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Isolation and Identification of Japanese Encephalitis Virus from Piglets in Thakayta Township, Yangon

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Japanese encephalitis is a vector-borne zoonotic disease caused by the bite of *Culex* mosquitoes. Japanese encephalitis virus circulates among the wild birds, and transmitted to porcine, equine and human. In 2010, there were two reported cases of Japanese encephalitis infection in Thakayta Township, Yangon and the study was initiated within a month to get the JE virus isolate. A small pig farm was selected which was close (<2 km) to the home of JE cases. Blood samples were collected from five piglets in a selected pig farm, weekly for 14 times. JE virus was isolated in C6/36 mosquito cells, identified by Immunofluorescent Assay and Reverse Transcriptase-Polymerase Chain Reaction, and then sequenced by DNA sequencer. The isolate was found to be genotype I. In 2009, the first JE virus isolate in Myanmar was genotype III. This study was the second isolation of JE virus from piglets in Myanmar.

Key words: Japanese encephalitis, Virus isolation, Gene sequencing, Phylogenetic tree

INTRODUCTION

Japanese encephalitis virus (JEV) is the most important cause of epidemic encephalitis worldwide, with an estimated 35,000 to 50,000 cases and 10,000 deaths annually.[1] The case fatality rate of JE virus infections is approximately 25%, with 50% of survivors developing permanent neurological and psychiatric sequelae.[2] JEV is transmitted between vertebrate hosts by *Culex* mosquitoes, principally of the *Culex tritaeniorhynchus.*[3, 4] In Asia, pigs as well as birds are important natural hosts for Japanese encephalitis virus because these animals are often kept close to human dwellings, they serve as amplifying or bridging hosts that transmit the virus to humans.[5, 6]

It is an RNA virus of the genus *Flavivirus*, family *Flaviviridae.* It has a genome of 11 kb which encodes for three structural and seven nonstructural proteins. The envelope (E) protein is the most important among the structural proteins because it has neutralizing properties to the virus. According to phylogenetic analysis, there are five known genotypes of JEV worldwide according to the envelope (E) gene sequence.[7, 8]

In Myanmar, in 1982, a study on vector, amplifier, and human infection with JEV was done in a Yangon community (Dawbon Township). JEV infection was detected in 52.1% of pigs. The known JEV vector mosquito species, especially *Culex tritaeniorhynchus,* were found in the study area but no concurrent human JEV infections were elicited.[9] An investigation on JEV infection in Bogalay Township, Myanmar was done in 1999. Findings showed that JEV antibodies were detected in 33% of the pigs. They also found the Culex vector mosquitoes especially *C. vishnui* followed by *C. tritaeniorhynchus.*

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JE virus antibodies were not detected among the villagers during the study.[10] Non-epidemic investigation was done in Yangon, 1968. Two hundred and thirty-eight human sera were tested at Virus Research Centre in Poona, India. Sixteen percent of the sera had detectable neutralizing antibody against JE virus.[11] Attempts to isolate virus from the blood of patients with flavivirus encephalitis are usually unsuccessful because of transient viraemia and low titers. But, it is occasionally isolated from cerebrospinal fluid of patients who do not yet have antibody, particularly those who subsequently die,[12, 13] and from post-mortem brain tissue.[13- 15]

In the 2006 JE outbreak in China, isolation and sequencing of JE virus was attempted from CSF and mosquitoes. Eleven sequences were obtained and further analysis showed genotypes I and III.[16] JE virus was isolated from human and human brain in Chiang Mai (Northern Thailand) in 1964 and 1982, respectively. They were found to be genotype III. But in 1984 and 3-year survey (2003-2005) in pigs changes in JE virus genotype pattern showed genotype I.[17]

JE is a vaccine preventable disease and currently used vaccines are derived from JEV strain representative of genotype III. Clinical trials with genotype III vaccines have demonstrated effectiveness in areas where heterologous JEV genotypes cause human disease, supporting the hypothesis that immunity induced by genotype III vaccines protects against infection with JEV belonging to other genotypes.[18] In Myanmar, isolation of JE virus from pigs in DikeOo pig farm was done in 2009 and the isolate was identified as genotype III.[19]

MATERIALS AND METHODS

Study site

A small pig farm within a 2 km radius from houses of confirmed human cases of JE virus infection was targeted for sample collection (Fig. 1).

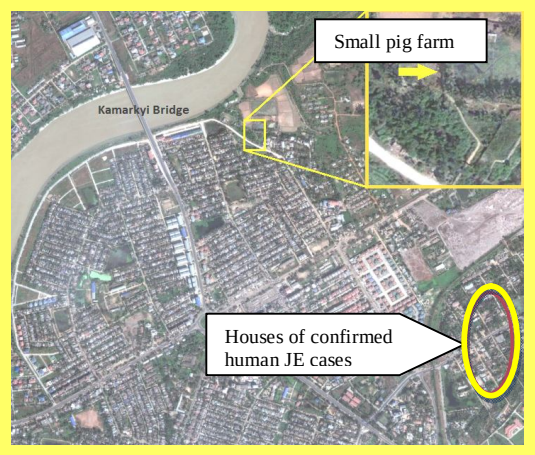

Fig. 1. A Google map showing the location of pig farm where the JEV was isolated, and houses of confirmed human JE cases

Sample collection

Blood samples were collected weekly from five piglets starting from 8 weeks of age to 22 weeks. Two to three milliliters of blood sample were collected from anterior vena cava of each piglet. A total of 70 blood samples were collected during 14 weeks.

Antibody testing by JE ELISA

Antibody was checked by Dengue-JE IgM Combo ELISA kit, Panbio Australia.

Virus isolation and identification

Each pig serum (50 µl) was diluted in 1,000 µl of serum free Modified Eagle's Medium, and 200 µl of diluted serum was inoculated onto 3 day old C6/36 mosquito cell line in a 25 cm^2 culture flask and incubated at 32ºC for 90 minutes. During incubation, culture flasks were kept on gentle rocking by a rocking machine. Then, 5 ml of MEM media was added to the flask and incubated in an incubator at 32ºC for 7 days. On day 7 of incubation, media was aspirated and one side of the flask was tapped gently to detach some cells.

Then, the loosened cells were fixed onto glass slide by using acetone for 10 minutes. Virus detection was carried out by indirect immunofluorescent staining using anti-flavivirus monoclonal antibody (4G2) and anti-JE monoclonal antibody as the first antibodies and, FITC conjugated goat anti-

mouse IgG as the second antibody. Then, the flasks were replenished with MEM 5 ml and incubation was continued, then the above steps were repeated for the next two consecutive weeks.

Gene sequencing

RNA extraction was done, by using QIA amp Viral RNA extraction kit (QIAGEN, Germany) according to the manufacturer's protocol, from a supernatant of JEV-positive cell culture, after the first passage, according to manufacturer's protocol as well as RNA reverse transcription-PCR (RT-PCR) [TPersonal (Biometra)]. RT-PCR was performed on 4 µl of cDNA template by using 2.5 units of Ampli-Taq Gold DNA Polymerase [RobusTT^{TM}II RT-PCR Kit (Finnzymes)]. Overlapping of JEV E gene fragments was amplified with 2 sets of primers: Ea forward primer, Ea reverse primer; and Eb forward primer and Eb reverse primer, respectively. The 1,500 nucleotides generated partial sequences of the JEV E gene that were compiled by using Sequence-Alignment Editor software version 5.0.9; pair-wise genetic distances were calculated with MEGA software version 4.0.

RESULTS AND DISCUSSION

After ELSA testing, two out of five serum samples collected at 14th week were JE antibody positive. The OD value of one sample was higher among the two. After virus isolation, one sample from 13th week showed cytopathic effect (CPE). This sample is also the one which was ELISA positive and with the higher OD value. After IFA testing, the sample that shown CPE was Flaviviral polyclonal antibody positive and dengue polyclonal antibody negative. After confirmation with JE monoclonal antibody and RT-PCR, the isolate was Japanese encephalitis virus.

After sequencing, the new Myanmar JEV sequence was analyzed with a group of 27 previously published JEV strain sequences which include 11 from Thailand, 5 from Japan, 6 from Indonesia, 2 from Malaysia, and one each from Korea, Australia and China. A phylogenetic tree was generated, and the new Myanmar JE virus isolate fits into the same GI cluster (Fig. 2).

This strain was associated with another subcluster (GIb) strain isolated in 2005 from Southern Thailand. But, eight Thai strains also isolated in 2005 formed a subcluster to Myanmar JE virus. In Myanmar 2009, the first JE virus sequenced was genotype III which is 99.58% identical with Beijing, China strain. Isolate was obtained from a pig farm about 70 miles from Yangon.[19]

But, in this (2012) study, the isolate was genotype I. In these two isolation studies, most of the techniques were similar except sample collection pattern. In 2009 study, the serum samples were randomly collected, but in this study more systematic collection was made because the chance of getting JEV isolation was very low. A study found that the viraemia to primary JEV infection lasted for one to three days, and was detected from day 2 to day 5 post infection.[20]

In Myanmar, there have been a lot of sero-prevalence studies regarding JE virus, but there is no genotypic identification study within the last three decades. The first isolation of JE virus from the brain of a dead horse was done in 1977 and the isolate was confirmed as JEV by the WHO reference center in Poona, India.[21] In this study, the isolate was genotype I. Regarding preventive measures, Japanese encephalitis disease is a vaccine preventable disease and currently used vaccines are derived from JEV strain representative of genotype III, and this vaccine is still effective against four genotypes (I, II, III and IV).[18]

To date, the Muar strain, which was the first isolated genotype V JEV from specimens of brain tissues of patients with viral encephalitis in Malaya in 1952,[22, 23] the second finding of genotype V was from *Cx. tritaeniorhynchus* collected in Tibet, China (2009),[24] and the third report of JEV genotype V was from Republic of Korea (2010).[25]

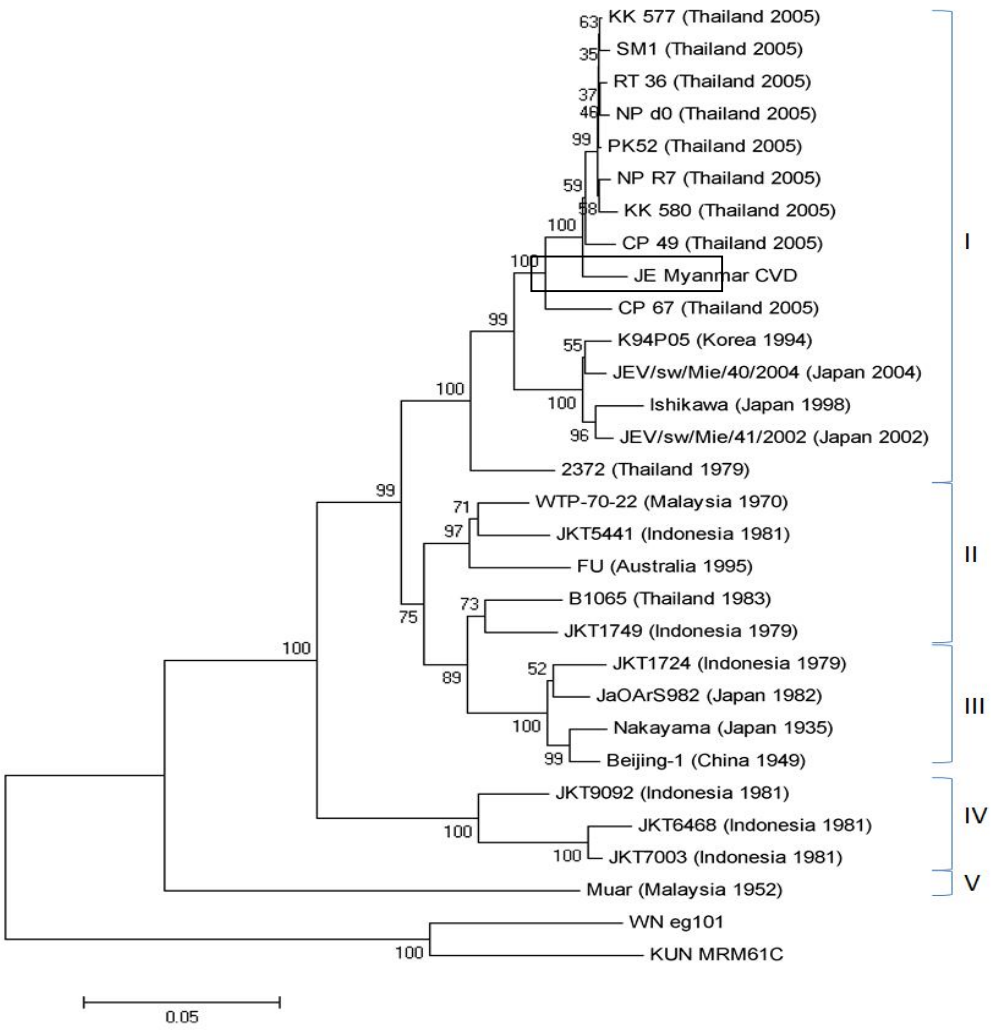

Fig. 2. Phylogenetic relationship of Japanese encephalitis viruses done by E gene (1,500 nt) analysis by MEGA version 4 software, using the neighbor-joining (p-distance) method. The length of the tree branches indicates the percentage of divergence; the percentage of successful bootstrap replicates is specified at the nodes (1,000 replicates). West Nile and Kunjin virus prototype sequences were included to root the tree.

Therefore, the genotype V is reemerged within the last three years, however, the protective efficacy of current vaccine against JEV genotype V has not been studied yet. In Myanmar, two strains of JE virus genotype I and III had been identified but it could not be concluded whether there is co-circulation or changes in genotype because of small sample size.

Conclusion

In Myanmar, there are cases of JE infection which are still reporting and, in 2008 there were 8 cases of JE ELISA confirmed patients in Yangon Children's Hospital. Among 8, 2 patients were expired, 1 suffered deafness and 1 had undergone blindness.[26] However, fever develops in only a small proportion (about 1:300) of those exposed.[27] On the other hand, the ratio of cases to undetected is 1:300.

Thus, in Myanmar, there must be a lot of undiagnosed cases and thousands of sub-clinical infections. In comparing to the dengue infection, the mortality rate is low but the neurological sequelae may be a burden to their family. Like other infectious diseases, vaccination is the most effective preventive measure against JE. In Thailand,

introduction of JE vaccine into children immunization started in 1990.[28] But, implementation of JE vaccine into Expanded Program on Immunization necessitates strong data of JE prevalence, natural immune status among the normal population, and mortality among the confirmed JE cases.

It was concluded that as long as there is no JE vaccination program in Myanmar, prevalence and genotypic studies should be continued to get the baseline data. And, to know the changes in genotype is also important because the new emerging JE virus strain may not be protected by the current JE vaccine.

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