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Isolation and characterization of a California encephalitis serogroup orthobunyavirus from Finnish mosquitoes

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ABSTRACT

The mosquito-borne California encephalitis serogroup viruses of the genus *Orthobunyavirus* (family *Bunyaviridae*) include several causative agents of encephalitis in humans. Until recently, Inkoo virus (INKV) was the only orthobunyavirus isolated in Finland, showing high seroprevalence in the population. In this study, we recovered five orthobunyavirus isolates from mosquitoes collected in eastern Finland in the early autumns of 2007 and 2008 by inoculation of Vero cells. The isolates were determined by S, M and L segment sequences to represent the California encephalitis virus species but distinct from INKV (68% polyprotein amino acid (aa) identity). In genetic analyses, isolates clustered together with a number of westernmost Chatanga virus isolates (98% polyprotein aa identity) reported from Russia, forming a distinct phylogroup. However, the sequence homology of this phylogroup to the majority of Chatanga isolates, comprising three different geographically clustered phylogroups, was considerably lower (89–92% polyprotein aa identity). The five new isolates were designated as Möhkö isolates of Chatanga virus, according to the village of origin. The isolates were closely related to Snowshoe hare virus (SSHV) and La Crosse virus (LACV) with an aa identity of 87% and 82% within the M segment polyprotein, respectively. The genetic relatedness of Möhkö isolates to a number of human pathogenic orthobunyaviruses warrants further investigation on their potential disease associations and further serological analysis is needed to compare them to other Chatanga virus isolates and SSHV to determine their true antigenic relation.

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1. Introduction

Orthobunyaviruses of the family *Bunyaviridae* are mainly arthropod-borne viruses, and include several important human and veterinary pathogens. Many orthobunyaviruses causing disease in human belong to the California encephalitis virus species (King et al., 2012). While the majority of human infections are either asymptomatic or present with a mild flu-like disease, and therefore usually remain unrecognized, virus infections of this serogroup can also cause severe central nervous system infections. In the US, approximately 80–100 cases of neuroinvasive California encephalitis serogroup virus infections are reported annually in those aged <16 years, with most cases associated with La Crosse virus (LACV), and to a lesser extent with California encephalitis, Jamestown Canyon (JCV), Snowshoe hare (SSHV) and Trivittatus viruses (LeDuc, 1987). In Europe, circulation of Tahyna (TAHV), Inkoo (INKV) and SSHV/Chatanga viruses of the California encephalitis virus species (serogroup) have been described. While all these viruses are reported in western Russia (Vanlandingham et al., 2002), INKV circulates in Northern Europe, and TAHV principally in central Europe and also in Norway (Hubalek, 2008). Only limited published information is available on human INKV and SSHV infections, whereas TAHV is a well-known causative agent of febrile illness with hundreds of published cases since the virus was first isolated (Hubalek, 2008).

Altogether 48 different species are identified in the genus *Orthobunyavirus*. The negative-sense RNA genome of orthobunyaviruses consists of three segments, designated as small (S), medium (M) and large (L). The S segment encodes a nucleocapsid protein (N) and a non-structural protein (NSs); the M segment encodes two glycoproteins (Gn and Gc) and a non-structural protein (NSm); and the L segment encodes an RNA-dependent RNA

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polymerase. The segmented RNA genome allows for segment reassortments, which have been described among orthobunyaviruses (Reese et al., 2008), although only within the same serogroup (King et al., 2012). The pathogenicity of a reassortant orthobunyavirus can resemble that of the original virus, as is the case with e.g. Iquitos virus, reassortant of Oropouche virus, both causing febrile disease (Aguilar et al., 2011). In some cases, however, the pathogenicity can dramatically change through reassortment, such as with Ngari virus, a reassortant of Batai and Bunyamwera virus, known to cause severe human illness throughout East Africa (Briese et al., 2006).

Orthobunyaviruses are continuously being found in new geographical areas. In Russia, a close relative to SSHV, Chatanga virus (Lavrent'ev et al., 2008) was isolated from mosquitoes collected in the late 1980s. Within the past decade, the discovery of South river virus in Mexico (Blitvich et al., 2012), BATV in Germany (Jöst et al., 2011) and Italy (Calzolari et al., 2010), and TAHV in China (Lu et al., 2011) all represent previously undocumented geographical areas for their circulation. The recent discovery of Schmallenberg virus, causing malformation and reduced milk production in cattle and livestock in central Europe (Hoffmann et al., 2012), highlights the emergence of orthobunyaviruses.

While old serological evidence of Batai virus antibodies in cows exists (Brummer-Korvenkontio, 1974), for decades, INKV was the only prominent representative of the genus *Orthobunyavirus* in Finland. Recently, however, Schmallenberg virus has rapidly spread in Europe, and was also found in Finland, from a malformed lamb (ProMED-mail, 2013). A 30–50% prevalence of antibodies to California encephalitis serogroup virus is found in the Finnish population (Brummer-Korvenkontio, 1973; Putkuri et al., 2007), yet clinical alert and the use of specific laboratory diagnostics have been very limited. While most human INKV infections are presumably subclinical, a small number of unpublished cases associate INKV with a neuroinvasive disease (personal communication Brummer-Korvenkontio, M.). The entire serogroup has become underdiagnosed or largely forgotten by the medical community. Furthermore, only few if any entomological studies on mosquito species have been carried out in Finland since the 1970s (Utrio, 1976). Therefore, no reliable estimates can be made as to distribution and epidemic potential of diseases caused by California encephalitis serogroup viruses in the country.

In this study, the screening of mosquito-borne viruses in Finland by virus isolation in cell culture led to the discovery of several similar isolates of California encephalitis serogroup orthobunyaviruses. These isolates were related to SSHV and the previously reported Chatanga virus from Russia, which had not been found in Finland or other European regions beyond Russia prior to this study. We present here the isolation, as well as the genetic and serological characterization of these isolates designated as Möhkö isolates of Chatanga virus according to the place of origin.

2. Materials and methods

2.1. Mosquito collection

Altogether 11430 mosquitoes and 670 mosquito larvae were collected during 2004–2009 in Finland. Collections took place in six different municipalities in autumn (in 2005 also in mid-summer). Mosquitoes were collected using hand nets (human bait) or the Biogents BG-sentinel mosquito trap system (luring substance bait) at forested wetland areas, mainly in the morning and evening, occasionally also overnight. In 2007–2008, collections took place in eastern Finland and 1940 individuals were captured. Collected mosquitoes were briefly frozen alive at −20 °C, tubed, temporarily stored on dry ice, and finally placed at −70 °C.

2.2. Virus isolation attempts from mosquitoes collected in 2007 and 2008

Pools of 10 mosquitoes were homogenized with pestle in Dulbecco's bovine serum albumin (BSA). Green monkey kidney cells (Vero cells, ATCC **CCL-81™)** and *Aedes albopictus* clone C6/36 cells were used for cell-culture isolation screening. Vero cells were grown at 5% CO_2 at 37 °C and C6/36 cells at room temperature to an 80–90% confluence of cell monolayer in 25 cm^2 flasks. Prior to infection, two homogenized mosquito pools were combined. After 1 h incubation with 200 μl of this suspension on cells, fresh medium was added. The medium used for Vero cells was Eagle's Minimum Essential Medium (EMEM) supplemented with fetal bovine serum (FBS) to a final concentration of 2%, glutamine, penicillin and streptomycin, whereas for C6/36 cells, L-15 medium was used with 2% fetal bovine serum and antibiotics. Media were changed after 24 h. In contrast to our previous studies (Sane et al., 2012), unfiltered suspension was used in Vero cell inoculation and no amphotericin B was used. Cells were observed daily for cytopathic effect (CPE). If no CPE was observed earlier, the supernatant was collected after two weeks, stored at −70 °C, and cells were studied with immunofluorescence assay (IFA).

2.3. Immunofluorescence assay

Slides were prepared and stained using a selected panel of antisera to arboviruses of the genera *Alphavirus* (Sindbis virus), *Flavivirus* and *Orthobunyavirus* (California serogroup; Tahyna polyclonal and Bunyamwera serogroup; Batai polyclonal) as described earlier (Huhtamo et al., 2009). Briefly, Vero cells were harvested, washed with PBS three times, and cell suspension was placed on IFA slides and air dried. After acetone fixation, slides were stained by incubating first with antisera followed by anti-human IgG or anti-mouse FITC conjugate. The slides were investigated with a fluorescence microscope.

2.4. Sequencing, genomic analysis and phylogenetic tree reconstruction

Initial virus identification was conducted with RT-PCR specific for the California serogroup S segment, followed by agarose gel electrophoresis, and if positive, with sequencing. Total RNA was extracted from supernatants of the pools positive in orthobunyavirus IFA, using QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using Expand Reverse Transcriptase with S segment specific PCR primers; Ink-S-FP 5′-agt agt gtg ctc cac ttg aat act and Ink-S-RP 5′-agt agt gtg ctc cac tga ata cat tt. The PCR was performed using *Taq* DNA polymerase and *Taq* Extender with standard PCR protocols and the purified PCR products were directly sequenced by Sanger sequencing. The identity of the nucleotide fragments to California serogroup was confirmed by BLAST searches carried out using the NCBI server (http://ncbi.nlm.nih.gov) with all available databases.

The open reading frame (ORF) of the M and L segments were sequenced using a next generation sequencing (NGS) approach and direct Sanger sequencing. For this purpose, all isolates were grown in Cellmaster roller bottles to gain maximum amount of virus in the supernatant. Supernatant was ultracentrifuged for 20 h with 24000 rpm in SW28 rotor and virus pellet was eluted to 200 μl TNE buffer (10 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl). RNA was extracted and reverse transcription was performed as described above. Complete M and beginning of L segment PCR products were produced with specific primers (M segment primers from Campbell and Huang, 1999; L segment primers: L-548 5′-tga aag tcg cac atg gtg at and L-3801 5′-tgc gca atc tcc tac aga tg) and

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