International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 1 (2014) pp. 407-413**
http://www.ijcmas.com

Original Research Article

Comparison of CPE NT and PRNT assays for estimating Neutralizing antibody titres against Japanese Encephalitis Virus

Gupta BP[1*], Mishra SK[2], Manandhar KD[1], Malla R[1], Tamarakar CS[3,4], Bajarcharya A, Rauniyar R[5], Raut PP[6], Sah SK[7] and Pokhrel S[8]

[1]Central Department of Biotechnology, Tribhuwan University, Kirtipur, Kathmandu, Nepal
[2]National Public Health laboratory, Teku, Kathmandu, Nepal
[3]Dean, Science and Technology, Tribhuvan University
[4]Director, Bir Hospital Trauma Centre, Kathmandu, Nepal
[5]Dayananda Sagar Institutions, Bangalore
[6]Civil Service Hospital of Nepal, New Baneshwor, Kathmandu, Nepal
[7]B.P.Koirala memorial cancer Hospital, Bharatpur, Nepal
[8]Universal College of Medical Sciences, Nepal
Corresponding author

A B S T R A C T

Sero-epidemiological studies are required to identify population susceptible to Japanese Encephalitis. The presence of neutralizing antibodies to Japanese encephalitis virus (JEV) would be expected to be the most reliable indication of past infection and presumed immunity to JEV. The neutralizing antibody (NAb) titres can be determined by plaque reduction neutralization test (PRNT) and cytopathic effect -based neutralization test (CPE -NT). The gold standard method for estimation of neutralizing antibody is the plaque reduction neutralization test (PRNT). It is technically demanding and small number of serum sample can be tested in 24 well plates in duplicate for seroprevalence study and testing vaccine efficacy for JE vaccine. This study has been carried out to find out the correlation between two methods, cytopathic effect -based neutralization test (CPE- NT) was done and validated against PRNT using 296 pre vaccinated and 230 post vaccinated serum sample of children. The samples were tested in duplicate by CPE NT and PRNT in 10 and 4 separate assay runs, respectively and 50% neutralizing antibody titres calculated using the Karber formula. Correlation between the two neutralization assays was observed ($r=0.74$ for pre vaccinated and 0.73 for post vaccinated sample with $R^2 = 0.55$, $p<0.0001$). CPE NT can be used as assay of choice for undertaking studies on serosurvey and response to vaccine at mass vaccination settings as this method is cost effective, less time consuming and less labor intensive.

Keywords

JEV;
PRN -T;
CPE -NT.

Introduction

Japanese encephalitis virus (JEV), a mosquito-borne pathogen of the genus *Flavivirus*, family *Flaviviridae*, is main cause of viral encephalitis. It was first isolated from human brain tissue in Japan in 1935 (Yun *et al.*, 2003) and is

transmitted by bloodsucking Culex mosquitoes - predominantly Culex tritaeniorhynchus. Three billion people live in the endemic areas and at least 50,000 clinical Japanese encephalitis (JE) cases occur each year, which are a great burden to the affected populations (Japanese encephalitis vaccines, WHO, 1998). The clinical to subclinical ratio of JE is 1:300 (Kitchener *et al*., 2008). It has spread across Asia and has become the most important cause of epidemic encephalitis worldwide (Solomon *et al*., 2003).The disease reported in Southeast Asia mainly India, Bangladesh, Sri Lanka and Nepal (Erlanger *et al*; 2009). In India, first case of JE was reported from Vellore in 1955 (Saxena V and Dhole T N 2008).A major outbreak resulting in 42.6% case-fatality rate was reported from Bankura district of West Bengal in 1973 (Chakravarty *et al*., 1975).There was extensive outbreak of Japanese Encephalitis in 1981 (Rao *et al*., 1988) & prevalence of neutralizing antibodies against JEV was 49.17% in south Arcot district Tamil Nadu & Pondicherry (Risbud *et al*., 1991). The outbreaks also occurred in Bihar, Uttar Pradesh, Assam, Andhra Pradesh, Tamil Nadu and Karnataka (Anuradha SK *et al*., 2011). A major outbreak of Japanese encephalitis occurred in the eastern UP during 1977-78 and 2005 with 5,737 cases & with 1,344 deaths (Parida *et al*., 2006).

In the past, haemagglutination inhibition (HI) assay was the most widely used technique for this purpose, because it is rapid, and simple to perform, and HI antibodies have been shown to correlate well with neutralizing antibodies (Erlanger *et al*., 2009). However, an alternative test with increased sensitivity and specificity that does not require the use of primate red cells would be desirable. Due to the presence of cross-reactive antibodies during JEV serocomplex infections within the same geographic regions poses problems for development of serological assays for serosurvey study. The PRNT and CPE NT with enhanced sensitivity for detecting low levels of JEV neutralizing antibody was developed. The serosurvey of JEV infections and vaccine efficacy is commonly achieved by using this assay. This assay is more sensitive than HI and ELISA (Solomon *et al*., 2000).

The PRNT and CPE NT assay measures the level of JE neutralizing antibodies in a serum sample by determining the ability of dilutions of serum to block the production of viral plaques in PRNT and morphological changes of cell in CPE NT on BHK-21cell monolayer by a known amount of JEV. Although Plaque reduction neutralization test (PRNT) is considered the "gold-standard" assay for measurement of neutralizing antibody, it is technically demanding and small number of sample can be screen. Thus for screening of large number of sample in sero prevalence study CPE based NT in 96-well plates would be preferred.

Materials and Methods

BHK-21 cells, passage 13-21, were used throughout the study. The cells were grown in growth medium (GM) consisting of 1 x Dulbecco's modified Eagle's medium (DMEM; GIBCO Invitrogen, Paisley, UK) supplemented with 10% FBS, 5% TPB,13.3 ml/L of (7.5% w/v) NaHCO3, 5 ml/L L-glutamine , Penicillin, Streptomycin and Gentamycin – 250 µl/l, 400 µl/l and400 µl/l respectively.The cell concentration was adjusted to $0.25x10^6$ cells /mL, and either 1mL was added to each well of a 24-well plate or 100 mL to each well of a 96-well plate. Plates were incubated for 42 h to allow the formation of a monolayer before use.

The Virus strain (JEV 057434) adapted to BHK-21 cell, belongs to Genotype III was isolated from human in Gorakhpur in 2005 were used throughout the study. Virus pool were prepared by infecting confluent monolayer of BHK cells was with 0.1 m.o.i (multiplicity of infection) at 37^0C with 5% CO_2 and harvested when 80-90 % of cells showed cytopathic effect (CPE), observed. Virus suspension were distributed in cryovials and stored at -70^0C till further use. The virus titre was determined by PRNT and CPE NT.Virus dilutions giving $10^{-2.6}$ per well in a 96-well plate (CPE NT) and 40-50 plaque per well in a 24-well plate (PRNT) were chosen for all further experiments.

The serum samples were collected under the project on serosurvey and antibody response to single dose attenuated JEV SA-14-14-2 vaccine by National Institute of Virology, Gorakhpur Unit during December 2010-January 2011.The study was conducted in the rural and urban settings in Eastern UP. Children between 1-15 years of age were included in the study. All the seven districts of Gorakhpur and Basti District were included in the baseline serosurvey. The total pre vaccinated sample size was 2100. The post vaccinated sera were obtained from 1-15 years children immunized with JEV SA 14-14-2 live attenuated vaccine on 1 month post vaccination. The serum was stored at -20^0C. Altogether 296 pre vaccinated and 230 post vaccinated sera were chosen for this study.

Serum samples to be assayed were thawed and placed in ice bath. Required aliquots were taken out and first dilution was carried out in MEM with 2% FBS in eppendorf tubes. Tubes were incubated at 56^0C water bath for 30 minutes for inactivation of complement. Serum was further diluted in 96 well plates as required by the assay procedure.

For CPE NT, Serum samples were diluted 1:4 after inactivation. Samples were then diluted in 96 well plates in duplicate in 60μL volume. A predetermined virus dilution was added capable of giving 2 log TCID50 virus dose. Virus antibody mixture was incubated for 1 hr at 37^0C with 5% CO_2. The virus antibody mixture was added on preformed monolayered of BHK cells. Plates were incubated at 37^0C with 5% CO_2. A known positive control and virus control without serum was always included in the each plate. Samples were tested in duplicate. A virus titration was performed in each plate for assessing the dose of the virus. CPE readings were taken when the virus dose showed 2 logs $TCID_{50}$ titre. After reading, the plate were washed with PBS and approximately 100 μL of 0.1% Amido black was added to each well and allowed to stain for 1 hour. Finally, the cell monolayer in each well was rinsed and the plates allowed to air dry. The dried plates can be stored for several months without any adverse effect. Neutralizing antibody titres were assigned after staining the plates and determined to reciprocal of antibody dilution capable of giving 50% inhibition of virus dose.

For PRNT, Serum was diluted 1:5 dilution and inactivated. Sera were further diluted in 96 well plates in fourfold dilutions starting from 1/5 to 1/320. To make fourfold dilutions 20 μL of the 1/5 dilution was transferred to the next well containing 60 μL of diluents and so on. The sera were tested in duplicate to minimize inter-assay variations and to help achieve more reliable results. An aliquot of challenge virus was thawed and the working dilution prepared in maintenance medium and kept covered from light and on wet ice until

needed. An equal volume of diluted challenge virus (60 μL) was added to the volume of serum dilution in each well (60 μL). This resulted in the final dilution of test serum starting from 1/10 to 1/640. The plates with virus/serum dilution mixtures were incubated at 37^0C for 1 h to allow neutralization to take place. The controls were included in each assay run; at least two wells were incubated with 60μL of diluted challenge virus in the absence of serum as a virus control to determine the average plaque count per well and at least one well and preferably one row of wells were inoculated with media (without virus or serum) to serve as cell controls. At the end of the 1 hour incubation period, 100 μL volumes of each virus/serum dilution mixture, virus controls and cell control were added to the appropriate well of a 24-well cell cultured plate. At the end of this incubation period the medium was carefully removed and 1mL of overlay medium was added to each well. The plates were returned to the 37^0C incubator and incubated for a further 3 days. At the end of the 3 day incubation period, the overlay medium was carefully decanted into disinfectant. The wells were washed with PBS and approximately 500 μL of 0.1% Amido black was added to each well and allowed to stain for 1 hour. Finally, the cell monolayer in each well was rinsed and the plates allowed to air dry. The dried plates can be stored for several months without any adverse effect on the ability to count plaques.

Results and Discussion

Neutralizing antibody titres can be determined by PRNT or by CPE NT. In principle both the tests are similar. However, it is always believed that PRNT test is more reliable as it gives mathematical end points for antibody titres. CPE test is more prone of subjective variations. Many sera tested in this study were already tested by CPE titration for N'Ab titres. Comparison and the correlation coefficient calculation for pre vaccination and post vaccination sera revealed that both assays were significantly correlating with each other. In CPE NT as the upper limit was set to be about 200 higher titres were not determined accurately in CPENT. Figure 1 and 2 shows the correlation coefficient and the p -values for each of the data set.

Cost of assessing the antibody response against virus in mass vaccination set up is high as very large number of samples needed to be assayed. Both assays employ the 50% inhibition as end point determination of antibody titres. In CPE NT 100 $TCID_{50}$ is used as virus dose while in PRNT about 50 plaques are used as virus dose. Cost of undertaking PRNT is at least 200 times more than that of CPE NT. In assessing the response in mass vaccination, collection of substantial amounts of serum samples also becomes a difficult task. Also in these situations, a rough estimate of seroconversion is sufficient rather than having highly accurate serum titres. Keeping this in mind neutralizing antibody titres carried out by PRNT was compared with that of done by CPE NT, It was observed that both assays correlated extremely well (Correlation coefficient 0.73, $p<0.0001$). This was observed in both pre and posts vaccination sera samples also. It can be concluded that in future CPE NT may be used as assay of choice for undertaking studies on response to vaccine at least in mass vaccination settings.

Japanese encephalitis (JE) is a major public health problem in several countries in Southeast Asia including India.

Figure.1 Correlation of Pre vaccinated sera by CPE NT and PRNT

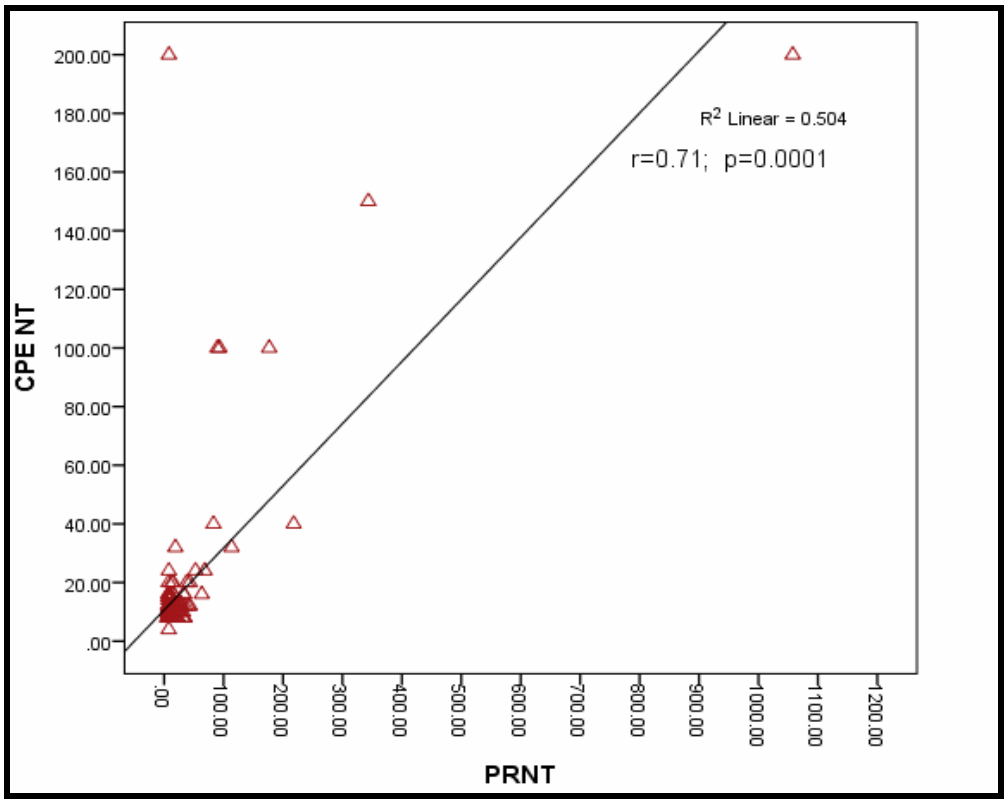
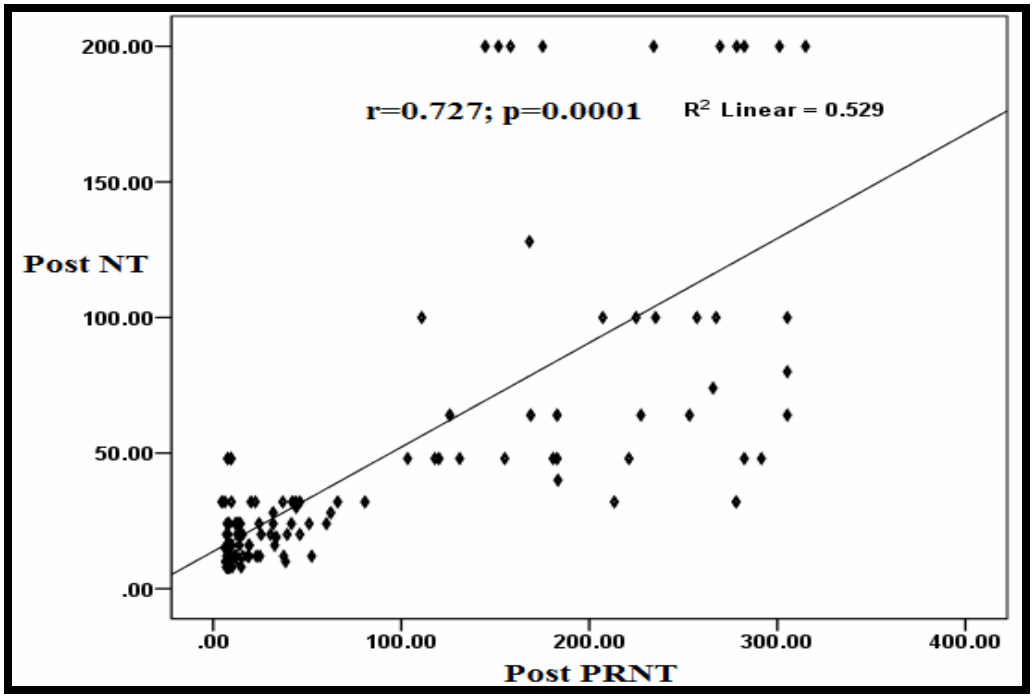


Figure.2 Correlation of post vaccinated sera by CPE NT and PRNT



Owing to the very high number of cases in eastern Uttar Pradesh, a seroprevalence study in children aged 1-15 years from seven districts of eastern Uttar Pradesh was undertaken by National Institute of Virology Gorakhpur unit.

Serum samples from Gorakhpur (High endemic) and Basti (Low endemic) districts were tested for JE virus neutralizing antibodies by plaque reduction neutralization test. In addition, seroconversion one month after single dose of live attenuated SA-14-14-2 vaccine was also studied. Comparisons of CPE NT and PRNT assays for determination of neutralizing antibody were also performed. A titre of 1:10 was considered seropositive. Seroconversion was defined as seropositivity in 1 month post vaccination sera in vaccines having serum titre <1:10 at baseline. Antibody titre rise was mentioned as two-fold and four-fold in relation to baseline antibody titre. Comparison of CPE NT and PRNT assay for estimating neutralizing antibody titres against JE virus showed a correlation coefficient of 0.73.In mass vaccination settings, CPE NT may be used as an assay of choice for undertaking studies on antibody response.

Acknowledgement

This work has been done as part of M.Sc. dissertation. The authors would like to thank all the staff of National Institute of Virology, Gorakhpur Unit.

References

Albrecht, P., Herrman K, and Burns, G.R. 1981. Role of virus strain in conventional and enhanced measles plaque neutralisation test. J. Virol.Methods.3(5):251-60.

Beasley DW, Lewthwaite P, Solomon T.2008. Current use and development of vaccines for Japanese encephalitis. Expert Opin Biol Ther 8: 95–106.

Bunning, M.L., R. A. Bowen, C. Bruce Cropp *et al*., , 2002. "Experimental infection of horses with West Nile virus," Emerging Infectious Dis. 8(4): 380–386.

Burke, D.S., A. Nisalak, and M. A. Ussery, 1985 "Kinetics in IgM and IgG responses to Japanese encephalitis virus in human serum and cerebrospinal fluid," Journal of Infectious Diseases,

CDC.1993. Inactivated Japanese encephalitis virus vaccine. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 42: 1–15.

Clarke, D.H., and J. Casals. 1958. "Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses," The American J. Trop.Med. Hygiene.7(5): 561–573

Cohen, B.J., Parry RP, Doblas D, Samuel D, Warrener L, Andrews N, *et al* 2006. Measles immunity testing: comparison of two measles IgG ELISAs with plaque reduction neutralisation assay. J .Virol. Methods.131 (2):209—12

Endy, T.P., and Nisalak, A.2002. Japanese encephalitis virus: ecology and epidemiology. Curr. Top. Microbiol. Immunol. 267: 11–48.

Erlanger, T.E., Weiss S, Keiser J, Utzinger J, and Wiedenmayer K.2009. Past, present, and future of Japanese encephalitis. Emerg.Infect .Dis. 15: 1–7.

Griffin, D.E. Measles virus. In: Knipe DM, and Howley, P.M, 2001editors. Fields Virology. Philadelphia: Lippincott Williams & Wilkins;. p. 1401-41.

Hiscox, A., C. H. Winter, P. Vongphrachanh *et al*., 2010. "Serological investigations of flavivirus prevalence in Khammouane Province, Lao People's Democratic Republic, 2007-2008," The American J. Trop. Med.Hygiene.83(5): 1166–1169.

Shlim, D.R., and Solomon, T .2002. Japanese encephalitis vaccine for travelers: exploring the limits of risk. Clin. Infect. Dis. 35: 183–188.

Solomon, T., Dung NM, Kneen R, Gainsborough M, Vaughn DW, *et al*. 2000. Japanese encephalitis. J Neurol. Neurosurg. Psychiatry. 68: 405–415.

Van den Hurk, A.F., Ritchie, S.A, and Mackenzie, J.S. 2009. Ecology and geographical expansion of Japanese encephalitis virus. Annu .Rev. Entomol. 54: 17–3

vol. 151, no. 6, pp. 1093–1099.

WHO, 2006. Japanese encephalitis vaccines. Wkly. Epidemiol Rec. 81: 331–340.

WHO,1998. Japanese encephalitis vaccines. Wkly. Epidemiol Rec. 73: 337–344.

Yamanaka, A., K. C. Mulyatno, H. Susilowati *et al*., 2010 "Prevalence of antibodies to japanese encephalitis virus among pigs in Bali and East Java, Indonesia, 2008," Japanese .J.Infectious Dis.63(1): 58–60.