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STRATEGY FOR DETECTION OF JAPANESE ENCEPHALITIS VIRUS GENOTYPE WITH COMBINATION MAC-ELISA, NS1-ELISA, AND REALTIME RT-PCR

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ABSTRACT

Japanese encephalitis virus (JEV) is a mosquito-borne virus, that causes acute encephalitis syndrome (AES). Suspected viral AES patients in General hospital Bac Giang province, 2004-2017 were selected 310 patients, clinical samples were collected for JE diagnosis by NS1-ELISA and MAC-ELISA as a strategy to select positive samples for genotyping by real-time RT-PCR and sequencing. The results showed that in acute samples, 81/320 (25,31%) JE cases were detected by MAC-ELISA, but only 3,75% (12/320) JE cases by NS1-ELISA. Thus, the sensitivity of NS1-ELISA is far from MAC-ELISA (k=0.4). But it is useful for selecting samples for the genotype of JEV. By Realtime RT-PCR yielding 4/12 CSFs were positive with JEV primer sets I and III, showing JEV genotypes I and III cause human disease in Bac Giang province from 2004 to 2017. The sequence of whole E gene genotype III of an isolate coded 04VN76 (isolated from CSFs coded 04 CSF98) was decoded and submitted on Genbank.

INTRODUCTION

Japanese encephalitis virus (JEV) is one kind of Arboviruses that is transmitted by *Culex* species. The virus belongs to the Flavivirus genus in the

Flaviviridae family. The Flavivirus genus is including dengue, West Nile, and Murrey Valley viruses... (1–3). As previously described, JEV can cause human diseases ranging from asymptomatic infection or febrile illness to severe meningitis or encephalitis in terms of acute encephalitis syndrome (AES). Besides, other pathogens also cause AES with similar symptoms as AES caused by JEV (4–6). Even though, JEV was recorded as a leading etiology causing AES for children in Asia and the Pacific Ocean (7,8). Thus, the diagnosis of AES caused by JEV can not be based on clinical symptoms, it should be done by specific diagnosis in the laboratory.

The most specific diagnosis is done by the isolation of JEV or the detection of RNA of JEV in clinical samples, but the short duration of viremia and low virus titers during JEV infection and early representation of the neutralization antibody after the infection event in the acute phase of illness, the results of the isolation of JEV as well RT-PCR will be obtained nothing. If using a molecular technique such as Real-time PCR to identify the genetic material of the virus is also less effective in the diagnosis, but quickly identifies JEV genotype from clinical samples, thus this technique is commonly applied in rapid detection of JEV genotype, although the cost of the test is higher than serology test (9–11). As a classification, JEV has only one serotype but has 5 genotypes. All 5 JEV genotypes were detected from mosquitoes and reservoirs. But from human (patients) only JEV genotype III and I were widely detected in Asia in the decades 50s and 70s, respectively. But in North Australia, a new genotype of JEV was detected in early 2001 showing genotypes I and II were circulating in this continent (12). The evidence of JEV genotype IV causes the human disease is poor, but recently genotype IV was detected in a fatal Australian in 2019 (13). Surprisingly JEV genotype V was detected in mosquitoes in China and Korea in recent decades (10,14). It raises the question of whether JEV genotype I and V will co-circulate in Asian countries in the future or if JEV genotype V will be soon “quiescent” as JEV genotype III after JEV genotype I appears (15) and how about the role of JEV genotype IV cause human disease? If so, which JEV genotype is infected humans yearly is a question that needs to be detected.

So far, the sensitive technique for JEV diagnosis was widely recommended by World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) Fort Collins in the acute phase of JEV infection being MAC-ELISA, but this technique can not be genotype JEV (16–18).

As documented, NS1 antigen is related to viral RNA replication, which is a biomarker of natural infection (4). Several researchers pointed out that NS1 appears before the IgM antibody in the early stage after the infection of the virus. Hence the NS1-ELISA is widely applied for the detection of dengue virus infection in the early stage. NS1 antigen appears when the viral replication, it is a marker of JEV replication. Thus, when NS1 antigen was detected in CSFs, RNA or JE virus will exist in those samples (19,20). If so, NS1-ELISA will be a useful technique for screening and selecting clinical samples for the detection of RNA virus by molecular method for genotyping as well for the isolation of the virus, alone serology techniques can not do.

In this study, MAC-ELISA and NS1-ELISA were carried out for screening JE cases among viral AES patients in Bac Giang province during the 2004 to 2017 period to choose clinical samples which were confirmed positive by both techniques. The confirmed positive CSF samples by ELISA were genotyped by real-time RT-PCR and nucleotide sequence with the target E gene of JEV.

MATERIALS AND METHODS

Clinical samples

Clinical samples were collected from viral AES patients, who were admitted General Hospital of Bac Giang province with clinical symptoms of acute onset, fever, altered sensorium, neurological signs, and symptoms. Clinical samples were collected from 310 AES patients in Bac Giang province only in June during the epidemic season, 2004-2017 based on the target of JE diagnosis according to WHO standards (18). For each patient, 3 kinds of samples were collected which include CSF samples (CSFs) in the acute phase and sera samples in acute and convalescent phases. Collected CSFs alone were aliquoted into 2 tubes; one kept at -20^{o}C used for the detection of IgM antibody and NS1 antigen, the other at -70^{o}C used for the detection of viral RNA as well as for the isolation of JEV. All sera samples were kept at -20^{o}C for JEV diagnosis by MAC-ELISA and NS1-ELISA techniques.

METHODS

The detection of IgM antibodies to JEV was performed by a commercial MAC-ELISA kit produced by the National Institute of Hygiene and Epidemiology, Vietnam.

NS1 antigen detection is based on the ELISA principle and reagents, which were developed by Li et al (2012), procedure technique was performed following their protocol (3). The first monoclonal antibody (clone 8F1) against the NS1 antigen of JEV was used for coating the F96 Nunc-Immuno plate. Sera samples were diluted to 1/100 in PBS (-), CSFs diluted to 1:10 in PBS (-), and after that loading diluted samples were into wells as diagram. After incubated at 37^{o}C for 1 hour, washed plate 5 times with PBS-T, and then added working antibody (clone 3E10)-HRPO conjugates attached to NS1-JEV antigen. The plate was incubated at 37^{o}C for 1 hour and washed. To detect the complex reaction of NS1 antigen and antibodies, a colorless TMB substrate system (TMB/Hydrogen peroxide) was added. The substrate will be hydrolyzed by the enzyme HRPO and changed to a green color. Stopping the enzymatic reaction was done by $4N.H_2SO_4$, which convert green to yellow color. The optical density (OD) is directly proportional to the amount of JE NS1 antigen present in the tested samples. The OD was read by an ELISA reader at 450nm/620nm wavelength; the ratio between OD of samples and negative control of more than 2 were confirmed as positive samples.

All positive CSFs by NS1-ELISA were screened JEV genotype by real-time RT-PCR. From positive CSFs, one part of the samples was extracted using the QIAamp Viral RNA Mini Kit (QIAGEN Sciences, Germantown, MD, USA) to obtain total RNA. The RNA was reverse transcribed and amplified using

Realtime RT-PCR SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) and specific primers and probes to JEV genotype I and genotype III (4). The other part of CSFs was confirmed positive by real-time RT-PCR to be inoculated into C6/36 cells for the isolation of JEV. Observation of cell culture every day, JE virus was propagated in C6/36 cells, total RNA was extracted from infected culture fluid and RT-PCR was performed to amplify the complete E gene of JEV.

RT-PCR's product was purified by the QIAquick PCR Purification kit (QIAGEN). The nucleotide sequences of the purified PCR products were determined using BigDye Terminator Cycle Sequencing reaction kits, reading nucleotide sequence results by ABI 310 Sequencer (Applied Biosystems).

DATA ANALYSIS

The performance of each technique which is related to the ability to detect tested positive samples by NS1 or IgM ELISA techniques and positive qualitative results of these two techniques is evaluated by the Kappa index (k), which is applied to compare statistical conformity. The correlation coefficiency between two techniques to be described how the difference in the equivalence of diagnostic ability. In case of the same categorical variable, It is no agreement if k is between 0.0-<0.2; The agreement is too low if k is between 0.2–0.4; The agreement is medium if k is between 0.4–0.6; The agreement is better and very good if k between 0.6 - 0.80 and 0.80 upward to 1, respectively (21).

Analysis nucleotide sequence of E gene by DNA Star (Lasergene) and contigs were arranged and compered by Clustal Wv.1.83 and submit data on the Genbank to obtain the code number of total E gene of isolated JEV in Vietnam.

Medical ethnic:

This study was accepted by the Ethnic Council in Medicine at NIHE, Code number: IRB-VN01055-14/2016.

RESULTS

Table 1. Detecting JE cases in clinical samples of AES patients by NS1-ELISA and MAC-ELISA for selecting positive CSFs in acute phase

Clinical samples	Detection of IgM/NS1 antigen	No of tested samples	Positive cases	Positive proportion
Acute CSFs	IgM to JEV	310	79	25.48%
	NS1 antigen	310	12	***3,87%***
Acute sera	IgM to JEV	310	79	25.48%
	NS1 antigen	310	12	***3,87%***
Convalescent sera	IgM to JEV	310	82	26.45%
	NS1 antigen	310	0	***0,00***

In total 310 AES cases in the General hospital of Bac Giang province were suspected of viral infection in the summer season only from June 2004 to 2017 (Table 1). Three kinds of clinical samples were collected: Acute CSFs in the first five days of illness (acute phase), pair of sera samples which include one in the acute phase and the other in the convalescent phase. Using MAC-ELISA to detect IgM antibody specific to JEV, to confirm 79 JE cases, the average positive proportion is 25.48% (79/310); When using NS1-ELISA for the detection of NS1 antigen of JEV in 310 CSFs as well as in 310 acute sera samples, only 12 positive samples were found for each kind of samples, the average positive proportion to detect JEV NS1 antigen in acute samples to be 3.87% (12/310). Whereas JEV NS1 antigen was found nothing in 310 convalescent sera samples, 82/310 (26.45%) JE confirmed cases were done by MAC-ELISA.

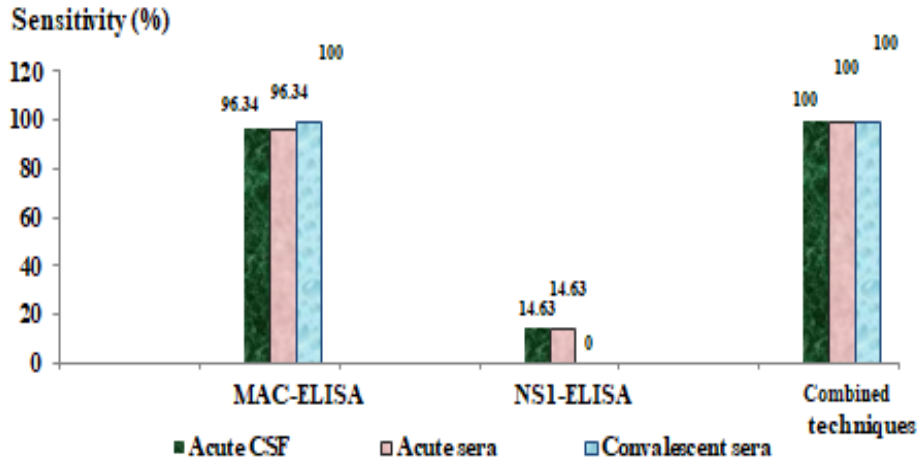

Figure 1. Evaluation of sensitivity for JE diagnosis diagnosis in acute and convalescent samples by MAC-ELISA and NS1-ELISA

Table 2. Sensitivity of NS1-ELISA and MAC-ELISA in JE diagnosis with acute cerebroispinal fluid of JE patients, in Bac Giang province

Cerebroispinal fluid		NS1-ELISA		Total
		Positive	Negative	
MAC-ELISA	Positive	9	70	79
	Negative	3	228	231
Total		12	298	310
Sensitive of NS1-ELISA		3.87% (12/310)		k < 0.4
Sensitive of MAC-ELISA		26.45% (79/310)		p<0.0001

Using MAC-ELISA and NS1-ELISA for JE diagnosis in acute samples, the positive proportion by MAC-ELISA was 25.48% and by NS1-ELISA to be 3.87% yielding the positive proportion at 26.45% (82/310) if combine the results of the two techniques (Table 2). It could be said that the sensitivity of NS1-ELISA for JE diagnosis in the collected acute phase was 14.63%, by

MAC-ELISA to be 96.34% showing the correlation conference by kappa value with the agreement too low (k=0.4) if using these techniques to detect positive cases among clinical acute-phase samples (Figure 1) ($p<0.0001$). In case testing in clinical convalescent sera samples, there is no agreement between two techniques. The positive proportion by MAC-ELISA was 26.45% and by NS1-ELISA to be nothing. It could be said that NS1 JE antigen was absent in the collected convalescent sera phase, it is no meaning when using NS1-ELISA for JE diagnosis. It could be said that the sensitivity of MAC-ELISA for JE diagnosis in collected convalescent sera phased is 100.00%. But 12 detected positive NS1 antigen samples in CSFs were oriented for detecting RNA of JEV by Realtime RT-PCR with specific primer sets to genotype 1 and genotype 3.

Table 3. Using Realtime RT-PCR for determination of JEV genotype in acute CSFs of JE patients in Bac Giang province, 2004-2017

Code of sample	Year	Diagnosis by	
		Realtime RT-PCR	NS1-ELISA
04CSF98	June 2004	**G III**	Positive
07CSF215	June 2007	**G I**	Positive
07CSF235	June 2007	**G I**	Positive
17CSF02	June 2017	**G I**	Positive

Among 12 clinical acute, CSFs were confirmed by NS1-ELISA, which were kept at -70^{o}C, one part of CSFs was used for the detection of RNA of JEV by real-time RT-PCR with specific primer sets of JEV GI and GIII. There were only 4 positive samples confirmed by Realtime RT-PCR with specific primer sets of JEV GI and JEV GIII, showing JE patients in Bac Giang province were infected by JEV genotype I&III from 2004 to 2017 (Table 3).

Thus the other part of CSFs were put on C6/36 cells for the isolation of JEV, all supernatant of culture samples were checked by real-time RT-PCR for detecting RNA of JEV, yielding only one JEV strain coded 04VN76 was isolated from CSFs coded 04CSF98. The whole E gene of the 04VN76 strain was decoded and compared 1500 nucleotide sequences with the other 12 isolated JEV genotype III, showing the nucleotide sequence identity among genotype III in Vietnam to be 0.3%-6.8% from 1989 to 2004. The results of Realtime RT-PCR were agreed with Sequencing and nucleotide sequence JEV genotype III code 04VN76 and submitted to the GenBank database obtained assession number [XXX] (Table 3).

DISCUSSION

JE virus has 5 genotypes, most of the human cases due to genotypes I and III, the other genotypes cause human disease being poor data to confirm due to demonstrating which genotype causes human disease yearly seems to be difficult from acute clinical samples (15). All though there are several methods for confirming JE cases such as the isolation of the virus, or the detection of viral nucleic acid (RNA), antigen, or antibody, each method has advantages and limitations such as JE diagnosis by the detection of RNA method or the isolation of virus is most specific, but less sensitivity, need expertise, facility

for cell culture, time-consuming; JE diagnosis by the detection of NS1 antigen is early diagnosis, but also less sensitivity; JE diagnosis by the detection of IgM antibody method is useful for confirmation of acute infection, least expensive, easy to perform (2,17,22). The choice of diagnostic method depends on the purpose of the test is done, the type of laboratory facilities and technical expertise available, costs, and the time of sample collection. For the detection of JE virus genotype in CSF samples, it can be done directly by Real-time RT-PCR with specific primer sets to genotype I and III have been reported or put CSF into C6/36 cells for the isolation of JE virus and then detection of JE virus genotype by sequencing (23,24). Which way is best for the detection of JE virus genotype cause human disease is a question?

As we know, after JEV infection in human, three kinds of antibodies will be responded including hemagglutination inhibition, complement combination, and neutralizing antibodies, which were named by the phenomenon to identify the antibody in *In vitro*. As a chemical structure, those immunoglobulin antibodies consist of 5 classes: IgA, IgD, IgE, IgG, and IgM. Among those IgG and IgM are major antibodies in the humoral immune. IgM appears early after infection and exists for around 30-90 days, which depends on the primary or secondary infection. After the JEV infection, neutral antibodies to JEV responded earliest to the clear virus, yielding the isolation of JEV as an exception (1,16,24). Whereas, NS1 antigen is a biomarker of natural infection, relating to viral RNA replication. As information relates to NS1-ELISA used for JEV diagnosis is poor data, so far. But for dengue diagnosis, the NS1-ELISA technique is applied to early diagnosis in the early stage of dengue fever/dengue hemorrhagic fever (DF/DHF) patients (3,4,20).

According to WHO guidelines, MAC-ELISA is considered a standard technique for JE diagnosis based on serodiagnosis to detect IgM anti to JEV in sera or in CSF, which gives the evidence of viral infection recently (17). Normally, IgM is un-existed in CSF, but it will be passed the “blood-brain” barrier with protective function only. In general, IgM antibody appears early in blood and CFS of JE cases. Thus, the detection of IgM to JEV from CFS is considered a “golden standard” not only for JE diagnosis but also for neu-tropic viral infections. Thus, the detection of IgM specific to JEV by the MAC-ELISA technique is applied widely in the laboratory for early diagnosis of JEV since the decade 1990s (10,17,18). Thus CSF/sera samples were collected in acute and convalescent phases of illness for the detection of IgM antibody as well as NS1 antigen of JEV based on the theoretical immunology as IgM antiviral JE appeared early event in the first day of onset of illness, NS1 antigen appeared within 5 days of the acute phase and then JEV will be cleared after day 5 of the infection (4,16,17).

In this study, clinical samples were collected from 310 AES cases in Bac Giang province from 2004 to 2017 for the detection of the JE virus genotype based on the strategy to detect positive samples by MAC-ELISA and NS1-ELISA. And then, selecting positive samples was confirmed by NS1-ELISA for the detection of viral RNA by Real-time RT-PCR and oriental for the isolation of JE virus for E gene sequencing.

With acute CSF/sera samples of JE diagnosis by MAC-ELISA, the proportion positive was confirmed at 25.48%, higher than NS1-ELISA only 3.87. It is interesting, that in convalescent sera 26.46% of JE cases were detected by MAC-ELISA, but none of the JE cases was detected by NS1 ELISA (Table 1); When the combination of positive results of 2 techniques for JE diagnosis in acute samples showing 79/310 positive samples (Table 1). Therefore, the sensitivity of MAC-ELISA and NS1-ELISA were evaluated based on 310 CSF/sera samples collected in both phases. To our results, if using NS1-ELISA, only 12/82 positive samples were detected in acute CSF/sera samples, and the sensitivity of NS1-ELISA was 14.63%. Meanwhile, if using MAC-ELISA for JE diagnosis, 79/82 positive samples were detected in acute-phase samples, and the sensitivity of MAC-ELISA for JE diagnosis was 96.34% and 100% in convalescent collected sera samples (Figure 1). It can be said that NS1-ELISA had lower effectiveness in JE diagnosis events using acute collected samples. But NS1-ELISA could detect NS1 antigen among 310 CSF/sera samples yielding 12 positive samples, the aliquoted CSFs samples were also used for the detection of RNA of JEV by Real-time RT-PCR, for the detection of the genotype of JEV causes AES, 2004-2017 in Bac Giang province. As our results, 4 positive samples in 2004, 2007, and 2017 were confirmed by Realtime RT-PCR by specific primer set genotype I and III (Table 3). Showing that, JEV genotypes I and III were etiology cause AES in Bac Giang province, 2004-2017. Among 82 JE patients confirmed by serology, most JE cases were non-JE vaccination or JE vaccination with only 3 basic doses without interval booster doses as manufacturer's instruction after three basic doses with inactivated vaccine.

In recent years, the emergence of several genotypes of JEV was detected such as JEV genotype I in most of the Asian countries during the 1960s-1990s and replacing JEV genotype III in which countries this genotype was circulated before in human, mosquitoes, and pigs (15,25,26). No longer after this, JEV genotype V was also detected in mosquitoes in China and Korea in 2009 and 2010, respectively after 60 years of absence (12,14). The emergence of JEV genotype V in recent years in several Asian countries, but the etiological of JEV genotype V had been still unknown (5). Thus, if JEV genotype I and genotype V will co-circulate, the monitoring of JEV genotype cause AES in patients is very important with the strategy of selecting positive CSFs in the acute phase by NS1-ELISA and MAC-ELISA showing it is useful for this purpose. In this study, among 4 positive CSF samples, which were detected RNA of JEV genotype I&III, using C6/36 cells for the isolation of the virus, yielded one JEV genotype III strain coded 04VN76 (from 04CSF98) and whole E gene of this virus strain was decoded and registered in Genbank database.

CONCLUSION

In this study, the JE virus genotype, which causes the human disease is feasible to detect in acute CSFs with the strategic use of several immunology and molecular techniques. Among 310 AES patients in Bac Giang province, 2004-2017, the sensitivity to detect JE patients in acute phase samples by MAC-ELISA was 96.34%, by NS1 ELISA was only 14.63% ($K=0.4$ with $p<0.0001$). Based on these results, Realtime RT-PCR was used to detect JEV

genotype I from 3 CSFs which coded 07CSF215, 07CSF235, 17CSF02 and genotype III from CSFs coded 04CSF98. But only one strain coded 04VN76 (isolated from CSFs coded 04CSF98) was decoded the whole E gene, reconfirming it is JEV genotype III.

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Conflicts of interest:

The author has declared no conflicts of interest

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