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Standardization of Indirect ELISA for Sero-Diagnosis of Japanese Encephalitis in Guinea Fowl and Chicken

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ABSTRACT

Japanese Encephalitis (JE) is an emerging zoonoses of public health significance and endemic in South-east Asia and India. Along with pigs, birds are important reservoirs of the JE virus and plays major role in disease perpetuation. In the present study, attempts were made to develop indirect ELISA for rapid serodiagnosis of JE in chicken and G. fowl. The optimum results were recorded at 50 ng well^{-1} of antigen, 1:100 serum dilution and 1:10000 goat anti bird IgG HRP conjugate. The present study indicated 12% prevalence of JE in Guinea fowl and 11% in broilers. The overall prevalence of JE in birds recorded was 8.07%. The sensitivity, specificity, accuracy, predictive value for positive test and predictive value for negative test of I-ELISA (IgG/IgY) recorded are 100.00, 92.10, 93.48, 72.72 and 100.00%, respectively, with virus neutralization test. It was evident from the results obtained that the developed indirect ELISA is highly sensitive, specific and accurate for diagnosis of JE in bird's sera. The concordance of indirect ELISA (IgG/IgY) vis-à-vis VNT also showed good agreement (kappa = 0.80) of standardized indirect ELISA.

Key words: Japanese encephalitis, I-ELISA (IgG/IgY), standardization, seroprevalence, chicken, guinea fowl

INTRODUCTION

Japanese Encephalitis (JE) is one of the important zoonotic diseases endemic in many parts of South East Asia. It is leading cause of childhood encephalitis (Solomon *et al.*, 2000) and the disease is endemic in almost 26 states of India. It is viral infection caused by JE virus of the genus *Flavivirus* of the family Flaviviridae. The virion is enveloped, single stranded positive sense RNA genome approximately of 11 kb. The JEV Nakayama strain was fist isolated in 1935 from human brain in Japan which is prototype strain used for development of JE vaccine. Beijing-1 strain is another important strain of JEV used for production of live attenuated vaccine designated as SA14-14-2 in China (Pugachev *et al.*, 2003). Phylogenetic studies suggested that JEV is continuously evolving in nature (Chen *et al.*, 1990). There are four geographically distinct genotypes (I, II, III and IV) widely distributed world over and Muar strain isolated from

Singapore may be the fifth genotype (Uchil and Satchidanandam, 2001; Ma *et al.*, 2003; Solomon *et al.*, 2003). Genotype III encompasses large temperate region of Asia including India.

It is a mosquito borne disease and main vector for disease transmission is mosquitoes of *Culex* species chiefly *Culex tritaeniorhynchus*. The virus has been isolated from several mosquito species but Culex is predominant. It is major cause of childhood encephalitis affecting children's. It is widespread in Asia and more than 60% world's population is living in these endemic regions (Solomon *et al.*, 2003). Swine population, water logging and paddy cultivations are the known factors for disease outbreaks but role of birds in the disease transmission has not been explored to the extent. Bird-mosquito-bird cycle is important cycle in disease transmission so as pig-mosquito-pig. Ardeid birds chiefly, pond herons and cattle egrets are known amplifiers of JEV but role of peridomestic and domestic birds needs to be explored.

Diagnosis of JEV infection is major constraint due to cross reactivity with other viruses of sero-complex by serological assays and low rate of virus isolation from clinical samples is another one. IgM capture ELISA is more sensitive and widely accepted standards for definitive diagnosis of Flaviviruses in human but there are very few reports on animals and birds side. In view of this, an indirect ELISA was developed and evaluated for rapid and sensitive sero-diagnosis of JE in chicken and guinea fowl.

MATERIALS AND METHODS

Present study on standardization of indirect-ELISA was conducted during the year 2008-2009. Known standard strains of JE and WN viruses were procured from National Institute of Virology, Pune (India) and revived and adopted in C6 36 *Aedes albopictus* mosquito cell line. The JEV antigen was purified according to the methods of Yang *et al.* (2006). It was prepared from cell culture grown formalin inactivated JEV by ultracentrifugation at 50000 g for 2 h at 4°C in Sovall Ultra Pro 80 ultracentrifuge (Kendro Lab., USA) using fixed angle rotor (A 621). The crudeconcentrates were then laid over 20-50% (w/v) discontinuous sucrose (Sigma) gradient and again centrifuged 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 was stored at -20°C and used for standardization of I-ELISA for birds. The WNV antigen was mouse brain derived antigen purified by sucrose acetone extraction method as described by OIE (2004).

A total of 288 sera samples (135 broilers, 50 cockrels and 100 guinea fowl) were collected from endemic (Bareilly, Uttar Pradesh) and non endemic (Nagpur, Maharashtra) areas and randomly processed for detection of JE specific antibodies by Virus Neutralization Test (VNT). Some of the samples were also screened by HI and sera samples strongly positive and negative both by VNT and HI were used as positive and negative control for standardization of indirect ELISA for detection of JE specific antibodies. The ELISA was standardized using checkerboards titration and positive to negative threshold was determined as per the methods of Dey *et al.* (2004). The indirect ELISA was performed in triplicates and mean of OD values obtained were calculated. Then one standard deviation unit was added in mean OD values of respective dilutions. The optimal antigen concentration and serum dilution was determined and analyzed. The samples showing positive to negative (P/N) ratio of ≥2 was considered positive by indirect ELISA.

The indirect ELISA was standardized as per the methods of Yang *et al.* (2006) with slight modifications and is summarized briefly. The microtitre ELISA plates were coated with varying antigen concentrations i.e., 50 μL/well of coating buffer containing 0.5, 1, 2 and 5 μg mL^{-1} JEV

antigen and incubation was done overnight at 4°C. Plated were washed five times with PBS containing tween- 20 and blocking was done with 400 μL 1% BSA in PBS-T. Incubation was done for 2 h at 37°C, plates were again washed as above and 50 μL of known positive, negative and test sera samples in varying dilutions from 1:50 to 1: 400 were added in triplicates. Then plates were again incubated at 37°C for 2 h and after washing 50 μL/well goat anti bird HRP conjugate (1:5000 to 1:15000) was added and again incubated at 37°C for 2 h. Plates were finally washed again and 100 μL OPD-substrate was added and plated were kept in dark for 5-10 min for colour development. The reaction was stopped by addition of 50 μL 4N H_2SO_4 and OD values were recorded at 490 nm (BIO-RAD 680 Microplate Reader, Japan).

All chicken and guinea fowl sera samples were subjected for screening of JE specific IgG/IgY antibodies by developed I-ELISA. The comparative efficacy of I-ELISA vis-à-vis VNT was calculated in terms of sensitivity, specificity, predictive values for positive test, predictive values for negative test, accuracy and *kappa* values according to Thrusfield (2005). Cross reactivity of standardized I-ELISA was also studied using WNV antigen.

RESULTS

The procured strain of JE virus was grown in cell culture and virus titer was estimated to be 1.86×10^8 PFU mL^{-1} by agarose overlay method as described by Gould and Clegg (1991). Extraction and purification of JEV and WNV antigens were done as per the standard methods and antigen concentrations recorded by ND 1000 Nanodrop spectrophotometer was 1.01 and1.52 mg mL^{-1}, respectively. Standardization of indirect ELISA for detection of IgG/IgY antibodies towards JE infection in birds showed optimum results at 1 μg mL^{-1} (50 ng/well) of antigen, 1:100 serum dilution and 1:10000 goat anti bird IgG HRP conjugate (Table 1, 2). The positive-to-negative (P/N) ratio of ≥2 was considered positive and the sera samples showing OD≥0.312 was considered positive for IgG/IgY antibodies to JEV in birds.

Assessment of cross reaction of standardized I-ELISA revealed slight cross reactivity with WN antigen. Of the 96 bird serum samples cross checked by I-ELISA (IgG/IgY) using JE and WN antigens, 12 (12.50%) and 6 (6.25%) samples, respectively, were positive for JE and WN antibodies. It was observed that all samples which showed positivity to WN were also positive for JE but the signals were observed to be weak for WN as compared to JE by an average 0.150 OD values.

Table 1: Optimization of antigen dilution for I-ELISA (IgG/IgY) in birds at 1:100 serum dilution and varying antigen concentrations

	Antigen conc. (μg mL^{-1})			
Mean OD values	0.5	1	2	5
For positive sera	0.247	0.312	0.330	0.374
For negative sera	0.150	0.148	0.200	0.199
For difference	0.097	0.164	0.130	0.175

Table 2: Optimization of serum dilution for I-ELISA (IgG/IgY) in Birds at 1 μg mL^{-1} antigen and varying serum dilutions

	Serum dilutions				
Mean OD values	1:50	1:100	1:200	1:300	1:400
For positive sera	0.410	0.312	0.292	0.156	0.128
For negative sera	0.220	0.148	0.164	0.141	0.111
For difference	0.190	0.164	0.128	0.015	0.017

Table 3: Comparative efficacy of indirect ELISA (I-ELISA) vis-à-vis virus neutralization test

Particulars	I-ELISA (IgG/IgY) vs. VNT (n = 46)
No. of true positive samples (a)	08
No. of false positive samples (b)	03
No. of true negative samples (d)	35
No. of false negative samples (c)	00
Sensitivity (%)	100.00
Specificity (%)	92.10
Efficiency/accuracy (%)	93.48
Predictive value for positive test (%)	72.72
Predictive value for negative test (%)	100.00

Table 4: Prevalence of Japanese encephalitis in Birds

		I-ELISA (IgG/IgY)		
Area	Species	No. of samples	Positive	Prevalence(%)
Bareilly retail shops	Broiler	100	11	11.00
CARI, Izatnagar	Guinea fowl	100	12	12.00
Nagpur	Broiler	35	00	00.00
	Cockrel	50	00	00.00
Overall prevalence		285	23	8.07

The sensitivity, specificity, accuracy, predictive value for positive test and predictive value for negative test of I-ELISA (IgG/IgY) recorded are 100.00, 92.10, 93.48, 72.72 and 100.00%, respectively, with virus neutralization test (Table 3). The concordance of indirect ELISA (IgG/IgY) vis-à-vis VNT also showed good agreement (*kappa* = 0.80) of standardized indirect ELISA (IgG/IgY) in birds. *Kappa* ranges from 1 (complete agreement beyond chance) to 0 (agreement is equal to that expected by chance), whereas, negative values indicate agreement less than is expected by chance. Arbitrary bench-marks for evaluating observed kappa values have been recommended as suggested by Altman (1991) are very good (>0.80), good (0.61 to 0.80), moderate (0.41 to 0.60), fair (0.21 to 0.40) and poor (20).

Of the 285 serum samples screened for JE specific IgG/IgY antibodies by standardized I-ELISA revealed overall 8.07% prevalence of JE in birds (Table 4). Out of 100 sera samples each of guinea fowl collected from CARI, Izatnagar and broilers from retail shops in Bareilly, 12 and 11 samples, respectively, were positive for Japanese encephalitis. The present study indicated 12% prevalence of JE in Guinea fowl and 11% in broilers. Of the 85 serum samples (Broilers 35, Cockrel 50) collected from Nagpur, none of the sample was positive for JE.

DISCUSSION

Definitive diagnosis of JE, WN or Dengue infections is very critical in serological assays and even by virus isolation. Success of virus isolation is very poor due to rapid development of neutralizing antibodies and it could only be isolated from cerebrospinal fluid (Burke and Monath, 2001). Serological test viz., HAI, CFT and VNT have limitations over each other as HAI test is pH sensitive and gives cross reaction with other Flaviviruses of JE sero-complex, whereas VNT is very time consuming and required much safety for handling of live virus. ELISA is more promising tool in recent past and various formats of ELISA are developed from time to time (Xingling *et al.*, 2005; Ravi *et al.*, 2006). IgM capture ELISA is gold standard test for diagnosis of JE, WN and Dengue

infections in human (OIE, 2004). But, there are very few reports on development of such assays for diagnosis in animals and birds. In this context in present study was designed to develop I-ELISA for diagnosis of JE in birds which could be used to explore the role of domestic and peridomestic birds in JE transmission.

Discontinuous sucrose gradient ultracentrifugation method which is widely used for viral antigen purifications revealed safe and reliable method for JEV antigen preparation (Yang *et al.*, 2006; Pyke *et al.*, 2004; Parida *et al.*, 2006). Sucrose acetone extracted Suckling Mouse Brain (SMB) antigens of JE and WN viruses were also found to be successfully used in serological assays (Johnson *et al.*, 2003; Ravi *et al.*, 2006). Virus/serum neutralization test which is gold standard test for serodiagnosis of Flaviviruses according to OIE (2004) was standardized for detection of neutralization antibodies against JE using C6 36 *Aedes albopictus* cells in 96 well tissue culture plates. The results were optimum using 200 PFU/0.1 mL JE virus in C6 36 cells (20000 cells/well) and were best read on 3rd day post infection. The neutralization titre of ≥4 was considered positive as suggested by Yang *et al.* (2006).

Although, IgM capture ELISA is the most accepted test for serodiagnosis, IgG which plays major role in humoral immunity also needs to be explored (Xinglin *et al.*, 2005). There are several reports on development of ELISA for diagnosis of JE in human (Solomon *et al.*, 1998; Cardosa *et al.*, 1993; Ravi *et al.*, 2006) and few in animals (Konishi and Yamaoka, 1982; Xinglin *et al.*, 2005; Yang *et al.*, 2006). But there are no reports on development of I-ELISA for birds. It is probably the first report from India on development of I-ELISA which is highly sensitive and specific for serodiagnosis of JE in chicken and guinea fowl. The present I-ELISA could detect low antibody titre as few of the samples negative in HI were positive by ELISA and VNT. Similar findings are reported by Chang *et al.* (1984), who developed biotin labeled antigen sandwich ELISA for detection of JE antibodies in human and animal sera. It could be further studied for serosurvey of JE in wild and peridomestic birds and may be important tool for early warning of disease.

Cross reaction with homologous viruses is major problem in serological assays (OIE, 2004). Cross reaction of JEV with WN and dengue is very common in HAI, however, ELISA is more sensitive (Solomon *et al.*, 1998). The cross-reactivity can be eliminated to the extent by use of purified JE antigen or recombinant antigen based ELISA (Xinglin *et al.*, 2005; Yang *et al.*, 2006). Purified JE antigen used in the present study must have eliminated cross reaction to greater extent. Slight positivity with WN antigen was recorded in cross reaction studies with WN. It is revealed that all sera samples which showed positive signals for WN antibodies were strong positive for IgG against JE and none of the sample which was positivity for WN was negative for JE. The difference in OD values of JE and WN positive samples was >0.150. IgG antibodies have high degree of cross reactivity than IgM to homologous and heterologous Flaviviruse antigens and these antibodies persist longer than IgM antibodies in the sera (Shu and Huang, 2004; 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., SLE, dengue, JE and Yellow fever infections. Ravi *et al.* (2006) also observed some degree of cross reactivity of conventional IgM Capture ELISA among closely related Flaviviruses. Some degree of cross reaction is observed between various Flaviviruses by conventional tests as well as ELISA developed using recombinant antigens (Cardosa *et al.*, 2002).

The prevalence rate of 11 and 12% was detected in chicken and guinea fowl, respectively, by indirect ELISA (IgG/IgY) with overall 8.07% prevalence of JE in birds. Seroprevalence of JE in chicken and other domestic and wild birds had been reported by various researchers. The sero-positivity to JEV antibodies has been reported in duck and night herons (Kumanan *et al.*, 2002); ducks from Nepal (Joshi and Gaidamovisch, 1981; Pant, 2006) pond herons and cattle egrets from Andhra Pradesh (Rodrigues *et al.,* 1985) ducks, fowls and peridomestic sparrows from Bihar (Loach *et al.*, 1983) and peridomestic birds from Bareilly (Sarkar *et al.*, 1995). In addition to seroprevalence, isolation of JE virus has also been reported from spleen and kidney of Japanese tree sparrow (Hasegawa *et al.,* 1975).

CONCLUSION

Presence of antibodies to JEV in the chicken and guinea fowl in the present study and similar supporting evidences of various researchers clearly underlines the need to elucidate role of these species along with other domestic and peridomestic birds in transmission of JE virus in nature. It can further be stated that these domestic birds can be used as sentinel for monitoring the imminent infection as has been suggested by Johnson *et al.* (2003). It was evident from the results obtained that the developed indirect ELISA is highly sensitive (100%), specific (92.10%) and accurate (93.48%) for diagnosis of JE in bird's sera with virus neutralization test.

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REFERENCES

Altman, D.G., 1991. Practical Statistics for Medical Research. Chapman and Hill, London.

Burke, D.S. and T.P. Monath, 2001. Flavivirus: Fields Virology. 4th Edn., Lippincott Williams and Wilkins, Philadelphia, pp: 1043-1126.

Cardosa, M.J., F.L. Hah, B.H. Choo and S. Padmanathan, 1993. Screening of pig sera for antibodies to Japanese encephalitis virus using a dot enzyme immunoassay and IgM capture ELISA: Comparison with the hemagglutination inhibition and plaque reduction neutralization tests. Southeast Asian J. Trop. Med. Public Health, 24: 472-476.

Cardosa, M.J., S.M. Wang., M.S.H Sum and P.H. Tio, 2002. Antibodies to prM protein distinguish between previous infection with Dengue and JE viruses. BMC Microbiol., 2: 9-14.

Chang, H.C., I. Takashima, J. Arikawa and N. Hashimoto, 1984. Bioton-labeled antigen sandwich ELISA (BLA-S-ELISA) for the detection of Japanese encephalitis antibody in human and a variety of animal sera. J. Immmunol. Methods, 72: 401-409.

Chen, W.R., R.B. Tesh and R. Reco-Hesse, 1990. Genetic variation of Japanese encephalitis virus in nature. J. General Virol., 71: 2915-2922.

Dey, S., C.M. Mohan, T.M.A.S. Kumar, P. Ramdass, A.M. Nairar and K. Nachimuthu, 2004. Recombinant Lip 32 antigen-based single serum dilution ELISA for detection of canine leptospirosis. Vet. Microbiol., 103: 99-106.

Gould, E.A. and J.C.S. Clegg, 1991. Growth, Titration and Purification of Alphaviruses and Flaviviruses. In: Virology: Apractical Approach, Mahy, B.W. (Ed.). Oxford University Press, Washington DC, pp: 43-76.

Hasegawa, T., Y. Takehas and K. Takahashi, 1975. Natural and experimental infections of Japanese tree sparrow with Japanese encephalitis virus. Arch. Virol., 49: 373-376.

Hogrefe, W.R., R. Moore, M. Lape-Nixon, M. Wagner and H.E. Prince, 2004. Performance of immunoglobulin G (IgG) and IgM ELISA using a WN virus recombinant antigen (prM/E) for detection of WNV and other flavivirus specific antibodies. J. Clin. Microbiol., 42: 4641-4648.

Holmes, D.A., D.E. Purdy, D.Y. Chao, A.J. Noga and G.J. Chang, 2005. Comparative analysis of immunoglobulin M (IgM) capture ELISA using virus like particles of mouse brain antigens to detect IgM antibody in sera from patients with evident flaviviral infections. J. Clin. Microbiol., 43: 3227-3236.

Johnson, A.J., S. Langevin, K.L. Wolff and N. Komar, 2003. Detection of anti West Nile virus immunoglobulin M in chicken serum by an enzyme linked Immunosorbent assay. J. Clin. Microbiol., 41: 2002-2007.

Joshi, D.D. and Gaidamovisch, 1981. Serological surveillance of virus encephalitis in Nepal. Bull. Vet. Sci. Anim. Husbandry Nepal, 10: 8-12.

Konishi, E. and M. Yamaoka, 1982. Evaluation of ELISA for quantitation of antibodies to Japanese encephalitis virus in swine sera. J. Virol. Methods, 5: 247-253.

Kumanan, K., A. Ramesh, R. Velmurgan, S. Jgannathan and V.D. Padmanabhan, 2002. Seroepidemiology of Japanese encephalitis among animals and birds in Tamil Nadu. Indian Vet. J., 79: 311-315.

Loach, T.R., K.G. Narayan and S.P. Choudhary, 1983. Serological evidence of persistence of Japanese encephalitis virus activity in Bihar, India. Int. J. Zoonoses, 10: 7-14.

Ma, S.P., Y. Yoshida, Y. Makino, M. Tadano, T. Ono and M. Ogawa, 2003. Short report: A major genotype of Japanese encephalitis virus currently circulating in Japan. Am. J. Trop. Med. Hyg., 69: 151-154.

Office of International Des Epizootics, 2004. Japanese encephalitis: Manual of diagnostic tests and vaccines for terrestrial animals. Part II, section 2.5, Chapter 2.5.14, pp: 1-8.

Pant, G.R., 2006. A serological survey of pigs, horses and ducks in Nepal for evidence of infection with Japanese encephalitis virus. Ann. N. Y. Acad. Sci., 1081: 124-129.

Parida, M., P.K. Dash, N.K. Tripathi, S. Sannarangaiah and P. Saxena *et al.*, 2006. Japanese encephalitis outbreak, India, 2005. Emerging Infect. Dis., 12: 1427-1430.

Pugachev, K.V., F. Guirakhito, D.W. Trent and T.P. Monath, 2003. Traditional and novel approaches to flavivirus vaccines. Int. J. Parasitol., 33: 567-582.

Pyke, A.T., D.A. Phillips, T.F. Chuan and G.A. Smith, 2004. Sucrose density gradient centrifugation and cross-flow filtration methods for the production of arbovirus antigens inactivated by binary ethylenimine. BMC Microbiol., 4: 3-8.

Ravi, V., A. Desai, M. Balaji, M.P. Apte and L. Lakshmane *et al.*, 2006. Development and evaluation of a rapid IgM capture ELISA (JEV-Chex) for the diagnosis of Japanese encephalitis. J. Clin. Virol., 35: 429-434.

Rodrigues, J.J., D.A. Gadkari, N. Shaikh and K.M. Pavri, 1985. A case of West Nile virus encephalitis. J. Assoc. Physicians India, 33: 500-500.

Sarkar, T., M.P. Mall and S.V.S. Malik, 1995. Seropositivity to Japanese viral infection among animals and birds in Bareilly area of Uttar Pradesh. Ind. J. Comp. Microbiol. Immunol. Infect. Dis., 16: 101-103.

Shu, P.Y. and J.H. Huang, 2004. Current advances in dengue diagnosis. Clin. Diag. Lab. Immunol., 11: 642-650.

Solomon, T., L.T. Thao, N.M. Dung, R. Kneen and N. Hung *et al.*, 1998. Rapid diagnosis of Japanese encephalitis by using an immunoglobulin M dot enzyme immunoassay. J. Clin. Microbiol., 36: 2030-2034.

Solomon, T., N.M. Dung, R. Kneen, M. Gainsborough, D.W. Vaughn and V.T. Khanh, 2000. Japanese encephalitis. J. Neurol. Neurosurg. Psychiatry, 68: 405-415.

Solomon, T., H. Ni, D.W.C. Beasley, M. Ekkelenkamp, M.J. Cardosa and A.D.T. Barrett, 2003. Origin and evolution of Japanese encephalitis virus in southeast asia. J. Virol., 77: 3091-3098.

Thrusfield, M., 2005. Veterinary Epidemiology. 3rd Edn., Blackwell Science Ltd., Oxford, UK.

Uchil, P.D. and V. Satchidanandam, 2001. Phylogenetic analysis of Japanese encephalitis virus: Envelope gene based analysis reveals a fifth genotype, geographic clustering and multiple introduction of the virus in to the Indian subcontinent. Am. J. Trop. Med. Hyg., 65: 243-251.

Xinglin, J., C. Huanchun, W. Xiang and Q. Changmin, 2005. Quantitative and qualitative study of ELISA to detect IgG against Japanese encephalitis virus in swine sera. Vet. Res. Commun., 29: 159-169.

Yang, D.K., B.H. Kim, S.I. Lim, J.H. Kwok, K.W. Lee, C.U. Choi and C.H. Kweon, 2006. Development and evaluation of indirect ELISA for the detection of antibodies against Japanese encephalitis virus in swine. J. Vet. Sci., 7: 271-275.