Title: Cache Valley virus in a patient diagnosed with aseptic meningitis

Running head: Aseptic meningitis due to Cache Valley virus

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Abstract

Cache Valley virus was initially isolated from mosquitoes and had been linked to CNS-associated diseases. A case of Cache Valley virus infection is described. The virus was cultured from a patient’s cerebrospinal fluid and identified with real-time RT-PCR and sequencing, which also yielded the complete viral coding sequences.
In mid September 2011, a 63-year old woman presented to an upstate New York (NY) hospital with complaints of fever, headache, neck stiffness and photophobia. One week earlier, she had noticed a macular, non-pruritic lesion on her right forearm, about 3cm in size with central clearing. Three days prior, as the first lesion was fading, a petechial rash developed on her lower extremities that spread to her torso. She then traveled to Pennsylvania for a weekend, and during this time she developed the mentioned fever and symptoms of meningitis. She returned home and went to the Emergency Department (ED) the next morning.

On physical examination, the patient appeared alert and oriented, with a blood pressure of 148/80 mmHg, a pulse of 87 per min, a respiratory rate of 18 per min, an oral temperature of 37.6 °C, and an oxygen saturation of 99% on room air. She had a scattered, bilateral, petechial rash on her thighs; meanwhile, the lesions on her back and abdomen were fading. She had moderate neck stiffness. Her neurologic exam was otherwise normal; there was no evidence of encephalitis, cranial nerve abnormalities, or focal findings.

Her medical history included hypertension, hypothyroidism, meningioma, migraine headaches, and a history of rheumatic fever during childhood. The patient stated that she and her husband frequently camped outdoors. Throughout mid- to late-August they camped in Wyoming and Livingston counties in NY and also embarked on a 5-day camping trip in Dansville, NY through Vermont and New Hampshire. She lived with her husband, had a cat, and frequently tended to her garden around her home. She had no knowledge of any sick contact.

Her white blood cell count on presentation was 5.7 thousand per microliter, with a normal differential. Hematocrit was normal at 42%, and platelets were normal at 212 thousand per
Computed tomography of the head without contrast showed mild atrophy without evidence of acute intracranial abnormality. Blood cultures were drawn, and she was sent home on doxycycline. The patient returned to the ED the following day with new complaints of nausea and vomiting in addition to the previously reported symptoms.

The patient was admitted to the hospital with a preliminary diagnosis of aseptic meningitis. A lumbar puncture was performed, and the cerebrospinal fluid (CSF) showed 216 nucleated cells with 91% lymphocytes, 7% monocytes, 1% basophils and 1% polymorphonuclear cells. CSF chemistries were normal, with glucose concentration of 60 mg/dL (56% of the serum level) and protein of 46mg/dL. No microorganism was seen in CSF by Gram stain. Magnetic resonance imaging of the brain showed only a small, stable meningioma. Virus culture of the CSF was initiated on several cell lines. Polymerase chain reaction (PCR) testing for enteroviruses in CSF and for herpes simplex virus in blood and CSF were performed. Serologic tests to detect antibodies against *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, *Anaplasma phagocytophila*, and *Rickettsia* (both typhus and Rocky Mountain spotted fever) in blood were also performed. All molecular and serologic test results were negative. Bacterial culture of the blood and CSF yielded no growth.

The patient gradually improved and was discharged four days after admission to complete a 10 day-course of doxycycline at home. On follow-up two months later, she reported transient difficulties in word-finding that occurred soon after the hospital discharge and had resolved at the time of the visit. Her headaches, including a migranous aura, were more severe following this hospitalization. She continued seeking medical care for her worsening headaches and memory loss a year later.
Six days after inoculation of the CSF, a questionable cytopathic effect (CPE) was noticed in the Buffalo green monkey kidney (BGMK) cell culture. Other inoculated cell lines included Rhesus monkey kidney (RhMK) and human male fetal lung (MRC5); both of these lines failed to produce any CPE. After three passages in BGMK, the amplified supernatant was filtered, inoculated, and successfully produced CPE in human colon adenocarcinoma (CaCo2), human lung carcinoma (A549), MRC5, and an African green monkey kidney cell line (Vero).

Characteristics of CPE involved initial cell rounding, followed by clumping and sloughing off the cell monolayer (Figure 1). No CPE was observed after the amplified stock was inoculated into RhMK cells, human female fetal lung (WI-38) cells, or human embryonal rhabdomyosarcoma (RD) cells.

Cultured virus from day 2 post-inoculated CaCo2 cells was harvested, and nucleic acids were extracted using the QIAcube automated nucleic acid extractor (Qiagen, Germantown, MD). A molecular panel for the detection of encephalitis viruses was performed on the extract (1). Included in this panel was a duplex real-time reverse transcription-PCR (RT-PCR) for the detection of Cache Valley virus (CVV) and the California serogroup viruses (2). The sample tested positive for CVV in this assay. All other PCR results were negative. For confirmation, cDNA was generated from the viral RNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA) and a conventional PCR that targets the S segment of the CVV genome (3). Sequence analysis of the 170-nucleotide amplicon showed 100% identity with several strains of CVV in GenBank.

To better characterize the CVV that infected the patient, the virus genome was subjected to additional sequencing. Briefly, viral RNAs were extracted, reverse-transcribed, and PCR amplified with random primers. The PCR products were sequenced using the 454 GS-FLX
Titanium platform (454 Life Sciences, Branford, CT), yielding a total of 139,321 reads, of which 100815 reads shared 60.4–100% identity to viruses in the family Bunyaviridae. All unknown and Bunyavirus-like reads were then employed to assemble contigs via the supplied Newbler v.2.6 software. A single contig of 905 nucleotides (nt) was constructed (>32x coverage) that matched 99% identity (99% coverage, e value: 0.0E+00) with the S segment of 4 Yucatan strains of CVV (BLASTN 2.2.27, 08.21.12). Five other contigs of length 138–2164nt were assembled and shared 92.5-97% identity with regions of the M segment of various CVV strains. Fourteen contigs of length 110-3014nt were also generated, sharing 71–100% identity with regions of the L segment of Tensaw, Batai, and Ngari viruses as well as of partially sequenced L segment of several CVV strains. Using targeted-primer RT-PCR followed by TOPO® cloning and Sanger sequencing, we closed the gaps between the contigs and confirmed partial sequences of 877nt for the S segment, of 4460nt for the M segment, and of 6871nt for the L segment of this CVV.

We next assessed sero-conversion in a pair of patient sera collected at the time of admission and at twenty-four days later. A neutralization assay in 24-well microplates was developed, using the patient strain harvested from BGMK then passaged twice through Vero cells. Briefly, test sera were serially 2-fold diluted in tissue culture medium, mixed with 100 TCID$_{50}$ of virus, incubated for 1hr at room temperature, and then added in duplicate onto the Vero cell monolayers that were 90% confluent. The cells were incubated in 5% CO$_2$ at 37°C for 5 days, and CPE was monitored by microscopic inspection during the incubation period. Complete protection of the cell monolayers was observed at the 1:160 dilution or lesser with a positive control serum, an anti-CVV mouse hyperimmune serum (Centers for Disease Control and Prevention, Fort Collins, CO), and at the 1:80 dilution or lesser with the patient convalescent
serum. By contrast, the acute serum failed to neutralize the virus at all tested dilutions, indicating that the patient was recently infected with CVV.

To confirm the neutralization data, an Immuno-fluorescence assay (IFA) was established to detect CVV-specific antibodies. Briefly, the BGMK-derived virus was used to inoculate CaCo2 cells, which were harvested at 2+ CPE and used to produce slides for the IFA test. Cells were trypsinized, washed, and resuspended in tissue culture medium; cell spots were then air dried and fixed in cold acetone for 30 minutes. Control slides were prepared in the same fashion with mock-infected CaCo2 cells. CVV-infected CaCo2 cells were incubated with the patient’s 24-day convalescent serum at dilutions ranging from 1:16 to 1:4096. To demonstrate specificity, CVV-infected CaCo2 cells were also incubated with an anti-CVV mouse serum, an anti-La Crosse encephalitis virus human serum (Wadsworth Center, Albany, NY), and a negative control human serum (Focus Diagnostics, Cypress, CA) all at the same dilutions. All described antisera were also tested in parallel at 1:16 on control slides. Slides that contained the patient convalescent and the anti-CVV mouse sera were reactive with a titer of 1:1024 (Figure 2). Neither the negative control human serum nor the anti-La Crosse virus human serum was reactive against the CVV-infected cells (<1:16). On control slides of mock-infected cells, no CVV-specific staining pattern was observed, validating the test results for the CVV-infected cells.

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Cache Valley virus is a negative sense, single-stranded RNA virus with a tripartite genome. It belongs to the Bunyamwera serogroup in the genus Orthobunyavirus and was named after a location in northern Utah where it was first isolated from mosquitoes (4). The virus has since been found widespread in North and Central America, infecting many species of domestic
and wild animals such as deer, cattle, sheep, and horses (5, 6, 7, 8). Epidemiologic studies indicate that CVV can be transmitted via a broad range of mosquito species (9), with white-tailed deer population as a potential natural reservoir (10).

CVV is a concern in animal husbandry due to its ability to cause abortion, stillbirth, and congenital defects of the musculoskeletal and central nervous system in affected ruminants (11, 12). In humans, CVV disease was first identified in a previously healthy young man from North Carolina following a deer hunting trip (13). Subsequently, a case of aseptic meningitis due to CVV in an adult male from Wisconsin was reported (14). Other human infections with CVV have been inferred by serologic studies of CVV in the endemic peninsula of Delmarva, USA and Yucatan, Mexico which reported infection rates of up to 18 % (15, 16).

We describe here a case of CVV disease in human. Similar to the Wisconsin case, this virus was isolated from the patient’s acute-phase CSF. Its positive identification was made via a laboratory-developed real time RT-PCR, followed by genomic sequencing. We also demonstrated a sharp rise in anti-CVV antibody titer between an acute and a convalescent serum drawn more than 3 weeks apart, indicating that the CVV infection was just acquired. In the absence of other etiology and coupled with previous observations that CVV infection can lead to CNS-associated diseases, it is reasonable to conclude that CVV was responsible for this patient’s illness. Due to the lack of molecular epidemiology of CVV in regions where the patient traveled, however, it was not possible to determine the time and place of her exposure.

Segemented RNA viruses can evolve over time through genomic reassortment. This characteristic has been reported in members of the Bunyamwera serogroup including CVV (17). Through the effort to identify the CVV strain involved, we obtained a sequence of 877nt of the
virus S segment, containing two open reading frames (ORF) of 233 and 101 amino acids (aa). Their translated sequences possessed 100% identity to the deduced N and NSs proteins, respectively, of 5 CVV mosquito isolates previously collected in the Yucatan area (18). We also sequenced 4460nt of the virus M segment, which encodes a hypothetical polyprotein precursor of 1434aa. It shares at most 94% nt identity and 98% aa identity with its homologues in 7 CVV strains isolated in North America. In addition, we deciphered the majority (6871nt) of the virus L segment. It contains an ORF for a protein of 2228aa, which shows 88% and 83% aa identity with the predicted RNA-dependent RNA polymerase of Tensaw and Batai viruses, respectively. At present, no complete ORF encoding the predicted RNA polymerase of CVV exists in GenBank. This report thus provides full nt sequence coverage for this protein.

In conclusion, we report a confirmed case of meningitis due to CVV infection. Since the discovery of CVV over half a century ago, there have been surprisingly few human cases reported. Given the wide geographic distribution of CVV and its transmission via a large number of mosquito species, we suspect that CVV infection is under-reported. A targeted study may be needed to assess the true scope of human infection with this virus. Additionally, inclusion of CVV in routine arbovirus surveillance is suggested. Knowledge of the endemic areas of CVV might benefit clinicians in the differential diagnosis of CNS-related diseases. And finally, this study reports complete coding sequences of a CVV, including the L segment-encoded RNA polymerase.

**Nucleotide sequence accession numbers:** The sequenced L, M, and S segments of this CVV (MNZ-92011 strain) have been deposited in GenBank under the accession numbers KC436106, KC436107, and KC436108, respectively.
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This study was approved by the Institutional Review Board of participating institutions.

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Figure legends

Figure 1: Cytopathic effect of the CVV-infected Vero cells.

Vero cells were seeded in 24-well plate, grown to 90% confluence, and were either mock-infected (panel A) or infected at 0.3 MOI (panel B) or at 30 MOI (panel C) with the MNZ-92011 strain, which was harvested from BGMK, amplified and titrated in Vero cells. Photographs were taken 4-day post-infection, using a Spot RT-KE monochrome camera connected to a Nikon Diaphot TMD microscope at 40x magnification, Spot 5.0 Basic software.

Figure 2: Detection of anti-CVV antibodies in the patient’s convalescent serum.

IFA of the patient’s 24-day convalescent serum (I), an anti-CVV mouse hyperimmune serum (II), and an anti-La Crosse virus human convalescent serum (III), using Caco2 cells that were either mock-infected (panel B) or at day 2 post-infection with the MNZ-92011 strain (panel A). FITC-conjugated dual anti-human/mouse IgG goat antibody (Focus Diagnostics, Cypress, CA) was used as secondary antibody. Images were taken with a Zeiss AxioCam MRc camera attached to AxioImager A1 microscope at 400X magnification, using Zeiss AxioVision 3.1 software. All sera were at 1:16 dilution with the exception of the anti-CVV mouse serum in panel IIA, where the serum was 1:1024 diluted.