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Short communication

Isolation and genomic characterization of Culex flaviviruses from mosquitoes in Myanmar

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ABSTRACT

An entomological surveillance of arboviruses was conducted in Myanmar in 2014. A total of 8357 *Culex* mosquito vectors were collected in the Mandalay area and virus isolation was done by using the mosquito cell line C6/36 E2. A total of eighteen strains of Culex flavivirus (CxFV) were isolated from *Cx. tritaeniorhynchus*, *Cx. vishnui* and *Cx. fuscocephala*. Like other insect-specific flaviviruses, CxFV can replicate only in mosquito cells but not in mammalian cells. These CxFV strains that were isolated in Japan from mosquitoes collected in Myanmar were closely related to the Wang Thong virus detected from *Cx fusocephalus* in Thailand and *Cx.theileri* flavivirus (CTFV) isolated from *Cx. theileri* mosquitoes in Portugal and Turkey. They encode a single open reading frame with 3357 amino acid residues. They have the characteristics of flaviviruses and have 95.62% amino acid identity with CTFV. This is the first report of CxFV in Myanmar with the characterized viral genome. This study illustrated that CxFV was circulating among the vectors of human pathogenic arboviruses in Myanmar but the impact of CxFV on other flaviviruses which are endemic in the study area still remains to be explored.

Flaviviruses are positive-sense and single-stranded RNA viruses that belong to the family *Flaviviridae*. They exhibit a wide geographical distribution (Blitvich and Firth, 2015). Most flaviviruses such as dengue viruses (DENV) and Japanese encephalitis virus (JEV) can cause human diseases but some viruses such as cell fusing agent virus (CFAV) (Stollar and Thomas, 1975), Kamiti River virus and Culex Flavivirus (CFV) isolated in Japan (Hoshino et al., 2007) have not been found to cause disease to humans and can replicate only in mosquitoes.

Myanmar is an endemic country for arthropod-borne flaviviruses such as DENV and JEV outbreaks have occurred (Swe et al., 1979; Thu et al., 2004). Entomological surveillance of these arboviruses is important for the detection of pathogenic as well as non-pathogenic flaviviruses. Therefore, virological surveillance of mosquitoes was conducted in Myanmar. In this study, non-pathogenic CxFV were isolated and characterized.

A total of 8357 mosquitoes mainly of the *Culex* species such as *Cx. tritaeniorhynchus, Cx. vishnui, Cx. fuscocephala* were collected by animal baited method during the rainy season (June–September) in 2014 in the Mandalay Region of Myanmar. Identification of mosquitoes based on morphology (Sirivanakarn, 1976) was done by medical entomologists and the mosquitoes were kept at −80 °C deep freezer before the experiments. All experiments were done at the Department of Virology, Institute of Tropical Medicine, Nagasaki University. According to the species and date of collection, about twenty female mosquitoes (18–25 number) were pooled into each tube. A total of 401 pools of mosquitoes were subjected to virus isolation. By a sterile pestle, each mosquito pool was homogenized in 1 ml of minimal essential medium (MEM) with 2% fetal calf serum (FCS), and then filtered through a 0.45 μm filter. Virus isolation and characterization was done using mosquito cells (C6/36 E2) and monkey cells (Vero) (Igarashi, 1978).

Viral RNA was extracted from infected culture fluids (ICFs) by using Viral RNA Mini kit (QIAGEN, Hilden, Germany). Screening for the presence of flavivirus genome was done by Prime Script ™ one step RT-PCR Kit (Takara Bio Inc., Shiga, Japan) using universal flavivirus primer (Tanaka, 1993). Samples that became positive were checked again by using primers specific for DENV, JEV and CxFV to identify the specific flavivirus isolated from the ICF. Only CxFV strains (18 strains) were isolated; no samples were positive for JEV and DENV. The whole

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polyprotein nucleotide sequences and NS-5 regions of the CxFV strains were amplified by specific primer sets that were constructed based on CTFV (Parreira et al., 2012). Sequence was performed by using the BigDye Terminator 3.1 ver and analyzed by ABI Prism™ Capillary Sequencer 3130-Avant Genetic Analyzer.

To identify the amino acid identity of the isolated viral strains from Myanmar, other reference insect-specific flaviviruses namely, *Cx. theileri* flavivirus from Portugal (HE574573), Mosquito virus from Turkey (KX652375), CFV (NC008604), Quang-Binh Virus (NC012671), and cell fusing agent (NC001564) were selected for comparison. The nucleotide sequences were aligned by DIALIGN-TX software for globally related multiple sequences (Subramanian et al., 2008) and the analysis of amino acid identities between CxFV isolated in Myanmar and other reference insect specific flavivirus strains were done by using Blast software (Camacho et al., 2009). The substitution model was selected by ProTest version 2.4 (Abascal et al., 2005) and JIT + G was chosen as the model (Guindon and Gascuel, 2003). Trees were drawn by Fig tree software, version 1.4.2 (FigTree).

Phylogenetic trees were constructed based on the whole polyprotein and NS-5 gene regions of the virus strains isolated from this study together with those from the neighboring countries of Myanmar as well as from other regions of the world. The DNA fragments encoding the near full-length genome and NS-5 protein of CxFV were submitted to GenBank (accession numbers from MF582365 to MF582382). This study was approved by the Institutional Ethical Committee of the Department of Medical Research (Upper Myanmar) with the approval code 12/Ethics/DMRUM/2014.

Nowadays, many new *Culex*-related flavivirus strains were explored dynamically and detected from wide geographical regions including Asia, Africa, Europe and America (Blitvich and Firth, 2015). In the present study, 18 CxFV strains were isolated from the pools of mosquitoes collected during field surveillance. This is a first report of CxFVs from Myanmar and we studied their characteristics. Five viral strains were isolated from *Cx. tritaeniorhynchus* mosquito pools, nine were from *Cx. fuscocephala* and four from *Cx. vishnui* mosquito pools. During this 2014 virological surveillance of mosquitoes, we failed to get the JEV.

Like other insect specific flavivirus, the viral strains isolated in Myanmar replicated only in mosquito cells (C6/36 E2) but not in mammalian cells (Vero). Viral RNA was detected only from the supernatant of infected mosquito cells but not from mammalian cells. These results were also similar to other previously reported viral strains such as CxFV from Japan, Quang-Bing virus from Vietnam and many others (Blitvich and Firth, 2015; Crabtree et al., 2009; Hoshino et al., 2007). Cytopathic effect (CPE) was observed as detachment and aggregation of C6/36 E2 cells after 5 days of post infection (dpi), however, no CPE was observed in Vero cells (Fig. 1). Some insect specific flavivirus strains such as CFA do not cause CPE on *Aedes aegypti* cells but can cause marked CPE on *Aedes albopictus* cells (Stollar and Thomas, 1975). Quang Binh virus (Crabtree et al., 2009) just like the viral strains isolated in Myanmar also causes marked CPE on the mosquito cell line C6/36 E2. The CPE could be related to the replication of the virus and adaptability of the virus to the host (Hoshino et al., 2007).

Sucrose gradient ultracentrifugation was used to get purified CxFV antigen and the procedure was performed according to the method described by Inoue et al. (2010). The purified virus particles were processed for transmission electron microscopy and were observed to appear as flavivirus-like particles with about 40–50 nm in diameter (Supplementary Fig. 1). The envelope proteins of the purified virus particles were checked by SDS-PAGE and we noted a band with molecular mass of about 60 kDa (Suplementary Fig. 1) (Hoshino et al., 2007), the mass of the viral E protein. The band was analyzed by Western blot, and the antibodies against JEV was used to check this structural protein however, no band appeared because the JEV antibody did not cross react with the CxFV protein.

Phylogenetic analysis based on NS5 gene showed that most strains have similarNS5 that cluster in one single genetic lineage. Therefore, only near full-length genome of two viral strains were amplified and analyzed

A single open reading frame (ORF) of CxFV isolated in Myanmar encoded 3357 amino acid residues. Only 11 amino acid residues differed (99% amino acid identities) between the two viral strains. Similar to other insect specific flaviviruses isolated from different parts of the world, the genomic characteristics were identical to those of the genus *Flavivirus* encoding a large ORF including three structural proteins and seven non-structural proteins with putative cleavage sites (Blitvich and Firth, 2015). Pairwise comparison done between CxFV strains isolated in Myanmar and in Portugal and Turkey showed an identical polyprotein size. The putative cleavage site of the polyprotein of the viral strain isolated in Myanmar and other representative cleavage sites of insect specific flavivirus are shown in Table 1. The isolated viral strain showed similar amino acid cleavage sites with those of CTFV isolated in Portugal and Turkey (Ergunay et al., 2016; Parreira et al., 2012). Like the CTFV isolated in Portugal and mosquito flavivirus isolated in Turkey, the viral serine-protease seems to be involved in the cleavage of the C/anchored C, prM/M, NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5, whereas a furin-like protease seems to play a role in the processing of the anchored C/prM, M/E, E/NS1 and perhaps the NS4A/NS4B, protein junctions (Parreira et al., 2012).

The gene sequences with the highest identities common between the viral strains isolated in Myanmar and other insect specific flavivirus strains were those of the structural protein genes PrM, E and non-structural protein gene NS-5 (Table 2). The isolated viral strains had 91% nucleotide and 95.62% amino acid identities with the CTFV isolated in Portugal. Both structural and non-structural proteins showed more than 84% amino acid identities with CTFV isolated in Portugal but compared with CxFV from Myanmar and other reference insect specific flaviviruses showed less than 84% identities. Kuno et al., reported that 84% amino acid identity can be used as criteria for determination of species of the members of the genus *Flavivirus* (Kuno et al., 1998). Therefore, we considered that the CxFVs isolated in Myanmar were of the same species with CTFV isolated in Portugal and Mosquito flavivirus from Turkey.

The phylogenetic tree based on partial NS-5 protein gene sequences from 35 flavivirus strains including the 18 strains of CxFV isolated in

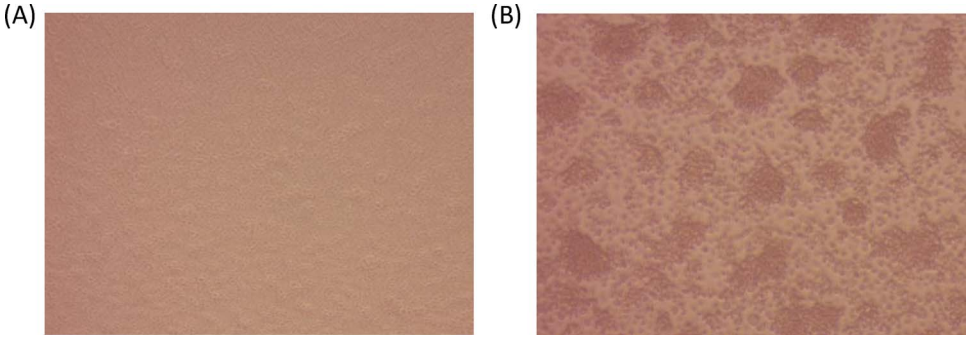

Fig. 1. Phase contrast photomicrographs of (A) C6/36 control cells and (B) cells at 7 days after infection by CxFV.