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Novel flaviviruses from mosquitoes: Mosquito-specific evolutionary lineages within the phylogenetic group of mosquito-borne flaviviruses

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ABSTRACT

Novel flaviviruses that are genetically related to pathogenic mosquito-borne flaviviruses (MBFV) have been isolated from mosquitoes in various geographical locations, including Finland. We isolated and characterized another novel virus of this group from Finnish mosquitoes collected in 2007, designated as Ilomantsi virus (ILOV). Unlike the MBFV that infect both vertebrates and mosquitoes, the MBFV-related viruses appear to be specific to mosquitoes similar to the insect-specific flaviviruses (ISFs). In this overview of MBFV-related viruses we conclude that they differ from the ISFs genetically and antigenically. Phylogenetic analyses separated the MBFV-related viruses isolated in Africa, the Middle East and South America from those isolated in Europe and Asia. Serological cross-reactions of MBFV-related viruses with other flaviviruses and their potential for vector-borne transmission require further characterization. The divergent MBFV-related viruses are probably significantly under sampled to date and provide new information on the variety, properties and evolution of vector-borne flaviviruses.

Introduction

Members of the genus *Flavivirus*, family *Flaviviridae* are enveloped viruses that have a positive-sense single-stranded RNA genome. The flaviviral genome contains a single open-reading frame encoding a large polyprotein that is cleaved and processed by viral and host enzymes to form the mature structural proteins found in virions, the capsid (C), membrane (M) and envelope (E). In infected cells, seven non-structural viral proteins have been identified (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Chambers et al., 1990a; Pletnev et al., 2011). Although flaviviruses show considerable conservation of their genome organization, they exhibit divergent host ranges. In general, the flavivirus groups are phylogenetically relatively closely related and have associations with specific vector and/or vertebrate hosts (Cook and Holmes, 2006; Gaunt et al., 2001; Grard et al., 2007, 2010). The mosquito-borne flaviviruses (MBFVs) are the largest group with currently over 20 recognized species that include some of the most important pathogens of human arboviral diseases. The MBFVs can be divided into two main groups based on their mosquito-vector associations (Gaunt et al., 2001). The flaviviruses transmitted by *Stegomyia* mosquito species, which include yellow fever virus (YFV) and dengue virus (DENV), have life cycles involving various vertebrate hosts, including primates. The flaviviruses transmitted by *Culex* mosquito species include West Nile virus (WNV), Japanese encephalitis virus (JEV) and St Louis encephalitis virus (SLEV), which are characteristically maintained in life cycles involving birds. Humans may be incidentally infected but are generally considered to be dead-end hosts. Some viruses that are genetically relatively closely related to YFV appear to have no known arthropod vectors, *e.g.* Entebbe bat virus (ENTV) and Yokose virus (YOKV), and it has been proposed that they may have lost this vector-dependence (Kuno et al., 1998).

The flaviviruses transmitted by ticks are associated either with small mammals or seabirds and include pathogens that infect humans, such as tick-borne encephalitis virus (TBEV). In addition

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to flaviviruses that are hosted by both vertebrates and arthropods, other flaviviruses are defined as no-known vector (NKV) viruses. These viruses are at present considered to be hosted exclusively by small mammals and include viruses associated with bats, such as Entebbe bat virus (ENTV) and Rio Bravo virus (RBV), and viruses associated with rodents, such as Modoc virus (MODV). Additionally, another group of flaviviruses that has been characterized in more recent years, the insect-specific flaviviruses (ISFs) are currently known to infect only insect hosts, primarily mosquitoes. These viruses include cell fusing agent virus (CFAV) (Cammisa-Parks et al., 1992; Stollar and Thomas, 1975), Kamiti River virus (KRV) (Crabtree et al., 2003; Sang et al., 2003) and many recently identified related viruses from different regions of the world (Cook et al., 2006, 2009, 2012; Crabtree et al., 2009; Farfan-Ale et al., 2009; Hoshino et al., 2007, 2009; Huhtamo et al., 2012; Kim et al., 2009; Morales-Betoulle et al., 2008). Interestingly, some of these ISFs appear to be capable of integrating their genomic sequences into mosquito genomes (Crochu et al., 2004). The additional flaviviruses, Tamana bat virus (TABV) (de Lamballerie et al., 2002) and Ngoye virus (Grard et al., 2006) appear to represent highly divergent genetic lineages not closely associated with any currently recognized flavivirus group.

Until recently, all flavivirus genomes were considered to contain a single ORF encoding the viral proteins. However, it has now been shown that through a ribosomal frameshifting mechanism, an alternative-sized NS1 protein (NS1′) is produced by some mosquito-borne flaviviruses within the Japanese encephalitis virus group (Blitvich et al., 1999; Firth and Atkins, 2009). Also, an additional protein designated “fifo”, encoded as an overlapping ORF in the NS2A/NS2B coding sequence, has been detected in some insect-specific flaviviruses (Firth et al., 2010). Whereas the NS1′ protein has been associated with pathogenic properties (Melian et al., 2010), the possible functions of “fifo” are currently unknown.

Recently, six novel flaviviruses isolated from mosquitoes were published and shown to be genetically related to the taxonomically recognized mosquito-borne flaviviruses (MBFVs) (Pletnev et al., 2011), namely Nounané virus (NOUV) (Junglen et al., 2009) from Côte d'Ivoire, Chaoyang virus (CHAOV) from China and South Korea (Lee et al., 2013; Wang et al., 2009), Lammi virus (LAMV) from Finland (Huhtamo et al., 2009), Marisma mosquito virus (MMV) from Spain (Vazquez et al., 2012), Nanay virus (NANV) from Peru (Evangelista et al., 2013) and Barkedji virus from Senegal and Israel (Kolodziejek et al., 2013).

Crucially, these viruses do not appear to infect vertebrate cells readily despite their apparent similarity to MBFVs, the latter which readily infect vertebrate hosts. Here we review the available information for the currently-known MBFV-related viruses and report the isolation and characterization of four strains of LAMV and four strains of a potentially novel virus species tentatively named Ilomantsi virus (ILOV).

Results

Virus isolation, identification and sequence analysis

Homogenates of the mosquito pools were tested in 68 virus isolation attempts, in parallel, on C6/36 and Vero cells. From these, eight virus isolation cultures from C6/36 cells were identified as positive for flavivirus antigen in IFA whereas the Vero cells infected with the same mosquito homogenates remained negative. These viral isolates caused mild CPE (rounding of cells and increasing numbers of floating cells) on infected C6/36 cells, approximately one week post-infection. A fragment of the NS3 gene was amplified and sequenced from each isolate. The derived sequences (536 bp) identified the isolates as flaviviruses representing two distinct groups, the isolates M0727, M0719, S0739 and M0728 were identical to each other and shared 98.6% nucleotide homology with the prototype strain of LAMV (FJ606789, corresponding to nucleotides 5104-5639). Following the criterion of defining a flavivirus species based on nucleotide sequence comparisons (Kuno et al., 1998), these isolates were considered strains of LAMV as they shared over 84% pairwise nucleotide homologies with LAMV prototype virus. The second group of isolates was also identical to each other, including isolates M0724, M0720, M077 and M0726. These isolates shared only 67.3% nucleotide homology with the prototype strain of LAMV, and 67.1% with the LAMV 2007 strains and were considered to represent a separate flavivirus species provisionally designated Ilomantsi virus (ILOV) based on the mosquito collection site.

One representative strain from each group, namely (M0719) designated Lammi virus strain Mekrijärvi 2007 (LAMV-M07) and ILOV (M0724), was chosen for further analysis. Electron microscopy was performed on concentrated ILOV samples, which showed spherical flavivirus-like virions of approximately 40–50 nm in diameter (not shown). The complete coding sequences of LAMV-M07 and ILOV demonstrated the characteristic organization typical of flavivirus genomes, encoding a long polyprotein. The coding sequence of ILOV was 10,353 bp, encoding a 3451 amino acid polyprotein (Genbank accession KC692067). Within the complete coding sequence, ILOV and LAMV were found to share only 62.7% pairwise identity at the nucleotide level and 64% at the amino acid level, demonstrating that they were separate virus species (Kuno et al., 1998; Pletnev et al., 2011). The ORF sequence of LAMV-M07 was 10,302 bp, encoding a polyprotein of 3434 amino acids (GenBank accession KC692068). The LAMV-M07 strain shared 98.8% homology at the nucleotide level and 99.6% at the amino acid level with the LAMV prototype strain. Similar to the LAMV prototype strain, the conserved cysteine residues found in most of the other flaviviruses including six cysteines in the preM, 12 in the E protein and 12 in the NS1 protein (Chambers et al., 1990a) were also found in LAMV_M07 and ILOV. The potential N-glycosylation sites in LAMV_M07 included two sites in preM, one site in E and four sites in NS1 protein that were identical to those of the LAMV prototype strain. In contrast to LAMV and LAMV_M07, which had one potential N-glycosylation site in the E protein, none were detected in the ILOV E protein. The putative fusion loop region of E the protein (residues 98–110) in the LAMV prototype and LAMV_M07 strain were found to be similar to those of other flaviviruses. However, in the case of ILOV, residue 110 was an arginine (R), whereas the corresponding residue in most of the other flaviviruses was a lysine (K).

Similar to LAMV and LAMV_M07, four potential N-glycosylation sites were predicted in the ILOV NS1 protein, three initial ones being at the same positions (LAMV polyprotein N884, N897, N981) and the last one being 4 residues before the location of corresponding LAMV N-glycosylation site (LAMV polyprotein N 1081). No DNA sequences corresponding to the genomic RNA of LAMV prototype virus, LAMV-M07 or ILOV were detected in the infected cells using primers targeted to the NS5 gene.

Novel flavivirus sequences that are related to LAMV and ILOV originating from different geographical locations are documented in public databases (Table 1), including Marisma mosquito virus (MMV) from Spain (Vazquez et al., 2012), Barkedji virus (BARKV) from Senegal (unpublished, GenBank accession EU078325.1) and Israel (Kolodziejek et al., 2013), Nounane virus from Cote d'Ivoire (Junglen et al., 2009), Donggang virus (DONV) (unpublished, GenBank accession NC_016997), Chaoyang viruses (CHAOV) from China (GenBank accession FJ883471) and South Korea (Lee et al., 2013; Wang et al., 2009) and Nanay virus from Peru