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ScienceDirect
Virology 359 (2007) 405–414
VIROLOGY
www.elsevier.com/locate/yviro

Genetic characterization of a new insect flavivirus isolated from *Culex pipiens* mosquito in Japan

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Received 30 June 2006; returned to author for revision 26 July 2006; accepted 22 September 2006
Available online 30 October 2006

Abstract

We found a new flavivirus that is widespread in *Culex pipiens* and other *Culex* mosquitoes in Japan. The virus isolate, named *Culex* flavivirus (CxFV), multiplied only in mosquito cell lines producing a moderate cytopathic effect, but did not grow in mammalian cells. The CxFV genome is single-stranded RNA, 10,834 nt in length and containing a single open reading frame encoding a polyprotein of 3362 aa with 5′ and 3′ untranslated regions (UTRs) of 91 and 657 nt, respectively. Phylogenetic analyses revealed that CxFV is closely related to the insect flaviviruses associated with *Aedes* mosquitoes, Cell fusing agent (CFA) and Kamiti River virus (KRV). The 3′ UTR of CxFV contains four tandem repeats, which have sequence similarities to the two direct repeats in the CFA and KRV 3′ UTRs. These results suggest that CxFV may be a new group of insect flaviviruses.

Keywords: Insect flavivirus; *Flavivirus*; Complete nucleotide sequence; *Culex pipiens*; Phylogenetic analysis

Introduction

Viruses in the genus *Flavivirus*, family Flaviviridae are small, enveloped viruses with an icosahedral nucleocapsid containing a 10- to 11-kb single-stranded, positive-sense RNA genome that encodes the viral proteins in a single open reading frame (ORF). The polyprotein encoded by the ORF is processed co- and post-translationally to produce three structural proteins, the capsid (C) protein, the membrane (M) protein which is cleaved from a precursor (prM) and the envelope (E) protein, as well as seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) that play important roles in replication, proteolysis and maturation (Lindenbach and Rice, 2003; Mukhopadhyay et al., 2005). The 5′ and 3′ untranslated regions (UTRs) of flaviviral RNA have conserved nucleotide sequence elements and secondary structures closely related to RNA replication and translation (Charlier et al., 2002; Markoff, 2003).

The majority of flaviviruses reported to date are known as disease agents causing encephalitis or hemorrhagic fever in vertebrates and are transmitted by blood-sucking arthropods, such as mosquitoes and ticks. Therefore, arthropod-borne flaviviruses are frequently classified into mosquito-borne and tick-borne groups. The other flaviviruses are categorized as "no-known vector (NKV)" group, currently with isolates only from vertebrate hosts. These three groups are distributed in many areas of the world, although the distribution of each flavivirus is geographically localized (Burke and Monath, 2001; Gould et al., 2003). The current status of these groups may be explained by divergent evolution involving adaptation to the host, the vector, and the associated ecology.

Among flaviviruses, the mosquito-borne flaviviruses comprise the largest group and are divided into *Aedes*- and *Culex*-borne clades based on differences in the main vector genus (Gaunt et al., 2001; Gould, 2002). These two mosquito-borne clades cause different clinical symptoms; e.g., hemorrhagic

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0042-6822/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.09.039

fever by *Aedes*-borne viruses and encephalitis by *Culex*-borne viruses. Encephalitis is also caused by tick-borne flaviviruses (Gould et al., 2004) and a *Culex*-borne flavivirus, West Nile virus (WNV), that can propagate in ticks (Lawrie et al., 2004). These findings may reflect an evolutionary relationship between the mosquito-borne and tick-borne groups. However, it is not clear which group (mosquito-borne, the tick-borne or NKV) is the most phylogenetically divergent (Billoir et al., 2000; Cook and Holmes, 2006).

Cell fusing agent (CFA) is a flavivirus originally isolated from a cell line established from the mosquito *Aedes aegypti* (Stollar and Thomas, 1975). CFA is classified as an "insect flavivirus" that propagates only in mosquito cells but not in mammalian cells (Cammisa-Parks et al., 1992). Kamiti River virus (KRV) is a CFA-related flavivirus isolated from field-collected *Aedes macintoshi* in Africa (Sang et al., 2003; Crabtree et al., 2003). These two insect flaviviruses were phylogenetically classified into the most outgroup lineage among the flaviviruses and considered as primordial forms of flaviviruses (Cook and Holmes, 2006). Another CFA-related DNA sequence is known as cell silent agent (CSA): this sequence is integrated into the genomes of *Aedes* spp. mosquitoes (Crochu et al., 2004). These findings suggest that insect flaviviruses have been closely related to mosquitoes, and that other insect flaviviruses associated with mosquitoes might exist in natural environments.

In the Far East area, mosquito-borne (Japanese encephalitis virus: JEV), tick-borne (tick-borne encephalitis virus: TBEV and Negishi virus), and NKV (Apoi virus: APOIV) flaviviruses have been isolated (Kuno et al., 1998; Cook and Holmes, 2006). In particular in Japan, mosquitoes in the genus *Culex* include important vectors of JEV and could be potential vectors of other flaviviruses, such as WNV which has emerged recently in North America (Mackenzie et al., 2004). At present in Japan, although *Aedes albopictus* and some other *Aedes* species are widely distributed, no *Aedes*-borne flavivirus has been identified. Yokose virus (YOKV), isolated from a bat in Japan, was phylogenetically classified in the *Aedes*-borne group, but its potential vector is not known (Tajima et al., 2005). In addition, the distribution of insect flaviviruses has not been reported in Japan.

A 2003 survey, involving collection of mosquitoes and detection of viruses, studied the distribution of mosquito-borne viruses in Japan. During the course of this nationwide survey, a new insect flavivirus was isolated from *Culex pipiens* and other *Culex* mosquitoes and designated "*Culex* flavivirus (CxFV)." In the present study, we investigated the morphology and structural proteins of CxFV and determined the complete nucleotide sequence of the genomic RNA of a CxFV isolate. Furthermore, to investigate the phylogenetic relationships among insect flaviviruses, the nucleotide sequences of the E genes of nine CxFV strains from *Culex* mosquitoes collected at different sites were compared with those of two already known insect flaviviruses CFA and KRV.

Results

Virus isolation and characterization

Homogenates of *Culex* and *Aedes* mosquitoes collected in Japan and Indonesia were used as the initial samples for virus isolation. The morphology of cells inoculated with cell culture supernatants after two or more serial passages was observed by phase contrast microscopy. In a number of infected cultures, moderate cytopathic effects (CPE) on C6/36 cells were observed from 4 days post-inoculation, although CPE was undetected in almost all cases of cells infected using the original inoculum or an inoculum after the first passage. No CPE was observed in Vero and BHK-21 cells in any of the samples. The C6/36 cells inoculated with a viral stock (passaged four times) showed only weak growth inhibition and cell aggregation in comparison with control cells (Fig. 1). Consequently, purification and titration of the causative agent by plaque formation assays were unsuccessful on C6/36 cells.

Flavivirus-like particles were seen by transmission electron microscopy of infected cells (Fig. 2). The enveloped virions were approximately 50 nm in diameter and seen in the lumen of endoplasmic reticulum. No such particles were observed in mock-inoculated controls. RT-PCRs with the flavivirus-specific primer sets (Table 1, described below) were performed on RNAs extracted from the culture supernatants of the inoculated cells. The PCR-amplified products were obtained only when

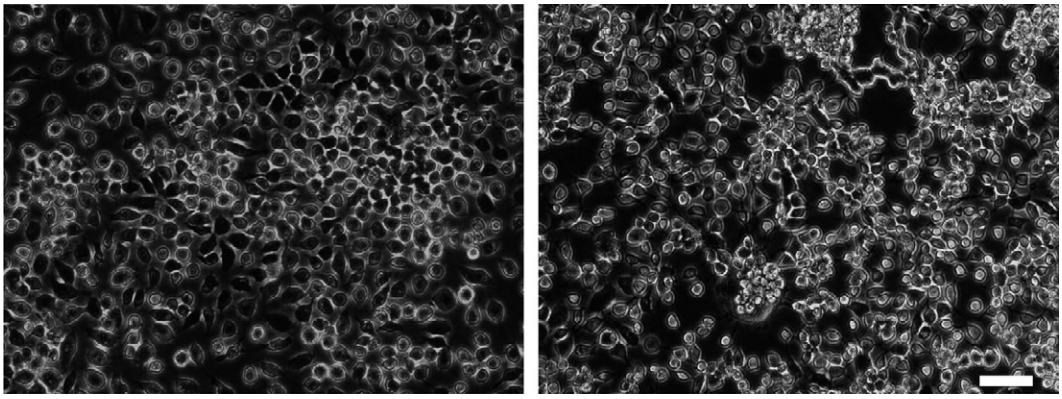

Fig. 1. Phase contrast micrographs of control C6/36 cells (left) and CxFV-infected cells (right) 84 h post-infection. The viral stock after four passages was used as the inoculum. Scale bar, 50 μm.