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RAPID COMMUNICATION

A Reassortant Bunyavirus Isolated from Acute Hemorrhagic Fever Cases in Kenya and Somalia[1]

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In late 1997 and early 1998, a large outbreak of hemorrhagic fever occurred in East Africa. Clinical samples were collected in Kenya and southern Somalia, and 27 of 115 (23%) hemorrhagic fever patients tested showed evidence of acute infection with Rift Valley fever (RVF) virus as determined by IgM detection, virus isolation, detection of virus RNA by reverse transcription-polymerase chain reaction (RT-PCR), or immunohistochemistry. However, two patients (one from Kenya and the other from Somalia) whose illness met the hemorrhagic fever case definition yielded virus isolates that were not RVF. Electron microscopy suggested these two virus isolates were members of the family *Bunyaviridae.* RT-PCR primers were designed to detect bunyavirus RNA in these samples. Regions of the S and L segments of the two isolates were successfully amplified, and their nucleotide sequences exhibited nearly complete identity with Bunyamwera virus, a mosquito-borne virus not previously associated with severe human disease. Unexpectedly, the virus M segment appeared to be reassorted, as the sequences detected exhibited 32–33% nucleotide and 28% amino acid differences relative to the corresponding M segment sequence of Bunyamwera virus. The association of this reassortant bunyavirus, proposed name Garissa virus, with severe disease is supported by the detection of the virus RNA in acute-phase sera taken from 12 additional hemorrhagic fever cases in the region.

The family *Bunyaviridae* contains five genera, *Bunyavirus, Hantavirus, Nairovirus, Phlebovirus,* and *Tospovirus* (*2*). Each genus is subdivided into serogroups. Hemorrhagic fever viruses, which cause febrile illness with hemorrhagic manifestations, have been described in the *Hantavirus, Nairovirus,* and *Phlebovirus* genera. An example of a bunyaviral hemorrhagic fever virus is Rift Valley fever virus (RVF, *Phlebovirus*), a mosquito-borne virus that causes hemorrhagic fever in humans and highly lethal, febrile disease in livestock characterized by hepatitis and abortion (*3*).

Viruses of the genus *Bunyavirus* are known to cause human disease but have not been associated previously with hemorrhagic fevers. The known clinical picture of bunyavirus-associated disease includes nondescript febrile illness, often with myalgia and arthralgia, and encephalitis (*4*). Bunyamwera virus, the prototype member of the *Bunyaviridae* family, which was first isolated from mosquitoes collected in Uganda in 1943 (*5*), causes febrile illness with headache, arthralgia, rash, and infrequent central nervous system involvement (*6*). Bunyamwera virus and 31 other bunyaviruses comprise the Bunyamwera serogroup within the *Bunyavirus* genus (*2*).

Bunyaviruses are lipid-enveloped viruses with a genome that consists of three single-stranded RNA segments designated S (small), M (medium), and L (large) (*7*), encoding the nucleocapsid protein and nonstructural protein, the two envelope glycoproteins (G1 and G2), and the viral polymerase, respectively. Genetic reassortment occurs in nature and in the laboratory by exchange of genomic segments between parental viruses sharing close antigenic and genetic relationships, i.e., within the same serogroup (*8, 9*). Genetic reassortment can profoundly affect RNA virus pathogenicity. For example, re-

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assortment among influenza virus strains (another RNA virus with a segmented genome) can result in new pandemic strains as a result of antigenic shift (*10*).

From October 1997 to January 1998, exceptionally heavy rains (60–100 times normal rainfall) caused severe flooding in Kenya and Somalia (*1*). Heavy rains and increased standing water have previously been linked to emergences of mosquito-borne diseases such as RVF (*3*). In December 1997, the Kenyan Ministry of Health and World Health Organization (WHO) received reports of 478 deaths due to hemorrhagic fever in northeastern Kenya and southern Somalia (*1*). The disease was characterized by acute onset of fever and headache, followed by hemorrhage (gastrointestinal and/or mucosal bleeding). High rates of spontaneous abortion and deaths from hemorrhage among domestic animals were also reported. Disease surveillance in the Garissa District, North Eastern Province, Kenya, was coordinated by the international RVF task force in December 1997 (*1*). Of 231 reported cases of unexplained, severe febrile illness, 115 met the case definition for hemorrhagic fever (fever with mucosal or gastrointestinal bleeding). Of these 115 patients, only 27 (23%) had evidence of acute RVF virus infection as determined by detection of IgM antibody, infectious virus, or virus RNA (by RT-PCR) in serum samples, or by immunohistochemistry (*11*) performed on liver specimens using an anti-RVF mouse hyperimmune ascitic fluid (*1*).

In addition to the RVF virus activity detected, two virus isolates, designated 9800535 and 9800521, were obtained from samples from hemorrhagic fever cases in Garissa, North Eastern Province, Kenya and Baardheere, Somalia, respectively. The viruses produced cytopathic effect in cultured Vero E6 cells 2 to 4 days after inoculation and did not react with anti-RVF immune mouse ascitic fluid by immunofluorescence assay. Electron microscopy of thin sections of infected cells revealed bunyavirus-like particles within vesicles in the cytoplasm and in the extracellular spaces (Fig. 1). Virions averaged 79 nm in diameter and consisted of a homogeneous core enclosed within the viral envelope and surrounded by surface projections. These findings are consistent with the ultrastructural appearance of other members of the family *Bunyaviridae* (*12, 13*), except that the average observed virion size was about 20% smaller.

Amplicons were successfully obtained by RT-PCR, and partial genomic sequences were determined for the L, M, and S segments of both isolates. The viruses were found to be virtually identical to one another despite their different geographic origins. The L segment fragments (582 nt) of the virus isolates differed by only a single nucleotide substitution; the M segment fragments (539 nt for 9800521, and 542 nt for 9800535) differed by only two nucleotide substitutions and the S segment fragments (515 nt for 9800521, and 525 nt for 9800535) by only one

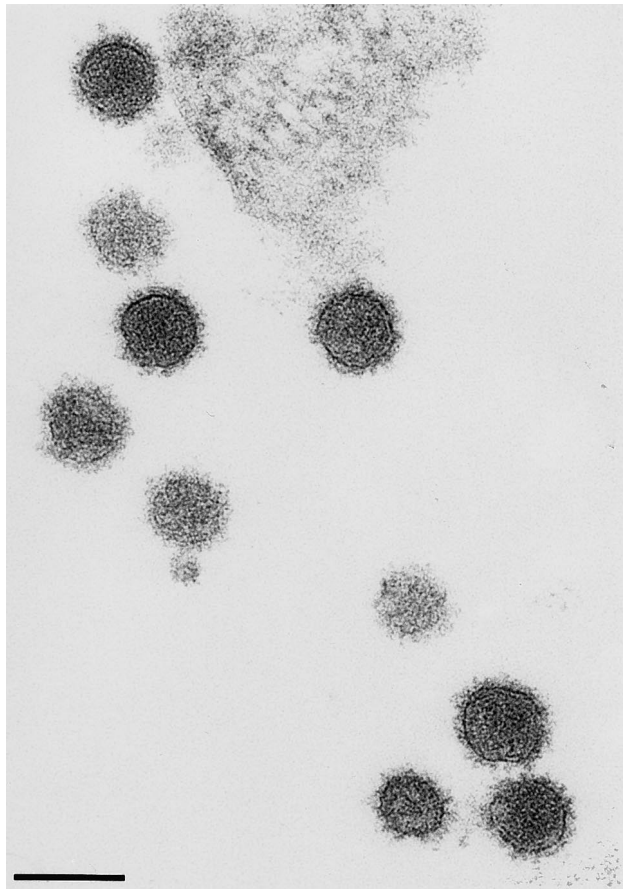

FIG. 1. Transmission electron micrograph of Garissa virus-infected Vero E6 cells. Extracellular virus particles show a homogeneous, ribonucleoprotein-containing core surrounded by the viral envelope, which is covered with prominent spikes. Bar, 100 nm.

substitution (GenBank Accession Nos. AF398345–AF398350).

The L segment fragment sequences of these isolates displayed greater than 97% nucleotide identity and 99.5% amino acid identity with those of Bunyamwera virus (*14*). Likewise, the S segment fragment sequences of the two isolates exhibited greater than 93% nucleotide and 100% amino acid identity with those of Bunyamwera virus (*15*). Unexpectedly, the M segment fragment sequences shared only 67–68% nucleotide and 72% amino acid identity with those of Bunyamwera virus (*16*). This amount of diversity is much greater than would be expected among Bunyamwera virus variants, based on the low level of diversity seen among variants of other Bunyamwera serogroup viruses, e.g., Cache Valley virus variants (see Fig. 2). This extent of difference is comparable to that observed between Bunyamwera virus and Germiston (67% nucleotide and 68% amino acid identity) or Cache Valley (66% nucleotide and 73–74% amino acid identity) viruses (*17, 18*), the only other Bunyamwera serogroup viruses for which M segment sequence data are currently available. These data demonstrate that the isolates represent strains of a reassortant bunyavirus,

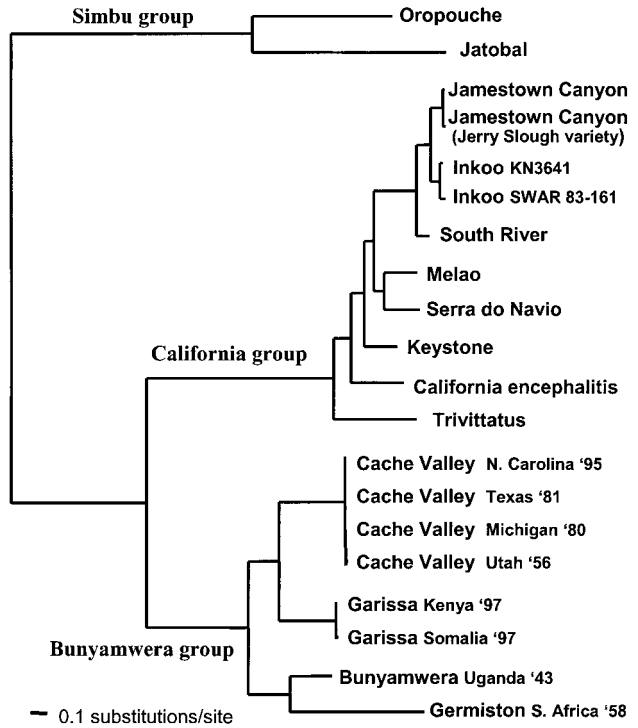

FIG. 2. Phylogenetic relationship of the M RNA segments of Bunyamwera group viruses relative to those of representative California group and Simbu group viruses. Maximum-likelihood analysis of the aligned sequences of a 544-nucleotide region of virus M segments was performed using PAUP4.0b5 (Sinauer Associates Inc.). Viruses included Garissa virus isolates 9800521 and 9800535 (GenBank Accession Nos. AF398348 and AF398347, respectively), Bunyamwera virus (BLCMA), Germiston (BLCMPOLY), Cache Valley virus (AF082576, AF186241–AF186243), Trivittatus virus (AF123491), California encephalitis virus (AF123483), Keystone virus (AF123489), Serra do Navio (AF123490), Melao virus (U88057), South River virus (AF123488), Inkoo virus (U88059-60), Jamestown Canyon (U88058, AF123487), Oropouche virus (AF312381), and Jatobal virus (AF312380). The analysis resulted in a −Ln likelihood of 6344.88883 with an estimated transition to transversion ratio of 1.658680 and γ shape of 0.483871.

with the genomic M segment coming from an unidentified Bunyamwera serogroup virus. We propose the name Garissa virus for this reassortant bunyavirus. The Garissa virus M segment has closer sequence identity to that of Cache Valley virus (72% nucleotide and 77% amino acid identity) than Bunyamwera virus. In addition, phylogenetic analysis of sequence differences suggests that the Garissa virus M segment shares a common ancestor with Cache Valley virus rather than with Bunyamwera or Germiston viruses (Fig. 2).

Consistent with the molecular identification of these viruses, results of enzyme-linked immunosorbent assays (ELISAs) done at the same time showed that antigens prepared from Garissa virus-infected cell lysates reacted strongly with polyvalent "grouping" antisera (antisera prepared against Bunyamwera serogroup bunyaviruses) (Table 1). In addition, antiserum specific for Bunyamwera virus reacted strongly with infected whole cells by indirect immunofluorescense assay (data not shown).

Of 70 serum samples tested by using a nested PCR assay, Garissa virus was detected in serum samples from 14 hemorrhagic fever cases, including the two patients who yielded the isolates. The broad geographic distribution of these cases is shown in Fig. 3. Of these 14 cases, 6 had serologic evidence of past RVF infection (i.e., anti-RVF IgM and/or IgG) but were negative for RVF virus by isolation or diagnostic RT-PCR. The other 8 cases exhibited no evidence of RVF infection; only Garissa virus was detected. In addition, the proportion of bunyavirus-positive cases was approximately equal (19% vs 21%) in the RVF seropositive (32 cases) and RVF seronegative (38 cases) groups, respectively, suggesting that Garissa virus infection and associated hemorrhagic fever disease in these cases were occurring independent of RVF infection. From the same group of 70 samples, RVF virus was detected in 9 cases by PCR assay and only one of these cases displayed serologic evidence of past bunyavirus infection. These findings strongly suggest that Garissa virus was an etiologic

TABLE 1

Reaction of ELISA Antigens from Virus Isolates 9800521 and 9800535 with NIAID or CDC Typing Fluids

Grouping fluids	Result
NIAID Group A1	−
NIAID Polyvalent Anoph. A	−
NIAID Polyvalent B1	−
NIAID Group B Master	−
NIAID Group Bunyamwera	+
CDC Broad Bunyamwera	+
NIAID Polyvalent Bwamba	−
NIAID Group California	−
NIAID Polyvalent Congo	−
NIAID Group Kemerevo	−
NIAID Polyvalent Palyam	−
NIAID Group Phlebotomus	−
NIAID Polyvalent Quaranfil	−
NIAID Polyvalent 5	−
NIAID Group Simbu	−
NIAID Group Tacaribe	−
NIAID Group VSV	−
NIAID Polyvalent 1	−
NIAID Polyvalent 4	−
NIAID Polyvalent 7	−
NIAID Polyvalent 8	−
NIAID Polyvalent 9	−
NIAID Polyvalent 10	−
NIAID Polyvalent 12	−
CDC Yellow fever MAB	−
No antibody control	−
CDC Normal mouse ascitic fluid	−
CDC Normal monoclonal (SP1) ascites control	−
CDC Polyvalent Old World Arenavirus HMAF	−
CDC Polyvalent New World Arenavirus HMAF	−
CDC Polyvalent Ebola HMAF	−
CDC Marburg HMAF	−

Note. +, positive; −, negative.

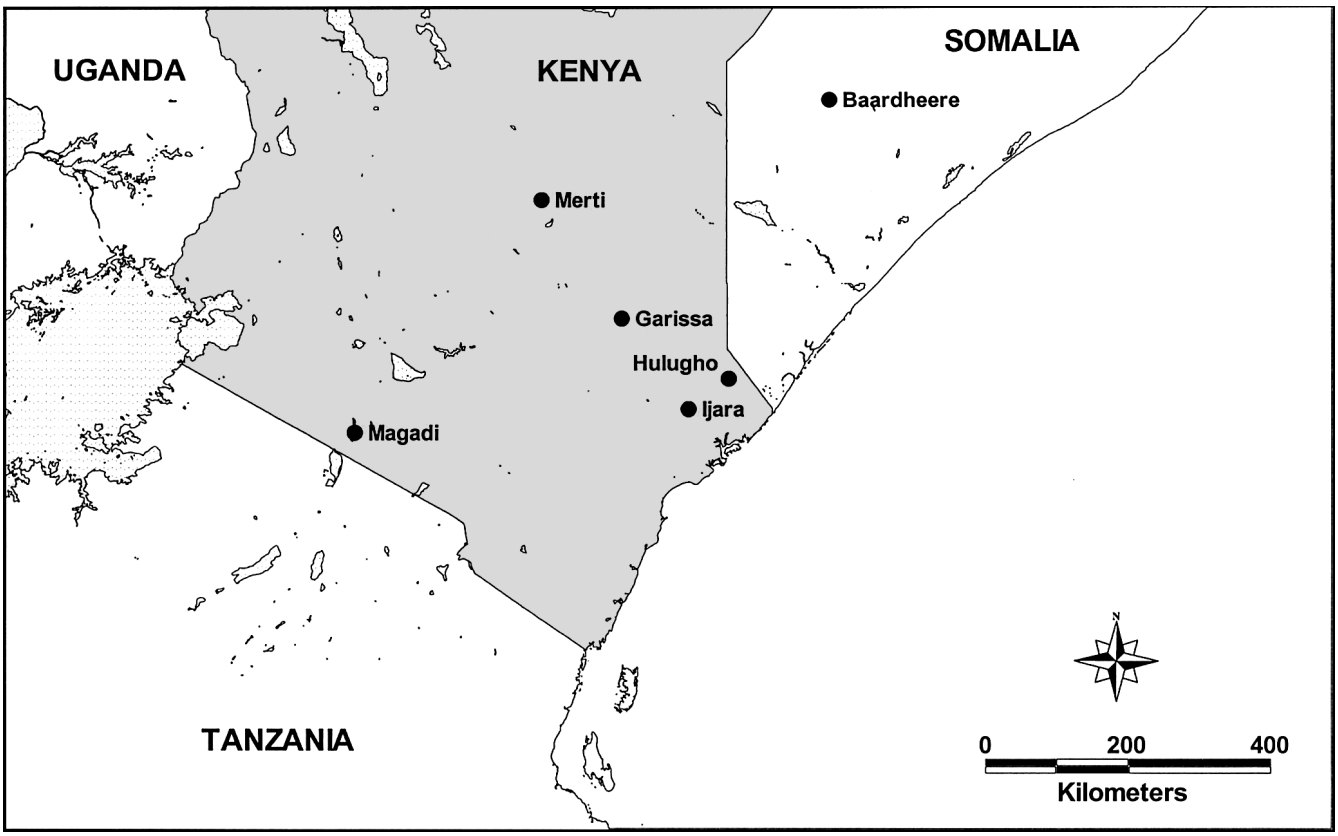

FIG. 3. Geographic distribution of hemorrhagic fever cases associated with Garissa virus.

agent of hemorrhagic fever in the 1997–1998 outbreak. As the hemorrhagic disease associated with these cases is quite unlike the neurological disease known to be associated with Bunyamwera virus infections, it is possible that this property is associated with the reassorted M segment present in Garissa virus. However, one cannot discount the possibility that the altered phenotype maps to amino acid differences in the L segment or regions of the S or L segments not sequenced in this study. Studies with reassortant California serogroup bunyaviruses have shown that the ability of La Crosse virus to reach the central nervous system after peripheral inoculation maps to its M segment (*19*). The study presented here represents the first evidence suggesting that natural genetic reassortment among bunyaviruses has produced a human pathogen with newly identified disease properties.

Methods: Virus Isolation. Serum samples from human hemorrhagic fever cases were inoculated into 25-cm^2 cell culture flasks containing confluent monolayers of Vero E6 cells maintained in Earle's minimal essential medium with Hanks' salts, 2% fetal bovine serum, and antibiotics. Cell cultures were observed daily for evidence of cytopathic effect. When cytopathic effect was observed, cells were removed for indirect immunofluorescence assay, and the cells and culture medium were frozen at −70°C. Virus seed pools were prepared from culture medium and cells were lysed by thawing of the frozen flasks.

Electron Microscopy. Infected Vero E6 cells were washed with 0.2 M phosphate buffer, fixed with 2.5% glutaraldehyde, and then pelleted by centrifugation. Cell pellets were postfixed in buffered osmium tetroxide, stained with uranyl acetate, dehydrated, and embedded in Epon substitute and Araldite resins (*20*). Thin sections were stained with uranyl acetate and lead citrate and viewed using a Philips EM410-LS transmission electron microscope.

Indirect Immunofluorescence Assay. Virus-infected cells were tested for the presence of RVF virus antigens by indirect immunofluorescence assay essentially as described previously (*21*), except that cells were incubated with anti-RVF immune mouse ascitic fluid.

ELISA. Antigens to the unknown viruses were prepared by detergent extraction of infected cells (*21*) and tested against arbovirus grouping antisera (NIAID Reagent and Reference Repository, Rockville, MD) in an IgG ELISA format (*21*).

IgG and IgM Detection in Patient Sera. Patient sera were tested for antiviral IgG by using Garissa virus-

infected cell antigens in an ELISA format essentially as described previously (*21*). IgM antibody titers were determined by IgM antibody capture ELISA using Garissa virus-infected cell slurry prepared as described previously (*21*).

Genetic Characterization. RNA was extracted from culture medium harvested from infected Vero E6 cells by using TriPure reagent according to the manufacturer's instructions (Boehringer Mannheim Corp.). Oligonucleotide primers were designed to target conserved regions in the three genomic segments of California and Bunyamwera serogroup bunyaviruses. S RNA primers were as follows: BUNYA1 (5′-GTCACAGTAGTGTACTCCAC-3′) and BUNYA2 (5′-CTGACAGTAGTGTGCTCCAC-3′); M RNA primers M14C (5′-CGGAATTCAGTAGTGTACTACC-3′) and M619R (5′-GACATATG(CT)TGATTGAAGCAAG-CATG-3′) (*22*); L RNA primers M13CBUNL1C (5′-TGTA-AAACGACGGCCAGTAGTGTACTCCT-3′) and BUNL605R (5′-AGTGAAGTCICCATGTGC-3′). RT-PCR was carried out using an Access RT-PCR kit (Promega) and a Perkin–Elmer 9600 thermocycler. Each RT-PCR contained 1× AMV/*Tfl* reaction buffer; 1 μM primer BUNYA1 and 1 μM primer BUNYA2, or 1 μM primer M14C and 1 μM primer M619R, or 1 μM primer M13CBUNL1C and 1 μM primer BUNL650R; 500 μM $MgSO_4$; 200 μM dNTPs; 5 U AMV reverse transcriptase; 5 U *Tfl* DNA polymerase; and 10% of the RNA from a single RNA extraction. Reverse transcription was carried out for 1 h at 42°C, and the reactions were heated for 2 min at 94°C. The reactions were then subjected to 40 cycles of a temperature profile consisting of 94°C for 30 s, 42–50°C for 1 min, and 68°C for 2 min, followed by a final extension of 68°C for 7 min. Ten percent (5 μl) of each reaction was then electrophoresed on a 1% agarose gel in Tris–borate–EDTA buffer. After electrophoresis, the gel was stained with ethidium bromide and PCR products were visualized by UV translumination to assess product yield. Products were not obtained for the reaction using primers BUNYA1 and BUNYA2, so second-round PCR was carried out using primers BUNCAL1 [5′-CATTTTCC(A/T)GGIAAC(A/C)G-GAACA-3′; I = inosine] and BUNCAL2 (5′-CCCCTAC-CACCCACCC-3′). Each second-round reaction contained 1× PCR buffer with 150 μM $MgCl_2$ (Boehringer Mannheim Corp.), 1 μM primer BUNCAL1, 1 μM primer BUN-CAL2, 200 μM dNTPs, 5 U *Taq* polymerase (Boehringer Mannheim Corp.), and 1 μl of the first-round reaction. The reactions were then subjected to 40 cycles of a temperature profile consisting of 95°C for 30 s, 42°C for 1 min, and 72°C for 2 min, followed by a final extension of 72°C for 10 min. Product yield was assessed by agarose gel electrophoresis, as described previously.

To obtain sequence data for each virus, the remaining RT-PCR or PCR product was electrophoresed on a 1% agarose gel in Tris–acetate–EDTA buffer. After being stained with ethidium bromide, specific PCR products were located by UV translumination, sliced from the gel, and purified from gel slices using a Sephaglas Bandprep kit (Pharmacia Biotech). Dye terminator cycle sequencing reactions were carried out using 5 to 50% of the gel-purified product (depending on product yield), ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kits with AmpliTaq DNA Polymerase FS (Applied Biosystems), and 3.3 pM primer. Extension products were purified using Centri-sep spin columns (Princeton Separations) and sequenced on an ABI 377 automated DNA sequencer (Applied Biosystems). Chromatograms were analyzed using Sequencher 3.0 software (Gene Codes). Sequences were obtained from both strands of each RT-PCR product for verification. Sequences were aligned with the corresponding sequences of Bunyamwera group viruses including Bunyamwera virus (*14–16*) (S RNA, D00353; M RNA, M11852; L RNA, X14383), Germiston virus (*17*) (M RNA, M21951), Cache Valley viruses (*18*) (M RNA, AF186241, AF186242, AF186243, AF082576), and additional California and Simbu group viruses (see legend to Fig. 3) retrieved from the GenBank sequence database. Alignments and sequence database searches were performed using the GAP and FASTA programs, respectively, of the Wisconsin Package Version 9.1 UNIX (Genetics Computer Group). Phylogenetic analysis was carried out using PAUP4.0b5 (Sinauer Associates Inc., Sunderland, MA).

Diagnostic Nested PCR. RNA was extracted from 100 μl of patient serum as described above except that RNA was isolated from the aqueous layer by using an RNaid kit (Bio 101, Inc., La Jolla, CA) instead of isopropanol precipitation. RT-PCR was performed as described above using primers specific for conserved regions of the S RNA of Bunyamwera serogroup bunyaviruses, BUNS274C [5′-CTTAAC(C/T)TTGGGGGCTGGA-3′] and BUNS957R (5′-CCCCIACCACCCACCC-3′), and a temperature profile consisting of 94°C for 30 s, 50°C for 1 min, and 68°C for 2 min, followed by a final extension of 68°C for 7 min. If no visible products were obtained in the RT-PCR assay, second-round PCR was carried out using primers BUNCAL1 and BUNS743R (5′-CTIACGTTIGTTT-TCTCTCCA-3′). The target regions for these primers resided totally within the amplified region of the RT-PCR primers in a "nested" fashion. Second-round reactions were prepared as described previously and subjected to 40 cycles of a temperature profile consisting of 95°C for 30 s, 50°C for 1 min, and 72°C for 2 min, followed by a final extension of 72°C for 10 min. Product yield was assessed by agarose gel electrophoresis, and products were purified and sequenced as described previously.

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