as antigen instead of whole JE virus antigen was explored. The NS1 protein is secreted from JEV infected mammalian cells and hence considered as the most suitable antigen for use in ELISA system among the seven non-structural proteins of JEV (Konishi et al., 2004; Hua et al., 2013). It elicits protective immune response in the host body and has been used by research workers as a diagnostic candidate for screening horse and human Serum samples (Konishi et al., 2004; Konishi and Kitai, 2009; Li et al., 2012).

In our study, recombinant NS1 protein was expressed in insoluble form which might be due to high level of expression in cells or lack of post translational modifications. Earlier works on the expression of NS1 (Lim et al., 2007) and envelope (Shukla et al., 2009; Fan et al., 2010) proteins have also reported the tendency of the viral protein to form inclusion within the *E. coli* host. However, this did not affect the antigenicity of protein as revealed by the protein's reactivity in western blot analysis and ELISA. In the present study, indirect IgG ELISA for sero-diagnosis of JE in swine using recombinant NS1 protein was successfully developed with high diagnostic sensitivity and specificity of 91% and 97%, respectively. The high specificity of this ELISA may be attributed to the use of rNS1 antigen as compared to previous studies wherein 80% (Yang et al., 2006) and 78.94% (Kolhe et al., 2015) specificity was reported using whole JE virus antigen. Swine sera have been reported to produce relatively high non-specific signals in ELISA (Konishi and Yamaoka, 1982), which was reduced to greater extent in this study by optimizing the blocking buffer, serum dilution and diluent buffer for serum and conjugate. A reproducibility study is the fulcrum in validating diagnostic assays. Excellent reproducibility as shown by value of kappa statistics across the five laboratories and five institutes during validation studies ensured that the ELISA developed in the present study is a promising diagnostic for JE sero-surveillance in swine population. The primary aim while developing this assay was to develop a robust test for purpose of sero-surveillance of JE in swine. However, there is a scope of using the recombinant NS1 protein in developing assay for diagnosis of JE in humans due to its conserved nature which has also been proven previously by other researchers (Tripathi et al., 2012).

Sero-positivity of JE virus was found in swine population from all six zones of India confirming the circulation of virus in all geographical regions of the country. The probable reason of higher sero-positivity in South zone could be that unlike in northern and western part of India which experience extreme weather events, south India witnesses favourable weather throughout the year, which is conducive for mosquito activity. It is also supported by the fact that, south Indian states viz., Karnataka, Tamil Nadu, Telangana report human JE cases every year. Moreover in western region, population of pigs itself is less compared to rest of the India.

Several studies have demonstrated the correlation between JE virus activity in swine and outbreaks in humans (Singh et al., 2013; Dhanze et al., 2016). The continued circulation of JE virus in swine population increases the risk of outbreaks in humans and warrants efficient sero-surveillance program in swine population, which is a suitable sentinel for this disease. However, sensitive and cost-effective diagnostic for effective JE sero-surveillance in swine was not available indigenously. The rNS1 based ELISA developed in the present study is an affordable, sensitive and robust tool which will aid in better sero-surveillance of JEV in swine population and thereby aiding in prevention of disease outbreak in humans.

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Declaration of Competing Interest

The authors have no conflict of interests to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113705.

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