An In-house Assay for Zika Virus Detection: Utility of an IgM Assay in Screening for Zika Virus (ZIKV) Infection by Using Serum Samples

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Abstract - Zika virus (ZIKV) is a mosquito transmitted *Flavivirus*, in the family *Flaviviridae*, which is related to clinically important arboviral pathogens. Considering that these flaviviral diseases display characteristic symptoms similar to ZIKV, almost 80% of the ZIKV infections are asymptomatic and many individuals seek medical care beyond the detectable period of RNA, serological laboratory testing is paramount. Here we used an ELISA assay that has been modified to detect ZIKV IgM antibodies. A total of 640 febrile serum samples have been used. Samples from 18 ZIKV (Moi at al. 2017) were first used to test IgM assay. All of the samples that were positive for ZIKV by PRNT were also positive by IgM ELISA (100% sensitivity). Thus, the anti-ZIKV IgM ELISA assay was used for screening of 622 samples obtained from acute febrile patients. Of the 622 samples from patients with acute fever, 171 (27.5%) were positive for anti-ZIKV IgM antibody. Among 158 ZIKV positive samples, 35 (20.5%) were also positive for DENV IgM antibodies. While the IgM ELISA is a useful tool for the detection of recent ZIKV infection, virus RNA detection assays and neutralization test are needed for diagnosis confirmation.
Conclusion: ZIKV IgM ELISA is a useful test that can be used in screening patients.

Keywords: Zika virus, Anti-Zika virus IgM antibodies, ELISA, Seroprevalence

(217 words)

Introduction

Zika virus is a mosquito borne *flavivirus* belonging to *flaviviridae* family. For almost 60 years after its first discovery, less than 20 cases have been reported worldwide prior to the outbreak of 5000 infections on Yap islands in 2007 (Peterson et al. 2016). Before 2007 Zika virus was known to be associated with only mild disease characteristics as rash, moderate fever, conjunctivitis, and arthralgia. So it came as a great surprise when recent outbreaks resulted in microcephaly, Guillain-Barre syndrome and birth related child defects. A total of 235 laboratory confirmed cases including one microcephaly case has been reported since 2016 in Vietnam (Moi et al. 2017). Recent global increase in cases of microcephaly and neurological disorders potentially associated with Zika virus infection has led to an increase of need for laboratory testing for differential diagnosis of Zika virus infection (Peterson et al. 2016). The co circulation of Zika virus in the regions with Dengue virus endemicity that causes similar initial symptoms and having antibody cross reactivity is challenging for diagnosis and surveillance of Zika disease (Tyson et al. 2019). While molecular methods are highly sensitive and useful in the acute phase, molecular methods for the diagnosis of Zika virus is challenging in the later phases of the diseases when the viremia level is low. Moreover majority (~80%) of Zika infections are asymptomatic and many individuals undergo testing for Zika virus beyond the period RNA is detectable which emphasizes the importance of serological testing methods. In addition, serological methods are relatively less expensive and require lower sample volume.
Objective: Here, we aim to determine the utility of an in-house ELISA kit in the determination of anti-ZIKV IgM antibodies in febrile patients for screening and identification of ZIKV cases.

Methodology
Sample collection In Vietnam:
In this study, 640 serum samples were collected from febrile patients who resided in Vietnam. Patients reported fever or symptoms/signs suggesting dengue fever at the time of recruitment. All patients were negative for anti-dengue virus (DENV) IgM antibody and DENV NS1 antigen.

Laboratory testing
Preparation of cells:
Baby Hamster Kidney (BHK) cells were cultured in T75 flasks using Eagle's Minimum Essential Medium (EMEM) supplemented with heat-inactivated 10% fetal bovine serum at 37°C in 5% CO_2. Cells were seeded at $1x10^4$ -$2x10^4$ cells/well in 12-well plates one day prior to the infection assay.

ZIKV and DENV propagation:
Zika virus strain MR766-NIID and DENV (1-4) strains; DENV-1, 01-44-1HuNIID strain (GenBank accession no. AB111007), DENV-2, 00St22A strain, DENV-3 5528 strain (GenBank accession no. KP893718) and DENV-4 SLMC318 strain were used. Each of these viruses were infected in to Baby Hamster Kidney cell lines (BHK) in 12-wells plate. On day 5 post infection, infected culture fluid was collected and stored in –80°C.

Virus titration:
Zika virus or Dengue virus (1-4 mix) was diluted tenfold (1:10 to $1:10^5$) using EMEM 10% FBS. A total of 100 µl of either serially-diluted Dengue or Zika was added to the BHK cells monolayer in 12-wells plate. After 30 min of incubation at 37°C, 1 ml methyl cellulose was added to each well and the cell monolayer was incubated at 37°C for 5 days. After 5 days of incubation, the cells were fixed upon confirmation of plaque formation by naked eye. A total of 1 ml formaldehyde was used to fix the cells for 60 min at room temperature. The plates were washed with tap water and stained by using methylene blue. Plaques were counted under a light box.

DENV or ZIKV IgM ELISA:

An in-house ZIKV IgM capture ELISA and DENV IgM capture ELISA (Focus Diagnostics, Germany) was performed to confirm the presence of anti-ZIKV IgM antibodies and anti-DENV IgM antibodies in serum samples. For detection of ZIKV IgM, an in-house IgM capture ELISA kit was used (Kutsuna et al 2014, Kutsuna et al 2016). Both of the ZIKV and DENV IgM ELISA procedure were similar with the exception of the antigen used. First, human anti-IgM antibody coated wells were washed once with wash buffer solution. Next, 1µl of serum sample was diluted in 100µl of

diluent. Then diluted samples are added to wells in 8 well strips and incubated at 37°C for 1 hr. Nonspecific reactants were removed by washing three times with PBS. In DENV ELISA inactivated DENV(1-4) antigen (Focus Diagnostics, Germany) or in ZIKV ELISA 100 µl of ZIKV antigen, obtained from cell culture supernatant with PFU $1x10^5$ PFU/ml, was added to each well and incubated at room temperature for 1 hr. Unbound DENV antigen or ZIKV antigen was removed after three washes with PBS. Next, 100 µl of horseradish-peroxidase conjugated anti-flavivirus antibody was added to each well and the wells were incubated for 30 min at room temperature. Excess conjugate was then removed with a PBS wash. Then 100 µl of tetramethylbenzidine (TMB) was added to each well and the wells were incubated in dark for 10 min at room temperature. The reaction was terminated by adding 1N $H2SO_4$ after 10 min.

Optical density was measured at 450 nm according to manufacturer's instructions for ZIka virus and Dengue virus. For ZIKV IgM ELISA the positive control or the samples with OD450 1.1 times or higher than the cut-off value was considered positive (OD450 of positive control or samples / OD450 of cut-off calibrator ≥ 1.1). For Dengue virus IgM ELISA OD450 of sample/OD450 of negative control ≥2 was considered positive.

Plaque reduction neutralizing test (PRNT):

Seventeen serum samples from Vietnam were determined for the presence of neutralizing antibodies to ZIKV and DNEV by using plaque reduction neutralization test (PRNT). The samples were serially diluted in 2-folds from 1:20 to 1:2560 in EMEM supplemented with 10% FBS. A total of 25 µl diluted serum samples was mixed with 25 µl of ZIKV ($2.5x10^3$ PFU/ml) to allow virus-antibody immune complex formation. After 1 h incubation at 37°C, 50 µl of the virus-antibody mixture were inoculated onto BHK monolayer in 12-wells plate. The plates are then incubated for 60 min at 37°C in 5% CO_2 then 1 ml methylcellulose was added to the wells. The plates were incubated at 37°C in 5% CO_2 for 5-7 days until plaque formation was confirmed by the naked eye. The cells were then fixed with 1X formaldehyde solution for 30 min at RT and then stained with 6X Methylene blue. The number of plaque was counted under the light box. PRNT50 end point was calculated using the reciprocal of the final serum dilution showing a 50% or greater reduction in plaque counts in wells compared to the number of plaque from the negative control wells with no antibodies. Positive PRNT samples were defined as having a neutralizing titer of 20 or above to Zika virus (PRNT50 ≥ 20)

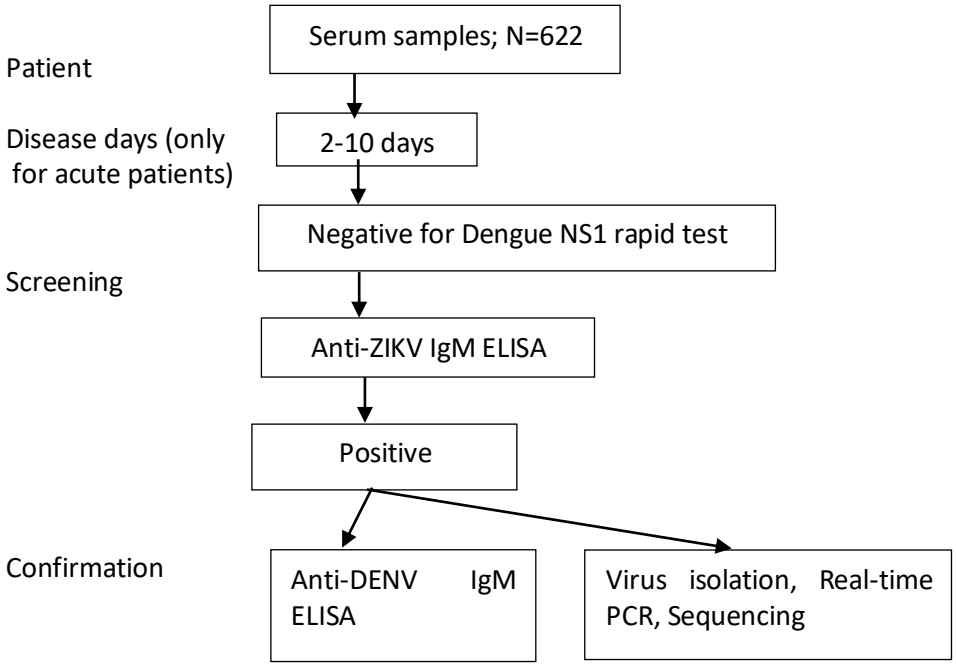

Figure 1: Screening algorithm that was used for the detection of ZIKV infection.
Algorithm for screening ZIKV infection in Vietnam by using ZIKV and DENV Immunoglobulin M (IgM) enzyme linked immunosorbent assays (ELISA)

Results and Discussion:

IgM ELISA for PRNT positive samples
For the validation of this IgM ELISA assays we performed DENV IgM ELISA and ZIKV IgM ELISA on serum samples that were confirmed by WHO criteria and tested negative for other flaviviruses. When ZIKV PRNT was performed the serum titer required to reduce viral plaques by 50% was 1:40-1:1280. All samples positive by PRNT were also positive by ZIKV IgM ELISA, P/N ratio varied from 2.2 - 19.5.

Detection of ZIKV cross-reactive antibodies in febrile patients
Table 1: Characteristics of the patients of the study:

Variable	
Age	0Y-97Y
Mean days(Disease)	5.5 +/-1.5 days
Sex	Male: 320 Female: 302
DENV IgM ELISA	35/171 (20.5%)

Among the residents, mean age was 35 years. Youngest and oldest age being 0 years to 97 years.

Of these 320 (51.4%) were male and 302 (48.6%) were female.
Table 2: Detection of anti-ZIKV IgM antibodies:

		ZIKV IgM ELISA (Percentage overall)		
DENV IgM ELISA		Positive	Negative	Total
	Positive	35 (5.6%)	14 (2.3%)	49 (7.9%)
	Negative	136 (21.9%)	437 (70.3%)	573 (92.1%)
	Total	171 (27.5%)	451 (72.5%)	622 (100%)

All 35 (35/171; 20.5%) early phase samples that were positive for both ZIKV and DENV IgM antibodies, but were negative for DENV NS1 antigen, suggesting that these patients were highly likely non-DENV patients. While our results suggest that the IgM ELISA assay is useful in screening for ZIKV patients, further diagnostic tests including PRNT are needed to confirm the ZIKV cases.

In this study, 622 serum samples that were negative for DENV NS1 antigen was used. Using the ZIKV IgM ELISA, of the 171 serum samples that were positive for anti-ZIKV IgM, 25 samples were positive for both ZIKV and DENV IgM. The results suggest of possible cross-reactivity between ZIKV and DENV. From the samples tested, 136 (21.9%) were positive for anti-ZIKV IgM ELISA but negative for anti-DENV IgM ELISA, suggesting absence of DENV cross-reactivity in these samples.

Conclusion

In this study, we have examined the utility of an in-house IgM ELISA assay in the determination of ZIKV cross-reactive antibodies. While the IgM ELISA is a useful tool for the detection of recent ZIKV infection, further tests are needed for disease confirmation due to potential IgM cross-reactivity between DENV and ZIKV. As such, the ELISA test should be performed in combination with virus RNA detection assays and neutralization test to increase the confidence of diagnosis. In all, the Zika virus IgM ELISA is a simple and inexpensive method which can be readily applicable in basic serology laboratories in endemic regions.

Acknowledgements

We thank the health care practitioners of the clinics and hospitals who supported the study. We would also like to thank all the members of Virology department in Nagasaki University for their support. This work was supported by e-Asia joint research program (e-Asia JRP). The ethics committee of the Institute of Tropical medicine, Nagasaki University, Japan (08061924-7) and National institute of Hygiene and Epidemiology, Hanoi, Vietnam (IRB-VN01057/2016) approved the study. We report no conflict of interest.

References

Moi, ML, Nguyen, T, Nguyen, C, Vu, T, Tun, M, Pham, T, Pham, N, Tran, T, Morita, K, Le, T, Dang, D and Hasebe, F 2017 '*Zika virus infection and microcephaly in Vietnam*', *The Lancet Infectious Diseases*, vol. 17, no.8, pp.805-806.

Petersen, L, Jamieson, D, Powers, A. and Honein, M 2016, Zika Virus. *New England Journal of Medicine*, vol.374, no.16, pp.1552-1563.

Tsai, W, Youn, H, Brites, C, Tsai, J, Tyson, J, Pedroso, C, Drexler, J, Stone, M, Simmons, G, Busch, M, Lanteri, M, Stramer, S, Balmaseda, A, Harris, E and Wang, W 2017 'Distinguishing Secondary Dengue Virus Infection From Zika Virus Infection With Previous Dengue by a Combination of 3 Simple Serological Tests', *Clinical Infectious Diseases*, vol. 65 no. 11, pp.1829-1836.

Tyson, J, Tsai, W, Tsai, J, Brites, C, Mässgård, L, Ha Youn, H, Pedroso, C, Drexler, J., Stramer, S, Balmaseda, A, Harris, E and Wang, W 2018, 'Combination of Nonstructural Protein 1-Based Enzyme-Linked Immunosorbent Assays Can Detect and Distinguish Various Dengue Virus and Zika Virus Infections' *Journal of Clinical Microbiology*, vol. 57 no. 2.

Yamanaka, A, Moi, M, Takasaki, T, Kurane, I, Matsuda, M, Suzuki, R and Konishi, E 2017, 'Utility of Japanese encephalitis virus subgenomic replicon-based single-round infectious particles as antigens in neutralization tests for Zika virus and three other flaviviruses', *Journal of Virological Methods*, vol. 243, pp.164-171.

World Health Organization 2017, Zika Virus Infection – India, Disease Outbreak News, viewed 08 May 2019.
https://www.who.int/emergencies/diseases/zika/india-november-2018/en/