Isolations of Potosi Virus from Mosquitoes (Diptera: Culicidae) Collected in Connecticut

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ABSTRACT Potosi virus (POTV) (Bunyaviridae: *Orthobunyavirus*) was first isolated from *Aedes albopictus* (Skuse) collected in Potosi, MO, in 1989, and subsequent isolations were reported from Illinois, Michigan, Ohio, and the Carolinas. To determine whether the distribution of this virus extends into the northeastern United States, we analyzed arboviruses acquired from mosquitoes collected in Connecticut from 1998 to 2004. In 2001, a bunyavirus was isolated from *Aedes vexans* (Meigen) that was different from other arboviruses known to occur in Connecticut by cross-neutralization and reverse transcription-polymerase chain reaction (RT-PCR) assays. Nucleotide and encoded amino acid sequences of a portion of the G2 envelope gene were 99 and 100% similar to POTV, respectively, yet distinct from indigenous strains of Jamestown Canyon (JCV), Cache Valley (CVV), and Trivittatus virus (TVTV). Viral isolates obtained from the statewide surveillance program were retested by RT-PCR coupled with restriction enzyme analysis to distinguish POTV from other bunyaviruses. POTV isolates, previously typed by neutralization, were correctly identified by RT-PCR; however, many isolates classified as JCV or CVV by enzyme-linked immunosorbent assay proved to be POTV by molecular assays. In total, 92 strains of POTV were isolated from 12 mosquito species in 2000, 2001, and 2003, whereas POTV was not detected in mosquitoes sampled during 1998, 1999, 2002, and 2004. Viral isolation rates were highest for *Anopheles punctipennis* (Say) (3.2–11.3 infection rate per 1,000 mosquitoes), whereas the greatest number of isolates came from *Ochlerotatus trivittatus* (Coquillett) (8–16 isolates). This finding represents the first detection of POTV in the northeastern United States where it infects a diverse array of mosquito species.

KEY WORDS Potosi virus, arbovirus, viral isolation, mosquitoes, Connecticut

A few viruses of the genus *Orthobunyavirus* (family Bunyaviridae) perpetuate in a cycle involving mosquito vectors and deer hosts. Jamestown Canyon virus (JCV) and Cache Valley virus (CVV) fill this ecological niche and are broadly distributed throughout the United States (Calisher et al. 1986). Human populations are frequently exposed to these viruses where deer are abundant (Grimstad et al. 1986, Mayo et al. 2001), and infection occasionally results in meningitis or encephalitis (Grimstad et al. 1982, Srihongse et al. 1984, Sexton et al. 1997). In 1989, another bunyavirus, Potosi virus (POTV), was isolated from *Aedes albopictus* (Skuse) in Potosi, MO (Francy et al. 1990), and accumulating evidence suggests that it also circulates in a mosquito–deer cycle. POTV infects a number of species of the genera *Aedes, Anopheles, Coquillettidia, Culex, Ochlerotatus,* and *Psorophora* (Mitchell et al. 1996), although only *Ae. albopictus* has been shown to be a competent vector in the laboratory (Mitchell et al. 1990, Heard et al. 1991). White-tailed deer, *Odocoileus virginianus*, are frequently infected by POTV in enzootic regions (McLean et al. 1996, Nagayama et al. 2001) and develop viremias sufficient to infect susceptible mosquitoes (Blackmore and Grimstad 1998).

POTV has been found primarily in the central United States with isolations restricted to mosquitoes from Missouri, Illinois, Michigan, Ohio, and the Carolinas (Francy et al. 1990, Harrison et al. 1995; Mitchell et al. 1996, 1998; Wozniak et al. 2001), and POTV antibodies were detected in deer from Arkansas, Missouri, Indiana, Iowa, and Colorado (McLean et al. 1996, Blackmore and Grimstad 1998). The geographic range of POTV may be much more extensive than reported previously. The involvement of deer hosts and a broad range of mosquito species suggest that other regions of North America also could support POTV transmission where these hosts are abundant. Indeed, the known distribution of related bunyaviruses, JCV and CVV, largely tracks that of their deer hosts and encompasses most of the continental United States, the southern provinces of Canada, and northern Mexico (Calisher et al. 1986). In Connecticut, deer populations have increased substantially in recent decades fueling the amplification of JCV and CVV (Main et al. 1979, Main 1981, Andreadis et al. 1994, Zamparo

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et al. 1997). It is not clear whether POTV also may coexist with these ecologically equivalent viruses in Connecticut, as observed in the Midwest.

In this report, we document the first isolation of POTV from mosquitoes collected in northeastern United States. In addition, we describe the patterns of POTV infection in field-collected mosquitoes to identify possible vectors and to discern the geographical and seasonal distribution of this virus in Connecticut.

Materials and Methods

Mosquito Collections. The statewide mosquito surveillance program in Connecticut was originally established to monitor eastern equine encephalomyelitis virus (EEEV) activity and then was expanded in September 1999 in response to the introduction of West Nile virus (WNV) in North America. Mosquitoes were trapped at up to 91 locations statewide from the beginning of June through the end of October 1998–2004, as described previously (Andreadis et al. 2004). Adults were transported back to the laboratory alive and sorted by sex, species, and trapping location on chill tables by using the keys of Darsie and Ward (1981) and Means (1979, 1987). Mosquitoes were combined into pools of ≤50 and stored at −80°C until virus testing.

Virus Isolation. Mosquito pools were homogenized in 1–1.5 ml of phosphate-buffered saline containing 30% heat-inactivated rabbit serum, 0.5% gelatin, and 1× antibiotic/antimycotic by using either a mortar and pestle or a copper BB and vibration mill. Mosquito homogenates were centrifuged at 4°C for 10 min at 520 × *g*, and 100 μl of the supernatant was inoculated onto a monolayer of confluent Vero cells growing in minimal essential media, 5% fetal bovine serum, and 1× antibiotics/antimycotics. Cells were maintained at 37°C in 5% CO_2 and examined daily for cytopathic effect from day 3 through day 7 after inoculation. Infected cell supernatants were stored in individual aliquots at −80°C until further testing.

Antigenic Typing. Viral isolates initially were identified by either the cell–lysate antigen enzyme-linked immunosorbent assay (ELISA) from 1998 to 2000 or by cross-neutralization tests (2001–2004) by using constant-serum varying-virus dilution techniques. For ELISA testing, antigen was prepared from infected Vero cell cultures as described previously (Ansari et al. 1993). Viral antigens were titered in dilutions from 1:10 to 1:1,280 and identified using hyperimmune mouse ascitic fluids (1:10) raised against JCV, CVV, La Crosse virus (LACV), Highlands J virus (HJV), EEEV, and WNV (provided by the World Health Organization Center at the Yale Arbovirus Research Unit, Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT). For neutralization tests, virus dilutions from 10^{-4} to 10^{-6} were incubated with hamster antisera (1:10) raised against either JCV, CVV, POTV, Trivittatus virus (TVTV), LACV, Snowshoe Hare virus (SSHV), or Keystone virus (KEYV), or hyperimmune mouse ascitic fluids (1:10) against EEEV, WNV, or HJV for 1 h at 37°C. The virus–antibody mixture was then assayed for neutralizing activity by infecting Vero cell cultures. Laboratory animals were immunized, bled, and euthanized in accordance to protocols approved by the Institutional Animal Care Use Committee.

Genetic Characterization. RNA was isolated from viral stocks by using the QIAamp viral RNA kit (QIAGEN, Valencia, CA) and eluted in a final volume of 70 μl of elution buffer, provided with the kit. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the Titan One-Tube RT-PCR system (Roche Diagnostics, Indianapolis, IN) and primers targeting conserved regions of the M segment of California and Bunyamwera serogroup Bunyaviridae: M14C (5′-CGGAATTCAGTAGTGTACTACC-3′) and M619Rnew (5′-GACATATGCTGATTGAAG-CAAGCATG-3′) (Bowen et al. 2001). RT-PCR reactions were prepared using two separate master mixes as recommended by the manufacturer. Master mix I contained 2 μl of extracted RNA, 500 μM ATP, 500 μM GTP, 500 μM CTP, 500 μM TTP, 12.5 μM dithiothreitol, and 1 μM of each primer (M14C and M619Rnew) in a final volume of 20 μl. This preparation was heated to 85°C for 5 min and then quick chilled on ice to denature RNA. Master mix I was added to a second master mix containing 5× RT-PCR buffer (10 μl) and Titan enzyme mix (1 μl) for a final volume of 50 μl. Amplification was performed as follows: one cycle of 45°C for 30 min and 94°C for 2 min, 10 cycles of 94°C for 15 s, 48°C for 30 s, and 68°C for 1 min, followed by 20 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 1 min + 5 s per cycle, and one cycle of 68°C for 7 min. Amplification products were digested with restriction enzymes *Apa*LI and *Pvu*II in separate reactions. Digestion products were separated on a 2.5% agarose gel and visualized by staining with ethidium bromide. Viral isolates were also screened for WNV and EEEV by RT-PCR and real-time PCR (Lanciotti et al. 2000, Beckwith et al. 2002, Lambert et al. 2003), and these results are described in previous publications (Andreadis et al. 2001, 2004).

Representative amplification products from Connecticut strains of POTV, TVTV, CVV, and JCV were purified using the PCR purification kit (QIAGEN) and commercially sequenced (Keck Center, New Haven, CT). To obtain sequence from CVV, we modified the RT-PCR protocol by substituting primer M619Rnew with M4510R (5′-ATCGCGTAGTAGTGTGCTACC-3′) and increasing the 68°C extension step of 1 min to 4 min. This resulted in the amplification of the entire M segment (≈4.5 kb). Sequence chromatograms were edited using the ChromasPro editing program (Technelysium Ltd., Tewantin, Australia). Sequence alignments were generated by the ClustalW algorithm, and phylogenetic relationships were analyzed by the neighbor-joining method using Mega 3.0 (Kumar et al. 2004).

Electron Microscopy. Infected Vero cells (3 d postinoculation) were fixed at 4°C in a 2.5% (vol:vol) glutaraldehyde/2% paraformaldehyde solution containing 0.1% (wt:vol) $CaCl_2$ and 1% (wt:vol) sucrose in 100 mM Na cacodylate, pH 7.4; postfixed in aqueous 1%

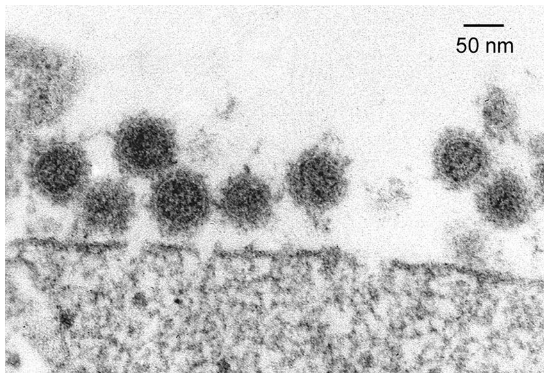

Fig. 1. Electron micrograph of virus strain CtAr S1312 grown in Vero cells.

(wt:vol) OsO_4; dehydrated through an ascending ethanol and acetone series; and embedded in a LX-112/ Araldite (Ladd Research Industries, Williston, VT) mixture. Thin sections were poststained with 5% (wt: vol) uranyl acetate in 50% (vol:vol) methanol followed by Reynold's lead citrate and examined in a Zeiss EM 10C electron microscope at an accelerating voltage of 80 kV.

Results

In 2001, we isolated a virus from a pool of *Aedes vexans* (Meigen) that could not be identified by serological or molecular assays. The isolate (designated as CtAr S1312) failed to react to antibodies raised against JCV, CVV, TVTV, LACV, SSHV, KEYV, HJV,

Fig. 2. Phylogenetic tree depicting relationships of bunyaviruses based on partial amino acid sequence of the G2 glycoprotein. Aligned sequences were analyzed using the Poisson correction for amino acid substitutions and neighbor-joining method in Mega 3.0. Numbers at nodes represent bootstrap support for 1,000 replicates. CT, Connecticut virus strains.

Table 1. Evaluation of RT-PCR and restriction enzyme analysis to distinguish POTV from other bunyaviruses

Prior test		No. isolates reacted w/ bunyavirus primers		No. amplification products cut by	
Assay	Result	Tested	Amplifed	*Apa*LI	*Pvu*II
Neutralization	POTV	11	11	11	11
	CVV	5	0		
	JCV	5	5	0	0
	TVTV	5	5	0	0
ELISA	CVV	41	19	19	19
	JCV	37	36	1	1

Viral isolates were initially typed either by ELISA or cross-neutralization tests and rescreened with bunyavirus primers (M14C and M619Rnew) and POTV-specific restriction enzymes (*Apa*LI and *Pvu*II).

WNV, and EEEV by neutralization and was negative for WNV and EEEV by real-time and RT-PCR. When examined by transmission electron microscopy, infected cell cultures revealed spherical virus particles measuring 80–90 μm in diameter (Fig. 1). Virions contained an electron-dense nucleocapsid core of 50–60 nm surrounded by an envelope with distinct surface projections. These characteristics are consistent with members of the family Bunyaviridae.

The virus was further characterized by RT-PCR using primers targeting a conserved region of the M segment of the genus *Orthobunyavirus.* Primer pair M14C/M619Rnew successfully amplified a fragment of ≈640 bp that was sequenced and compared with those on file at GenBank, in addition to Connecticut strains of JCV, CVV, and TVTV (accession nos. AY845246–AY845249). Isolate CtAr S1312 proved to be POTV on the basis of nucleotide and encoded amino acid sequences of a portion of the G2 glycoprotein that were 99 and 100% similar to the protoype strain of this virus. Phylogenetic reconstruction of amino acid sequences revealed two distinct clades representing the California and Bunyamwera serogroups (Fig. 2). POTV segregated with other members of the Bunyamwera group as expected. Connecticut strains of JCV, CVV, and TVTV were genetically distinct from POTV and grouped with their respective prototype strains.

To differentiate POTV from related viruses, we generated antisera against POTV (CtAr S1312) in hamsters to include in our panel of antibodies for routine testing by the cross-neutralization assay from 2001 to 2004. In addition, we developed an RT-PCR assay that distinguishes POTV from other bunyaviruses by identifying unique restriction enzyme sites of amplification products. *Apa*LI and *Pvu*II were predicted to digest only within the M14C/M619Rnew amplification products of POTV, yielding fragments 216 and 422 bp for *Apa*LI and 283 and 355 bp for *Pvu*II. The RT-PCR assay was tested against a sample of bunyaviruses previously classified by the cross-neutralization assay. RT-PCR coupled with restriction enzyme analysis confirmed that 11 isolates were POTV, identical to the cross-neutralization results (Table 1). None of the CVV isolates yielded amplification products, and PCR amplicons from JCV and TVTV isolates failed to digest with *Apa*LI or *Pvu*II. RT-PCR and cross-neutralization assays readily distinguished POTV from other bunyaviruses endemic to Connecticut.

We became aware of POTV as a distinct viral entity after the ELISA was replaced by the cross-neutralization assay for bunyavirus identification in 2001. To determine whether POTV may have circulated in Connecticut before 2001, we retested all of the bunyaviruses previously typed by ELISA from 1998 to 2000. Twenty isolates were reclassified as POTV by RT-PCR and restriction enzyme analysis (Table 1). All of these isolates were obtained in 2000 and all were previously typed as CVV by ELISA, except for one isolate that was misidentified as JCV. These newly identified POTV isolates were included in subsequent analyses.

POTV was isolated from a number of different mosquito species in 2000, 2001, and 2003 (Table 2). More than 90% of 92 isolations were recovered from *Aedes, Anopheles*, or *Ochlerotatus* mosquitoes, whereas relatively few were isolated from *Coquillettidia, Culex*, or *Psorophora.* Patterns of POTV infection were not consistent from year to year; however, in 2000 and 2003, infection rates were highest for *Anopheles punctipennis* (Say), and the greatest number of isolates came from *Ochlerotatus trivittatus* (Coquillett). Multiple isolations also were obtained from *Ae. vexans, Ochlerotatus cantator* (Coquillett), and *Ochlerotatus taeniorhynchus* (Wiedemann) in >1 yr. Finally, *Ochlerotatus canadensis* (Theobald) yielded 13 isolations in 2003. POTV was not detected in mosquitoes collected during 1998, 1999, 2002, and 2004.

The geographic distribution of POTV isolations from mosquitoes is presented in Fig. 3. In 2000 and 2003, POTV isolates were obtained throughout the

Table 2. POTV isolates from mosquitoes collected in Connecticut

Yr	Species	No. collected	No. isolates	Infection rate/1,000
2000	*Ae. cinereus*	545	1	1.8
	Ae. vexans	1,609	4	1.9
	An. punctipennis	174	2	11.3
	Oc. cantator	994	1	1.0
	Oc. taeniorhynchus	4,924	4	0.8
	Oc. trvittatus	1,945	8	4.4
2001	*Ae. vexans*	5,920	3	0.5
	Oc. cantator	1,577	2	1.4
2003	*Ae. cinereus*	8,710	8	0.9
	Ae. vexans	14,406	7	0.5
	An. punctipennis	1,563	5	3.2
	Cq. perturbans	11,627	2	0.2
	Cx. salinarius	10,377	1	0.1
	Oc. canadensis	22,354	13	0.6
	Oc. cantator	3,074	3	1.0
	Oc. sticticus	12,781	3	0.2
	Oc. taeniorhynchus	2,321	5	2.3
	Oc. triseriatus	683	2	2.9
	Oc. trivittatus	8,759	16	1.9
	Ps. ferox	5,239	2	0.4

Infection rates per 1,000 mosquitoes were calculated by pooling data for all trapping sites that yielded POTV isolates and applying the maximum likelihood estimator in the PoolInfRate (Biggerstaff 2003).

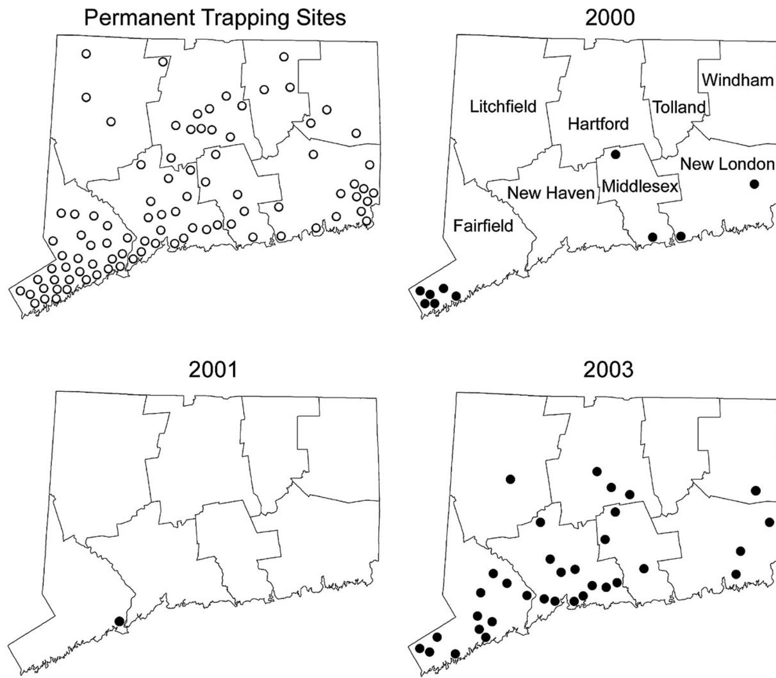

Fig. 3. Geographic location of POTV isolates from mosquitoes collected in Connecticut. Closed circles represent positive sites.

state, whereas in 2001, they were restricted to a single site in southwestern Connecticut. Viral isolations were concentrated in the southern portion of the state, largely reflecting trapping effort from that region. POTV activity predominated in rural and suburban habitats, including grasslands, deciduous forests, hardwood swamps, and coastal marshes. POTV was isolated as early as mid-August, followed by a sharp increase in the number of isolates during the second week of September, and a subsequent decline in October (Fig. 4). POTV seems to be broadly distributed throughout the state where it is transmitted from late summer to early fall.

Discussion

POTV has a wider distribution than previously recognized and now includes the northeastern United States. Repeated isolations of POTV over multiple years indicate that this virus is enzootic in Connecticut. Evidence suggests that POTV circulates in a cycle involving white-tailed deer and mosquitoes (Mitchell et al. 1990, 1996; Heard et al. 1991; McLean et al. 1996; Blackmore and Grimstad 1998); therefore, CT, with its abundant deer populations, provides an ideal habitat to support POTV transmission. Deer thrive in the suburban forests that comprise much of the northeastern United States, and by extension, we suspect that POTV is broadly distributed throughout this region.

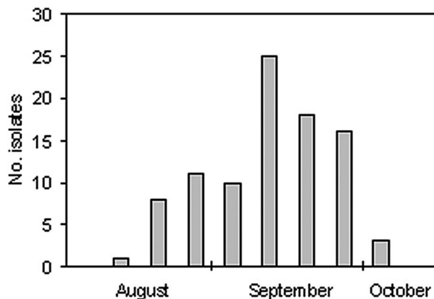

Fig. 4. Weekly isolations of POTV from field-collected mosquitoes 2000–2003.

Despite antigenic cross-reactivity between POTV and CVV, these viruses seem to cocirculate in Connecticut and the Midwest by infecting a common amplifying host. Experimental infections of white-tailed deer indicate that POTV and CVV confer partial cross-protection in their host (Blackmore and Grimstad 1998). POTV-immune deer were almost completely protected against subsequent CVV infection, whereas CVV-immune deer developed lower viremias upon challenge of with POTV. Nonetheless, cross-reactive immune responses do not mutually exclude these viruses from inhabiting the same geographical regions. Perhaps POTV and CVV are maintained by occupying slightly different ecological niches. In Connecticut, CVV transmission peaks in late summer and infects many of the same mosquito species as POTV (data not shown). How these viruses coexist in the same area is not clear and deserves further study.

POTV eluded our detection until the cross-neutralization assay was adopted in 2001. Both POTV and

CVV are members of the same serogroup (Bunyamwera) and could not be distinguished by ELISA. Retrospective analysis of bunyavirus isolates by RT-PCR revealed that POTV was present in Connecticut as early as 2000. It may be that this virus has always been maintained in this region and escaped detection because of inadequate sampling and/or lack of specific testing. POTV was first recognized in 1989 (Francy et al. 1990); therefore, virus-specific antibodies were not available until recently. Further analysis of earlier isolates will help to elucidate the history of this virus in North America. The RT-PCR assay used in our study provides convenient methodology to pursue this aim.

The majority of our POTV isolations were derived from woodland and saltmarsh mosquitoes of the genera *Aedes, Anopheles,* and *Ochlerotatus.* The most frequently infected species were *Oc. trivittatus* (24 isolations) followed by *Ae. vexans* (14), *Oc. canadensis* (13), *Oc. taeniorhynchus* (9), *Ae. cinereus* (Meigen) (9), *An. punctipennis* (7), and *Oc. cantator* (6). The adult population of these species extends into late summer and early fall, and they feed preferentially on mammals (Magnarelli 1977, Boromisa and Grimstad 1986, Irby and Apperson 1988, Apperson et al. 2002). Deer are the most abundant ungulate in the region and were the most frequent source of bloodmeals for these mosquitoes in ecologically similar sites in New Jersey and New York (Apperson et al. 2004). Interestingly, *Psorophora ferox* (Humbolt) and *Coquilletidia perturbans* (Walker) were infrequently infected by POTV, despite their host-feeding preference for deer. These species may be less susceptible to POTV infection than species of *Aedes, Anopheles,* or *Ochlerotatus.* Vector competence studies could help to clarify the role of these and other mosquitoes in the transmission cycle of POTV. POTV infection was not detected in ornithophagic mosquitoes such as *Culex pipiens* L., *Culex restuans* (Theobald), and *Culiseta melanura* (Coquillett), despite intensive testing of these species. However, a few isolates were derived from *Culex salinarius* (Coquillett), which may reflect this species propensity to feed on both mammals and birds.

The significance of POTV to public health remains unknown. Several related viruses of the Bunyamwera serogroup produce human disease ranging from a self-limiting febrile illness (Bunyamwera-BUN, Germiston, Ilesha, Batai, and Wyeomyia viruses) to central neurological system disease (BUNV and CVV) to acute hemorrhagic fever (Ngari virus) (Beaty and Calisher 1991, Nashed et al. 1993, Sexton et al. 1997, Gerrard et al. 2004). In addition, in utero infection of CVV causes congenital defects in lambs (Edwards 1994) and has been implicated in a similar condition in infants (Calisher and Sever 1995). Although POTV has not been associated with human disease, its presence in mammal-biting vectors suggests that humans may be frequently exposed to this virus. In enzootic regions, POTV should be considered in the diagnosis of arboviral illness when differentiating among closely related bunyaviruses.

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