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Identification of a novel subtype of South River virus (family *Bunyaviridae*)

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Abstract

We previously reported the isolation of South River virus (SORV) from a pool of mosquitoes collected in the Yucatan Peninsula of Mexico (Farfan-Ale et al. in Vector Borne Zoonotic Dis 10:777–783, 5). The isolate (designated SORV-252) was identified as SORV after a 197-nucleotide region of its small RNA genome segment was sequenced. In the present study, the complete small and medium RNA genome segments and part of the large RNA genome segment of SORV-252 were sequenced and shown to have 92%, 85% and 90% nucleotide sequence identity, respectively, to the homologous regions of the prototype SORV isolate (NJO-94F). To determine the antigenic relationship between SORV-252 and NJO-94F, cross-plaque reduction neutralization tests (PRNTs) were performed using sera from mice inoculated with these viruses. SORV-252 and NJO-94F were distinguishable in the cross-neutralization assays; there was a twofold difference in the PRNT titers in one direction and a fourfold difference in the other direction, suggesting that SORV-252 represents a novel subtype of SORV. Additionally, SORV-252 and NJO-94F have distinct plaque morphologies in African green monkey kidney (Vero) cells. In conclusion, we provide evidence that a novel subtype of SORV is present in the Yucatan Peninsula of Mexico.

All viruses in the genus *Orthobunyavirus* possess a single-stranded, negative-sense RNA genome comprised of three segments designated as small (S), medium (M) and large (L) [8, 10]. This genus can be subdivided into 18 serogroups, including the California (CAL) serogroup. One member of the CAL serogroup is South River virus (SORV), a poorly

characterized virus that is not a recognized cause of human or animal disease. The prototype isolate (designated NJO-94F) was obtained from *Anopheles crucians* in New Jersey in 1960 [12]. Two isolations were also made from *Aedes sollicitans* and *Culiseta melanura* in New Jersey in 1965. Additionally, SORV has been isolated on six occasions from *Culex salinarius*, *Cx. pipiens* and *Cs. melanura* in Pennsylvania in 1971 and 1972 [13] and on two occasions from *An. punctipennis* in Georgia from 2000 to 2008 (D.G. Mead, personal communication). More recently, an isolate of SORV (designated SORV-252) was obtained from *Ochlerotatus (Aedes) taeniorhynchus* in the Yucatan Peninsula of Mexico in 2008 [5].

There are limited sequence data for SORV. The complete S and M RNA segments (984 and 4509 nt, respectively) and a 410-nt region of the L RNA segment of NJO-94F have been sequenced [6]. Additionally, a 197-nt region of the S RNA segment of SORV-252 has been sequenced [5]. There are no sequence data available for any of the other SORV isolates. Thus, one objective of this study was to sequence the complete S and M RNA segments and part of the L RNA segment of SORV-252. Experiments were also performed to characterize the antigenic relatedness between, and to compare the plaque morphologies of, SORV-252 and NJO-94F.

Primers for the RT-PCR amplification and sequencing of SORV-252 were designed using the nucleotide sequence data of NJO-94F (primer sequences available upon request). Complementary DNAs were generated using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was performed using *Taq* polymerase (Invitrogen, Carlsbad, CA). PCR products were purified using a PureLink Gel Extraction Kit (Invitrogen, Carlsbad, CA) and sequenced using a 3730×1 DNA sequencer (Applied Biosystems, Foster City, CA).

The complete nucleotide sequences of the S and M RNA segments (with the exception of an estimated 23 nucleotides at the distal 5' and 3' ends of each segment) and a 364-nt region of the L RNA segment of SORV-252 were determined (GenBank accession nos. GU018050, JN815081 and JN815080, respectively). The nucleotide sequence of the S RNA segment has greatest identity to the homologous region of NJO-94F (91.8%), followed by Inkoo virus (INKV; 88.5%), Jamestown Canyon virus (JCV; 87.7%) and Jerry Slough virus (JSV; 87.6%). The M RNA segment of SORV-252 has greatest nucleotide identity to the homologous region of NJO-94F (84.6%), followed by JCV (81.5%), INKV (80.9%) and JSV (80.0%). The 364-nt region of the SORV-252 L RNA segment that was sequenced in this study has greatest nucleotide identity to the homologous region of NJO-94F (89.8%), followed by JSV (82.1%), INKV (81.0%) and JCV (80.8%).

Phylogenetic trees were constructed by the Bayesian method assuming the GTR model with gamma-distributed rates and using the complete nucleotide sequences of the S and M RNA segments and a 364-nt region of the L RNA segment of SORV-252 and selected other bunyaviruses. In the Bayesian tree constructed using S segment sequences, SORV-252 shares a close phylogenetic relationship with NJO-94F with high (1.0) posterior support (Fig. 1a). Phylogenetically, the S segments of these two isolates are most closely related to the homologous regions of INKV, JCV and JSV. These five isolates, together with the 12 other members of the CAL serogroup used in the analysis, comprise a distinct clade (denoted as I). Viruses in the Bunyamwera (BUN) and Simbu (SIM) serogroups comprise clades II and III, respectively. Three distinct clades (denoted as I, II and III) were also observed in the M segment tree (Fig. 1b). Clade I contains CAL serogroup viruses, clade II contains BUN serogroup viruses, and clade III contains SIM serogroup viruses. Phylogenetically, the M segment sequence of SORV-252 is most closely related to the homologous region of NJO-94F, and the posterior support for this topological arrangement is 1.0. All other branches in the tree also received maximal posterior support. The overall

topology of the L segment tree (Fig. 1c) was consistent with the trees constructed using S and M segment sequences in that the CAL, BUN and SIM sero-group viruses separated into three distinct clades (denoted I, II and III, respectively). Phylogenetically, the L segment sequence of SORV-252 is most closely related to the homologous region of NJO-94F, although this topological arrangement had only moderate posterior support (0.83).

Cross-plaque reduction neutralization tests (PRNTs) were performed to determine the antigenic relatedness of SORV-252 and NJO-94F. This test provides reliable information on the antigenic relationships among viruses in the genus *Orthobunyavirus* and is routinely used for taxonomic purposes [3, 7]. To produce immune sera for the cross-PRNTs, adult female Balb/c mice (in 2 groups of 3) were inoculated intraperitoneally with 25,000 plaque-forming units of SORV-252 or NJO-94F. Additional inoculations were administered 2 and 4 weeks after the initial injection. Two weeks after the final inoculation, blood was collected by cardiac heart puncture, allowed to clot and clarified by centrifugation. Cross-PRNTs were performed using African green monkey kidney (Vero) cells [1]. Titers were expressed as the reciprocal of the serum dilution yielding 90% reduction in the number of plaques (PRNT₉₀).

PRNT₉₀ titers for the SORV-252 immune serum in assays performed with SORV-252 and NJO-94F were 640 and 320, respectively. The PRNT₉₀ titers for the NJO-94F immune serum in assays performed with SORV-252 and NJO-94F were 320 and 1280, respectively. Thus, there was a twofold difference in PRNT₉₀ titers in one direction and a fourfold difference in the other. These data provide evidence that SORV-252 is a novel subtype of SORV since subtypes can be defined as two viruses that cross-react with less than a fourfold difference in one direction and with a fourfold or greater difference in the other direction in antigenic tests [2].

To compare the plaque morphologies of SORV-252 and NJO-94F, the abilities of these viruses to plaque in Vero cells was assessed. NJO-94F plaques are larger and could be visualized earlier than SORV-252 plaques. NJO-94F plaques were first visible with the naked eye at 3 days p.i. (data not shown) and were approximately 0.8 mm in diameter at 4 days p.i. (Fig. 2A). In contrast, SORV-252 plaques were first observed at 4 days p.i. and were approximately 0.1 mm in diameter at this time (Fig. 2B). At 6 days p.i., SORV-252 plaques had reached a size of approximately 0.3 mm in diameter (Fig. 2C). It is not known which nucleotide substitutions are responsible for these differential plaque morphologies. Experiments performed with La Crosse virus have shown that the L RNA segment is the major determinant of plaque morphology [4].

In conclusion, the sequence and serological data presented in this report indicate that a novel SORV subtype exists in the Yucatan Peninsula of Mexico. Although SORV is not a recognized pathogen of humans or other vertebrates, the discovery of this isolate is important. For example, we are currently performing a serological investigation to determine whether Cache Valley virus (a human pathogen) and Kairi virus (an equine pathogen) are causes of infection in vertebrates in the Yucatan Peninsula. It is important that SORV and other orthobunyaviruses that occur in the area are included in the PRNTs to avoid serological misdiagnosis.

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Fig. 1.

a Phylogenetic analysis of the S RNA segment of SORV-252. The displayed phylogeny was estimated by using the program MRBAYES, version 3.1 [9], and displayed using TreeGraph 2 [11]. The phylogenetic analysis is based on the complete nucleotide sequence of the S RNA segment of SORV-252 and 21 other orthobunyavirus isolates. Branches are labeled with posterior support (out of 1.0). SORV-252 is denoted in bold. GenBank accession numbers are as follows: Akabane virus (AB000851), Bunyamwera virus (D00353), Cache Valley virus (X73465), California encephalitis virus (U12797), Chatanga virus (HQ734825), Inkoo virus (U47137), Jamestown Canyon virus (EF681848), Jerry Slough virus (U12798), Keystone virus (U12801), La Crosse virus (AF528167), Lumbo virus (X73468), Main Drain virus (X73469), Melao virus (U12802), Morro Bay virus (U31989), Oropouche virus (AY237111), San Angelo virus (U47139), Serra do Navio virus (U47140), Snowshoe hare virus (EU294510), South River virus (NJO-94F, U47141), South River virus (SORV-252, GU018050), Tahyna virus (EU622820) and Trivittatus virus (U12803). **b** Phylogenetic analysis of the M RNA segment of SORV-252. The displayed phylogeny was estimated by using the program MRBAYES, version 3.1 [9], and displayed using TreeGraph 2 [11]. The phylogenetic analysis is based on the complete nucleotide sequence of the M RNA segment of SORV-252 and 19 other orthobunyavirus isolates. Branches are labeled with posterior support (out of 1.0). SORV-252 is denoted in bold. GenBank accession numbers are as follows: Akabane virus (AB100604), Bunyamwera virus (M11852), Cache Valley virus (AF082739), Chatanga virus (HQ734822), Inkoo virus (U88059), Jamestown Canyon virus (U88058), Jerry Slough virus (AF123487), Keystone virus (AF123489), La Crosse virus (AF528166), Lumbo virus (AF123484), Main Drain virus (EU004187), Melao virus (U88057), Oropouche virus (AF441119), San Angelo virus (AF123486), Serra do Navio

virus (AF123490), Snowshoe hare virus (K02539), South River virus (NJO-94F, AF123488), South River virus (SORV-252, JN815081), Tahyna virus (GQ386841) and Trivittatus virus (AF123491). **c** Phylogenetic analysis of the L RNA segment of SORV-252. The displayed phylogeny was estimated by using the program MRBAYES, version 3.1 [9], and displayed using TreeGraph 2 [11]. The phylogenetic analysis is based on a 364-nt region of the L RNA segment of SORV-252 and 14 other orthobunyavirus isolates. Branches are labeled with posterior support (out of 1.0). SORV-252 is denoted in bold. GenBank accession numbers are as follows: Akabane virus (AB190458), Bunyamwera virus (X14383), Cache Valley virus (EU004190), Chatanga virus (EU616897), Inkoo virus (EF687076), Jamestown Canyon virus (EF687074), Jerry Slough virus (EF687078), La Crosse virus (AF528165), Main Drain virus (EU004192), Melao virus (EF687079), Oropouche virus (AF484424), Snowshoe hare virus (EU203678), South River virus (NJO-94F, EF687075), South River virus (SORV-252, JN815080) and Tahyna virus (HM036219)

Fig. 2.

Plaque morphologies of NJO-94F and SORV-252 in Vero cells. Confluent monolayers of Vero cells in six-well plates were infected with (A) NJO-94F or (B–C) SORV-252. Cells were fixed using 10% formaldehyde, and plaques were visualized by staining with 0.25% crystal violet (w/v) in 20% methanol at (A–B) 4 and (C) 6 days post-inoculation. Images were transferred into Microsoft Photoshop, and plaque diameters were measured