Isolation and full genomic characterization of Batai virus from mosquitoes, Italy 2009

Eili Huhtamo,1† Amy J. Lambert,2† Stefano Costantino,3 Luca Servino,3 Letizia Krizmanic,3 Renzo Boldorini,4 Sara Allegrini,4 Ivan Grasso,5 Essi M. Korhonen,1 Olli Vapalahti,1,6,7 Robert S. Lanciotti2 and Paolo Ravanini3

1Department of Virology, Haartman Institute, Faculty of Medicine, PO Box 21, FI-00014 University of Helsinki, Helsinki, Finland
2Division of Vector-Borne Infectious Diseases, National Center for Zoonotic, Vector-Borne, and Enteric Diseases, Centers for Disease Control and Prevention, Public Health Service, Department of Health and Human Services, Fort Collins, CO, USA
3Laboratorio di Virologia Molecolare, UOA Microbiologia e Virologia, Azienda Ospedaliero-Universitaria Maggiore della Carità, Novara, Italy
4Dipartimento di Anatomia Patologica, Facoltà di Medicina e Chirurgia, Università Amedeo Avogadro del Piemonte Orientale, Novara, Italy
5Istituto per le Piante da Legno e l’Ambiente, Torino, Italy
6Department of Virology and Immunology, Helsinki University Central Hospital Laboratory, PO Box 400, FI-00029 HUS, Helsinki, Finland
7Department of Veterinary Biosciences, Faculty of Veterinary Medicine, PO Box 66, FI-00014 University of Helsinki, Helsinki, Finland

In 2009, 2589 mosquitoes were collected in northwest Italy and screened for orthobunyavirus RNA by RT-PCR. One pool of Anopheles maculipennis complex mosquitoes was found to be positive and a virus was isolated from that pool. The isolate was identified as Batai virus (BATV) by sequencing. Previously, BATV was detected in Italy, but limited data and no prior isolates existed. Full-length sequences of the S, M and L segments were determined for the newly isolated Italian strain. For comparison, partial sequences were also determined for the BATV strain Calovo (former Czechoslovakia, 1960). Phylogenetic analyses revealed clustering of the newly derived Italian BATV along with a recent isolate from Germany and the historic strain Calovo. To the best of our knowledge, this represents the first isolation of BATV from Italy, which confirms a broader geographical distribution of BATV in Europe than was previously verified by isolation.

The genus Orthobunyavirus of the family Bunyaviridae is a diverse group of arthropod-borne, negative-sense, single-stranded RNA viruses. Orthobunyaviruses are serologically classified into 18 globally distributed groups including the California, Bunyamwera, Simbu and group C serogroups that possess known human and animal pathogens. The orthobunyavirus genome is segmented with small, medium and large (S, M and L) segments generically encoding the nucleocapsid, envelope glycoprotein and polymerase protein, respectively. The reassortment of genomic segments between related orthobunyavirus species is well documented (Beaty et al., 1985, 1997; Borucki et al., 1999; Cheng et al., 1999; Briese et al., 2007) and has been associated with outbreaks of human illness (Bowen et al., 2001; Gerrard et al., 2004; Briese et al., 2006; Yanase et al., 2006). However, an overall understanding of the role of segment reassortment in the evolution of orthobunyaviruses is limited by a lack of comprehensive sequence data. Furthermore, an accurate estimate of the public-health impact of orthobunyaviruses is precluded by this lack of descriptive data, and the resultant inability to detect and identify many of these viruses by molecular methods.

A member of the Bunyamwera serogroup, Batai virus (BATV) was originally isolated from Malaysia in 1955.

†These authors contributed equally to this paper.

The GenBank/EMBL/DDBJ accession numbers for the S, M and L sequences of the Italian Caltignaga strain of BATV are KC168046, KC168047 and KC168048, respectively, and for the partial S and M sequences of the Calovo (former Czechoslovakia) strain of BATV are KC168049 and KC168050, respectively.

One supplementary table is available with the online version of this paper.
(Karabatsos, 1985) and later an antigenically identical virus was isolated in Calovo, Slovakia (strain Calovo, former Czechoslovakia, 1960; Bárdos & Čupková, 1962). The geographical distribution of BATV includes regions of Europe, Asia and Africa. The vertebrate hosts of BATV include domestic pigs, horses, ruminants (Geevarghese et al., 1994; Yanase et al., 2006) and wild birds (Hubálek, 2008). The transmission cycle occurs in agricultural ecosystems via Anopheles, Culex and Ochlerotatus species mosquitoes in a vertebrate–mosquito cycle (Yanase et al., 2006). The animal disease associations of BATV in Europe are not known, but the strain Chittoor was documented to cause mild disease in sheep and goats in India (Singh and Pavri, 1966), and the closely related American Cache Valley virus is known to cause stillbirths and congenital abnormalities in ruminants (Chung et al., 1990). In humans, BATV has been associated with self-limiting, influenza-like febrile disease (Bárdos et al., 1969; Sluka, 1969; Hubálek, 2008; Woodall, 1969; Nashed et al., 1993). Of paramount interest to public health, BATV has been implicated as a segment donor in the generation of the reassortant Ngari virus that has been associated with outbreaks of haemorrhagic illness in East Africa (Bowen et al., 2001; Gerrard et al., 2004; Briese et al., 2006; Yanase et al., 2006). However, neither of the Ngari virus parental segment donors, BATV and a serologically related Bunyamwera-like virus, has been associated with haemorrhagic disease (Elliott & Blakqori, 2011). This suggests that the presence of BATV in a given geographical region, under ecological conditions that include the co-circulation of a serologically related bunyavirus, might predicate a reassortment driven disease emergence event that may be characterized by an altered epidemiological outcome.

While historical data indicate that BATV is endemic to regions of eastern Europe (Hubálek, 2008), a broader geographical distribution of BATV in Europe remains largely unsubstantiated by virus isolation. Recently, a strain of BATV was isolated for the first time in Germany (Jöst et al., 2011). Here, we report what is to the best of our knowledge the first isolation of BATV from Italy where limited evidence of BATV circulation has been previously inferred through serology (Albanese et al., 1971) and RNA detection in mosquitoes (Calzolari et al., 2010). Full-length genomic S, M and L segment sequences were determined for the recently isolated Italian BATV strain and, for the purpose of comparison, partial sequences were determined for the BATV strain Calovo (former Czechoslovakia, 1960). Multi-segment phylogenetic analyses of the Italian BATV strain are presented. The data in this manuscript are provided to enhance the description of orthobunyaviruses in the public domain, and are intended to support better understanding of the geographical distribution and genetic diversity of BATV in Europe.

Mosquitoes were collected in rural and urban areas in Italy around Novara in the Piedmont region in 2009. The collection sites included different habitats: riverside in Bellinzago (45° 34’ 23” N, 8° 41’ 43” E), a rice field in

![Fig. 1. Map of the mosquito collection sites.](http://vir.sgmjournals.org)
Mosquitoes were collected overnight (from 5 pm to 9 am) using CO2 baited traps and identified using morphological characteristics. Mosquitoes were then sorted by species, date and site of collection, and pooled with a maximum number of 10 individuals per pool. Pooled mosquitoes were immediately frozen and stored at −80°C until processed. A total of 2589 female mosquitoes were collected, including *Culex pipiens* complex (*n* = 1470), *Anopheles maculipennis* complex (*n* = 786), *Culex modestus* (*n* = 163), *Ochlerotatus caspius* (*n* = 107), *Aedes vexans* (*n* = 58), *Ochlerotatus geniculatus* (*n* = 3), *Culiseta* (*n* = 1) and *Aedes albopictus* (*n* = 1) species divided in 273 pools (42 collected during early summer, 213 in middle summer, 18 in late summer). Mosquito pools were divided in 273 pools (42 collected during early summer, 213 in middle summer, 18 in late summer). Mosquito pools were divided in 273 pools (42 collected during early summer, 213 in middle summer, 18 in late summer).

For RT-PCR screening, cDNA was synthesized from extracted RNA using random hexamers with the RevertAid first strand cDNA synthesis kit (Fermentas), according to the manufacturer’s instructions. Two separate amplification methods (Kuno *et al.*, 1996; Lambert & Lanciotti, 2009), both targeted to the S segment, were applied to cDNAs with minor modifications for orthobunyavirus detection. RT-PCR screening generated one positive pool of *Anopheles maculipennis* complex mosquitoes, collected during middle summer on a cow farm near the village of Caltignaga (Fig. 1). The obtained PCR products were cut from agarose gels, and purified using the PureLink quick gel extract kit (Invitrogen). Amplicons were sequenced both directly and after cloning (CloneJET PCR cloning kit; Fermentas) in 5’ and 3’ directions using a BigDye terminator v1.1 cycle sequencing kit with an ABI PRISM 3130 system (Applied BioSystems). The obtained sequences were compared to those in GenBank using the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1990). Results of these analyses suggested that the amplified sequences were derived from BATV.

Virus isolation was attempted by adding the BATV RNA-positive mosquito homogenate onto freshly confluent BHK-21 (ATCC: CCL-10) and Vero E6 cells (ATCC: CRL-1586). A clear cytopathic effect was observed 2 days after infection in both cell lines, and the cells and supernatants were harvested on day 3 after infection. The infected cells were acetone-fixed on microscope slides and further tested positive by indirect immunofluorescence assay using a mouse polyclonal antibody against the Calvovo strain of BATV (Brummer-Korvenkontio, 1974) and FITC-labelled anti-mouse immunoglobulins (Dako). From the Vero E6 culture supernatants, RNA was extracted using a QIAamp nucleic acid extraction kit (Qiagen). From the infected cell supernatants, RNA was extracted using a QIAamp nucleic acid extraction kit (Qiagen).

For RT-PCR screening, cDNA was synthesized from extracted RNA using random hexamers with the RevertAid first strand cDNA synthesis kit (Fermentas), according to the manufacturer’s instructions. Two separate amplification methods (Kuno *et al.*, 1996; Lambert & Lanciotti, 2009), both targeted to the S segment, were applied to cDNAs with minor modifications for orthobunyavirus detection. RT-PCR screening generated one positive pool of *Anopheles maculipennis* complex mosquitoes, collected during middle summer on a cow farm near the village of Caltignaga (Fig. 1). The obtained PCR products were cut from agarose gels, and purified using the PureLink quick gel extract kit (Invitrogen). Amplicons were sequenced both directly and after cloning (CloneJET PCR cloning kit; Fermentas) in 5’ and 3’ directions using a BigDye terminator v1.1 cycle sequencing kit with an ABI PRISM 3130 system (Applied BioSystems). The obtained sequences were compared to those in GenBank using the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1990). Results of these analyses suggested that the amplified sequences were derived from BATV.

Virus isolation was attempted by adding the BATV RNA-positive mosquito homogenate onto freshly confluent BHK-21 (ATCC: CCL-10) and Vero E6 cells (ATCC: CRL-1586). A clear cytopathic effect was observed 2 days after infection in both cell lines, and the cells and supernatants were harvested on day 3 after infection. The infected cells were acetone-fixed on microscope slides and further tested positive by indirect immunofluorescence assay using a mouse polyclonal antibody against the Calvovo strain of BATV (Brummer-Korvenkontio, 1974) and FITC-labelled anti-mouse immunoglobulins (Dako). From the Vero E6 culture supernatants, RNA was extracted using a QIAamp nucleic acid extraction kit (Qiagen). From the infected cell supernatants, RNA was extracted using a QIAamp nucleic acid extraction kit (Qiagen).
viral RNA mini kit (Qiagen) and used as a template in nested RT-PCRs targeted to BATV S and L segments using primers designed based on BATV sequences available in GenBank (Table S1, available in JGV Online). The sequences obtained confirmed the isolate as BATV and the strain was designated ‘Caltignaga’ according to the mosquito collection site.

Full-length genomic sequences (S segment 943 nt, M segment 4440 nt, L segment 6870 nt – GenBank accession numbers KC168046, KC168047 and KC168048, respectively) were further determined using both primer directed and next generation sequencing (NGS) methods. Briefly, viral RNA templates were subjected to cDNA library preparation and NGS using the Ion Torrent Personal Genome Machine system (Life Technologies) and associated protocols for the generation of nucleic acid sequence data from RNA templates according to the manufacturer’s recommendations. NGS data were analysed using the DNASTAR NGen 4 software with de novo assembly parameters. Generated consensus sequences were then verified by spot sequencing using primer directed RT-PCR amplification and sequencing methods as previously published (Lambert & Lanciotti, 2008, 2009). Terminal ends were determined utilizing 5’ and 3’ RACE kits according to the manufacturer’s recommendations (Life Technologies). ORF determination was conducted using the EditSeq function of the Lasergene 9 package (DNASTAR), and basic analyses of both nucleotide and amino acid sequences were conducted using MEGA version 5 software (Tamura et al., 2011). For phylogenetic comparison, partial coding sequences (890 nt of the S segment and 682 nt of the L segment – GenBank accession numbers KC168049 and KC168050, respectively) were also determined for the Calovo strain of BATV (taken from the WHO reference collection of arboviruses that is maintained at the CDC in Fort Collins, CO, USA). A partial M segment coding sequence of the Calovo strain was previously determined by others (Briese et al., 2006; GenBank accession number DQ334335).

General characteristics of the newly derived Italian BATV sequences were as follows. For the S segment (943 nt), there were two ORFs, the nucleocapsid (N) and non-structural (NSs) ORFs that occurred in an overlapping organization and were 702 and 306 nt in length, respectively. The M segment (4440 nt) possessed a polyprotein precursor ORF of 4305 nt and the L segment (6870 nt) possessed an RNA-dependent RNA polymerase ORF of 6714 nt in length. All reported lengths were highly similar, if not identical, to those published by others for the full-length genomic description of BATVs of diverse origin (Dunn et al., 1994; Groseth et al., 2012). A hallmark of the family, the 5’ and 3’ termini of each genomic segment possessed highly conserved, complementary sequences (10 nt) that were identical to those published for other BATVs (Dunn et al., 1994; Groseth et al., 2012).

In GenBank along with the newly described sequences that are presented here (Fig. 2a–c). Alignments, restricted in length by partial sequences of S (702 nt), M (3152 nt) and L (~200 nt) segment sequences, were generated using the CLUSTAL W (Thompson et al., 1994) function of MEGA version 5 software (Tamura et al., 2011). Maximum-likelihood trees generated under the Tamura–Nei model of evolution (Tamura & Nei, 1993) and analysed with 1000 replicates for bootstrap testing (Felsenstein, 1985) are presented here (Fig. 2a–c).

Results of multi-segment phylogenetic analyses revealed that within all genomic segments, S (Fig. 2a), M (Fig. 2b) and L (Fig. 2c), the newly isolated Italian strain of BATV grouped together with a strain isolated the same year from Germany and the Calovo strain (former Czechoslovakia, 1960) with strong bootstrap support. The Italian strain also possessed high percentage nucleotide sequence identities of 99.4/97.0 % (S), 98.4/97.8 % (M) and 99.5/97.0 % (L) in compared regions with the German/Calovo (former Czechoslovakia, 1960) strains of BATV. Similarly, the newly derived Italian BATV possessed high percentage amino acid sequence identities of 100/100 % (N ORF), 100/99.0 % (NSs ORF) and 99.0/98.6 % (polyprotein ORF) in compared regions with the German/Calovo (former Czechoslovakia, 1960) strains of BATV. Taken together, these results suggest long-term circulation of closely related strains of BATV in Europe. There is no evidence of reassortment among these compared BATV strains (Fig. 2a–c). Of additional interest, all three phylogenetic trees showed European isolates generally grouping together, while Asian and African isolates of BATV appeared within their own strongly supported, independent groupings (Fig. 2a–c). These findings suggest a correlation between geographical and genetic diversity among the compared strains of BATV. Unfortunately, a more in-depth comparison of sequence data, including the identification of signature motifs that might be representative of a given geographical clade of BATVs, is restricted by a lack of available full-length sequences for the majority of BATVs included in this study.

The recent discoveries of BATV in Germany and Italy could be the result of enhanced research activity on mosquito-borne viruses that has caused an increased incidence of BATV detection. However, these discoveries could also be evidence of the emergence of BATV in a broader region of Europe than was previously verified by isolation (Hubálek, 2008). A non-descript clinical presentation and a lack of readily available diagnostics limit the detection of BATV and other orthobunyaviruses in clinical settings, likely contributing to their underestimation on a global scale. Furthermore, the lack of information on the hosts, pathogenic properties and distribution of BATV in Europe highlights the importance of further studies. Of unique interest, within Italy, the serological inference of BATV presence in the southernmost region of this country suggests potentially widespread circulation that justifies additional investigation (Albanese et al.,
1971). Significant efforts are currently targeted to study new and (re)emerging mosquito-borne viruses in Europe, such as West Nile, Usutu, Sindbis, dengue and Chikungunya viruses. In the future, efforts should also be devoted to further studies of orthobunyaviruses, such as BATV, that are possibly widespread in Europe, and have potentially underestimated veterinary and public-health impacts.

Acknowledgements
The authors thank Irina Suomalainen for expert technical assistance. This study was supported by grants from the Academy of Finland, HUSLAB and the Emil Aaltonen Foundation.

References


Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using

