Sero-Positivity of Japanese Encephalitis Virus in Equine Population of India Using IgG ELISA: Unraveling The Need for Vaccination

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Abstract

Japanese encephalitis (JE) is a mosquito borne flaviviral zoonoses, causing fatal disease in equines and humans. JE is endemic in most of the states of India with occurrence of human cases every year. The horses are not vaccinated against JE in India and thus they are at more risk of acquiring the disease. Due to non-availability of indigenously developed ELISA and high cost of imported kits, regular sero-surveillance is not being carried out to assess the true picture of JE virus in equine population of India. Therefore, a recombinant NS1 protein based indirect IgG ELISA was developed with the objective to assess the sero-positivity of JE virus in equine population of India. The diagnostic sensitivity and specificity of developed ELISA was 84.73 % and 86.70 %, respectively. The validation studies revealed good reproducibility of ELISA with kappa value ranging from 0.75 to 1 between the results of different laboratories. A total of 2069 horse serum samples were screened using the developed ELISA and 401 samples were positive for IgG against JEV with an overall sero-positivity of 19.38% in equine population of India. A sero-positivity of 25.90% and 12.22% was recorded in Himachal Pradesh and Jammu-Kashmir, both hill states of North zone of India for the first time, revealing the spread of virus to the non-endemic parts of the country. The high sero-positivity of JE virus recorded in equine population warrants the need for initiation of vaccination of horses in India to prevent the morbidity and mortality.

Introduction

Japanese encephalitis (JE) is a flaviviral zoonotic disease, responsible for causing fatal encephalitis in equine and humans. The vertebrate hosts such as pigs and birds play an important role in amplification and the maintenance of the virus, respectively, while mosquitoes are responsible for the transmission of virus (Halstead and Jacobson, 2008). Horses are the most affected animals, although other equids are also susceptible to JE virus (JEV) infection (OIE, 2016). Infected horses seldom develop fatal encephalitis with maximum infective mosquito bites resulting in subclinical infections. Equines infected with JEV may develop clinical signs such as fever, anorexia, depression, impaired locomotion, impaired vision, paresis, and paralysis. The mortality rate is high when JE infected horses show neurological symptoms and ranges between 5 to 30 per cent (Ihara et al., 1997; Yamanaka et al., 2006; Gulati et al., 2011).

JE is prevalent in most of the Asian countries including India. The vaccination against JE in equines has been practiced in several Asian countries including Japan, China and Korea leading to reporting of few sporadic cases instead of outbreaks in equine population of these countries (Yamanaka et al., 2006; Lam et al., 2005; Lian et al., 2002; Yang et al., 2008). The equines are not vaccinated against JE virus in India and thus they are at greater risk of acquiring infection, especially in states where JE outbreaks in humans have been reported (Dhillon and Raina, 2008). The cases of JE in humans have been reported almost every year from all six zones of India (NVBDGP, 2021) though limited sero-surveillance studies in equine population have been conducted till now (Gulati et al., 2011) .

A definitive diagnosis of Japanese encephalitis in horses depends on the isolation of the causal virus. However, the isolation rate of virus from diseased or dead horses is usually very low (OIE, 2016). Hitherto,

there is a single report of JEV isolation from horses in India (Gulati et al., 2012) though the actual burden is reported to be high as revealed by a serological survey conducted by Gulati et al. (2011), using virus neutralization test. Virus neutralization test (VNT), the gold standard test, is rather costly, cumbersome and involves risk of handling live virus. Earlier, ELISA using purified whole virus antigen (Ihara et al., 1997) and recombinant protein (Konishi et al., 2004) was described by research workers from Japan to detect JEV specific antibodies in equines. However, indigenous ELISA kit for sero-diagnosis of JE in equines is not available in India and imported kits are expensive. The regular surveillance of JEV in equine population is necessary to know the actual burden of the disease which would further aid in deciding the need for vaccination of equines in our country. In the present study we have developed an indirect IgG ELISA for sero-diagnosis of JE in equines and used it for the sero-surveillance of JE in equine population of India.

Materials And Methods

Standardization of indirect IgG ELISA

Indirect ELISA for detection of IgG against JEV in horses was standardized using the checker board titration method for determining the ideal blocking agent, optimum dilution of primary antibody (serum) and optimum dilution of HRPO conjugated secondary antibody. The recombinant NS1 protein of JEV earlier expressed in our laboratory (Dhanze et al., 2019) was used as antigen in the ELISA. The set of 24 JE positive and 24 JE negative horse serum samples previously confirmed by virus neutralization test (Kapdi et al., 2017) were used for standardizing the ELISA.

The concentration of recombinant NS1 protein was made to 10 µg/ml in carbonate buffer (pH 9.6) and 50 µl of it was added to each well of 96 well polystyrene maxisorp plate (Nunc, Denmark). The plate was incubated at 4°C for overnight. The un-adsorbed antigen was removed by washing the plate thrice with PBS-T (0.05 per cent Tween 20) manually. The ideal blocking agent was determined using two buffers including 5 per cent skimmed milk powder (Difco, US) in PBS-T and 1 per cent bovine serum albumin (SRL, India) in PBS-T. The blocked plate was incubated for 2 h at 37°C. After incubation, the blocking buffer was removed by washing thrice with PBS-T. The two dilutions of 1:100 and 1:200 of positive and negative sera in different diluent buffers including 5% SMP in PBS-T and 1% BSA in PBS-T were prepared and 50 µl of each was added to the wells of plate. After incubation at 37°C for 1 h, plate was washed three times with PBS-T. The goat anti-horse horse radish peroxidase (HRPO) conjugate (Novus, US) was diluted in two dilutions of 1:5000 and 1:10,000 in different diluent buffers including 5% SMP in PBS-T and 1% BSA in PBS-T and 50 µl of each dilution was added to the wells of plate. It was incubated at 37°C for 1 h and then plate was washed four times using PBS-T and pat dried. Further, 100 µl of freshly prepared substrate solution (10 ml citrate buffer, 10 mg OPD and 8 µl H₂O₂; pH 4.5) was added to each well and the color was developed by incubating the plate in dark at room temperature for 5 minutes. The reaction was stopped using 50 µl of stop solution and the absorbance was read at 492 nm using ELISA reader (Thermo Scientific, Multiskan Ex). The conditions that resulted in highest absorbance ratio between positive and negative sera (P/N value) were considered as optimal for further screening of field

samples by ELISA. The field sample was considered positive if S/N ratio is more than 2 where S stands for absorbance of test sample while N stands for absorbance of negative control. The positive and negative sera were included as controls while screening the field samples.

Evaluation of diagnostic efficacy of ELISA

The diagnostic efficacy of rNS1 protein based IgG ELISA in terms of sensitivity and specificity in comparison with virus neutralization test (VNT) was calculated as per Thrusfield, (2005). The VNT was carried out using porcine stable (PS) kidney cells, 300 TCID₅₀ of the JEV and heat treated test serum using the protocol described by Gulati et al. (2011). A total of 522 horse serum samples were screened using VNT. The reciprocal of the highest serum dilution that completely inhibited cytopathic effect (CPE) was expressed as the neutralization titer. The serum samples showing titer of 8 and above were considered as positive for JEV antibodies.

Validation of Equine IgG ELISA kit for the detection of JE

A batch of 26 coded samples including 17 positive serum samples and 9 negative serum samples was selected for validation. The equine IgG ELISA kit was validated according to OIE guidelines (OIE, 2014) in 3 different laboratories of our institute and in 3 different institutes across India. The Cohen's Kappa value was estimated to know the agreement between results of our laboratory and the other laboratories/institutes as per Thrusfield (2005).

Field Sample screening

A total of 2069 horse serum samples were screened for JE from 13 states of India including 1566 samples from North zone, 373 samples from West zone, 86 samples from North-east zone, 40 samples from East zone and 4 samples from central zone during April 2017 to May 2020, using in-house developed IgG ELISA.

Results

Standardization of indirect IgG ELISA for sero-diagnosis of JE in horses

The checkerboard titration revealed 1:100 dilution of horse serum and 1:10,000 dilution of goat anti-horse radish peroxidase (HRPO) conjugate to be optimum for the ELISA as P/N ratio was highest (14.3) with this combination when 5% skimmed milk powder (SMP) in PBS-T (0.05%) was used as blocking and diluent buffer (Fig. 1). The P/N ratio was 8.12 with 1:200 serum dilution and 1:10,000 conjugate dilution while it was 13.36 with 1:100 serum dilution and 9.12 with 1:200 serum dilution when 1:5000 conjugate dilution was used (Fig. 1). We observed that 5% skimmed milk powder in PBS-T is better blocking and diluent buffer in comparison to 1% BSA in PBS-T as P/N ratio was two times more with skimmed milk powder combination.

Diagnostic efficacy of developed IgG ELISA

The diagnostic sensitivity and specificity of developed indirect IgG ELISA was found to be 84.73% and 86.70%, respectively. The result of 522 horse serum samples screened by VNT and ELISA to calculate diagnostic efficacy of ELISA are presented in contingency table (table 1).

Validation of IgG ELISA kit

The results of inter-laboratory and inter-institutional validation studies revealed good reproducibility of the developed IgG ELISA kit. The results of our laboratory and different laboratories and institutes involved in validation were in excellent agreement with kappa value ranging from 0.75- 1.0.

Sero-positivity of Japanese encephalitis in equine population of India

Screening of 2069 field horse serum samples using standardized IgG ELISA showed a sero-positivity of 19.38% in equine population of India. The highest sero-positivity of 30.02% was observed in West zone followed by North zone (17.81%), Northeast zone (9.30%) and East zone (5%) (Table 2). In hill states of North zone including Himachal Pradesh and Jammu- Kashmir, the sero-positivity of 25.90% and 12.22%, respectively was recorded for the first time.

Discussion

The inevitable climate change has a significant impact on vector-borne viral diseases, possibly affecting vector population dynamics and disease transmission. The isolation of JE virus from horse in the year 2003 after a gap of 17 years in Japan (Yamanaka et al., 2006) and the first report of its isolation from horse in India in year 2011 (Gulati et al., 2012) are the glaring evidences for the re-emergence of this disease. Sero-epidemiological studies are critical for predicting potential outbreaks of vector-borne viral diseases among equines and provide data for establishing a system to prevent the diseases (Yang et al., 2008). The importance of such studies is more in JE endemic countries like India, wherein prophylactic JE vaccination is not yet practiced in equine population. The non-availability of indigenously developed IgG ELISA kit for sero-diagnosis of JE in equines till now has restricted the regular sero-surveillance activity. Albeit sero-positivity of JE virus in equines of India have been described in the past using haemagglutination inhibition test and virus neutralization test (D'Souza et al., 1978; Chakravarty *et al.*, 1981; Shankar et al., 1988; Rai et al., 1992; Mall et al., 1995; Raut et al., 2003; Gulati et al., 2011).

We have developed recombinant NS1 protein based indirect IgG ELISA for sero-diagnosis of Japanese encephalitis in equines with a diagnostic sensitivity and specificity of 84.73% and 86.70%, respectively. The NS1 protein has been reported to overcome the problem of cross reactivity and can differentiate between JE and related viruses (Shu et al., 2001; Kitai et al., 2007). Since the NS1 protein is secreted from infected mammalian cells, it can differentiate between the naturally infected population and population vaccinated with inactivated virus. The NS1 protein based ELISA using capture antibody was reported from Japan to detect subclinical infections in vaccinated horses with a sensitivity of 87.5% and a specificity of 83.0% (Konishi et al., 2004).

We observed that 5% SMP is better blocking agent than 1% BSA while developing ELISA for sero-diagnosis of JE in horses. Since BSA is a serum protein, in certain circumstances it could cause non-specific ELISA signals (Xiao and Isaacs, 2012). The diagnostic sensitivity of developed ELISA as compared to VNT was low and the obvious reason is the presence of multiple antigens in the whole virus used in VNT assay. The sensitivity of VNT is high but this test has several disadvantages including labour intensive, require skilled personnel, a minimum of five days to perform, and the handling of live virus (Peters, 2012), which can be overcome by performing the developed recombinant protein based ELISA.

The sero-positivity of 19.38 per cent was recorded in equine population of India while it was reported as 10 per cent from India in the last decade (Gulati et al., 2011). The sero-positivity is detected in 4 zones of India revealing the wide geographical circulation of JE virus in equine population. We do not have any plausible explanation for high sero-positivity in Western zone though it might be due to ardeid bird mosquito cycle of JEV as pig population is less in the Western zone. We have reported a sero-positivity of 25.90% and 12.22% in Himachal Pradesh and Jammu-Kashmir, respectively, while in the previous study conducted during 2006 to 2009, nil positivity was reported from these two states (Gulati et al., 2011). Hence, it is evident that the virus has spread its realm to non-endemic temperate areas of the country. The increase in sero-positivity of JE in equine population may have serious implications in terms of morbidity and mortality of horses. Our study clearly indicates an urgent need for initiation of vaccination program against JE for horses in the country to prevent outbreaks.

Declarations

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Statement of animal rights

The study involves screening of field horse serum samples for Japanese encephalitis. The study protocol was assessed and approved by Deemed University ICAR- Indian Veterinary Research Institute (IVRI) and by Institute Research Committee, IVRI. The field samples were collected by veterinarians adhering to the regulations and guidelines on animal husbandry and welfare after taking verbal consent of the owner.

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Conflicts of interest: Authors have no conflict of interest to declare.

Availability of data and material: All data generated or analyzed during this study are included in the manuscript.

Code availability: Not applicable

Authors' contributions:

1. Conceptualization: Himani Dhanze
2. Laboratory experiments, sample collection and screening: Aarti Kapdi, Vijayata Singh, Anamika Sahu
3. Original draft preparation: Himani Dhanze and Aarti Kapdi
4. Reviewing and editing: M. Suman Kumar
5. Funding acquisition: Himani Dhanze and B.R. Gulati
6. Supervision and mentoring: K.N. Bhilegaonkar and B.R. Gulati

Ethics approval: The study involves screening of field horse serum samples for Japanese encephalitis. The study protocol was assessed and approved by Deemed University, ICAR- Indian Veterinary Research Institute (IVRI) and by Institute Research Committee, IVRI. The field samples were collected by veterinarians adhering to the regulations and guidelines on animal husbandry and welfare after taking verbal consent of the animal owner.

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Tables

Table 1
Contingency table of horse serum samples
screened by VNT and ELISA

IgG ELISA	VNT		Total
	Positive	Negative	
Positive	111	52	163
Negative	20	339	359
Total	131	391	522

Table 2
Sero-surveillance of JE in equine population of India

Sl. No.	Zones of India	Number of equine serum samples screened	Number of positive samples	Sero-positivity (%)
1	North zone	1566	279	17.81
2	West zone	373	112	30.02
3	Northeast zone	86	8	9.30
4	East zone	40	2	5.0
5	Central zone	04	0	-
6	Total	2069	401	19.38

Figures

Figure 1

Bar graph representing the difference in OD of positive and negative samples and P/N ratio with different dilutions of serum and conjugate