Keystone Virus Isolated From a Florida Teenager With Rash and Subjective Fever: Another Endemic Arbovirus in the Southeastern United States?

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Keystone virus, a California-serogroup orthobunyavirus, was first isolated in 1964 from mosquitoes in Keystone, Florida. There were no prior reports of isolation from humans, despite studies suggesting that ~20% of persons living in the region are seropositive. We report virus isolation from a Florida teenager with a rash and fever.

Keywords. Keystone virus; Florida; Aedes atlanticus; skin rash.

The emergence of the Zika virus (ZIKV) and ongoing identification of Chikungunya virus and Dengue virus infections have generated increasing concerns about endemicity and spread of arboviruses in the United States. Keystone virus (KEYV; genus Orthobunyavirus, family Peribunyaviridae), a member of the orthobunyavirus California serogroup, was first isolated from mosquitoes in Keystone, Florida (near Tampa Bay), in 1964 [1]. Subsequent studies demonstrated widespread distribution of the virus in mosquitoes, particularly Aedes atlanticus, as well as in squirrels, raccoons, and whitetail deer, in coastal regions stretching from the Chesapeake Bay southward through Florida, and as far west as Texas [2–6].

In studies conducted in human populations in the 1960s, rates of seropositivity were in the range of 19–21% [7]. However, actual isolation of KEYV from humans has never before been reported, nor has KEYV been linked with a clinical syndrome in humans, despite its apparent endemicity in animals and mosquitoes.

CASE REPORT

A 16-year-old previously-healthy male presented in August, 2016, to an urgent care clinic in north-central Florida with a history of low-grade fever and a diffuse skin rash. The patient reported feeling "warm" the evening before, with a temperature estimated by his parents to be in the range of ~100°F. An erythematous papular rash appeared the morning of presentation, starting on his chest and progressively spreading to his abdomen, arm, back and face. There were no vesicles, and the rash was painless and non-pruritic. It was aggravated by heat and sunlight. The patient denied chills, headache, neck stiffness, or gastrointestinal symptoms. He did note mild fatigue and ankle discomfort, which he attributed to concurrent attendance at a summer camp for marching band participants, wearing new band shoes. The preceding week, the patient had been diagnosed with possible tonsillitis and had been placed on therapy with amoxicillin at 500 mg taken orally 3 times a day, which he had self-discontinued the following day. He had no relevant past medical or surgical history; no known immunosuppressive or congenital conditions; no known allergies; no recent travel outside central Florida; and no exposure to farms or farm animals. He had received all recommended childhood immunizations. No other family members were ill. The patient and his family had moved to Florida from Kansas (where Keystone virus has not been identified) 4 years before the reported illness. The band camp that he was attending continued into the evening, and he reported being bitten numerous times by mosquitoes, despite the application of diethyltoluamide (commonly known as DEET).

On examination in the clinic, he had a temperature of 98.5°F (36.9°C), pulse of 69 beats/minute, respiratory rate of 12 breaths/minute and blood pressure of 122/82 mm/Hg. A diffuse, erythematous, papular rash of the trunk, arm, back and face was noted (Figure 1). The patient's head, neck, respiratory, cardiovascular, and abdominal examinations were normal. A rapid Monospot test performed at the clinic was negative. With the consent of the patient and his parents, saliva and urine samples were collected. The rash resolved 2 days later, with no further fever. He subsequently has taken amoxicillin without appearance of a rash.

VIRAL IDENTIFICATION/ISOLATION

Reverse Transcription Polymerase Chain Reaction Screens

As the patient presented with a history of rash and fever during a period when a ZIKV outbreak was occurring in Florida, saliva and urine samples were tested and were negative for ZIKV, Chikungunya virus, and Dengue virus serotype 1–4 genomic ribonucleic acid (RNA) by reverse transcription polymerase chain reaction (RT-PCR) [8]. The possibility of an alphavirus or flavivirus etiology was further evaluated by performing RT-PCR using universal primers that detect alphaviruses and flaviviruses in Brazil [9]. Both saliva and urine samples tested...
negative for alphavirus and flavivirus vRNAs, whereas positive-controls worked as expected (data not shown).

Unbiased Reverse Transcription Polymerase Chain Reaction and Sequencing for Identification of Putative Viral Etiological Agent

A presumptive virus identity was obtained following a modification of a procedure we previously outlined [8]; detailed methods are provided in the Supplementary Data accompanying this paper. Viral genomic RNA (and/or deoxyribonucleic acid [DNA]) were extracted from virions using a QIAamp Viral RNA Mini Kit (Qiagen Inc.). PCR amplicons were purified (Qiagen QIAquick PCR purification kit), and A-tailed with Taq DNA polymerase (New England Biolabs). The A-tailed PCR amplicons were then TA-cloned, and the inserts sequenced using Sanger sequencing. Out of a total of 40 plasmids with TA-cloned inserts (20 inserts from urine, 20 from saliva), 1 from urine contained a 166-bp insert with a 99% identity of Keystone virus small-genome sequences deposited with GenBank (KT630293.1, U12801.1, and KT630290.1).

Direct Sequencing of Keystone Virus in Urine

As there are numerous orthobunyaviruses, and published RT-PCR primers for sequencing KEYV were not available, the virus was sequenced using purpose-designed primers based on 3 complete, complete Keystone virus genomes in GenBank. Sequencing was accomplished using a genome-walking strategy, with the PCR primers described in Supplementary Table 1; our complete methods are included in the Supplementary Data. The sequence was designated as KEYV/Homo sapiens/Gainesville-1/2016; the 3 genome segment sequences have been deposited in GenBank under the accession numbers MH016784 (large segment), MH016785 (medium segment), and MH016786 (small segment). The complete L genome sequence has 98 to 99% identities with the corresponding genomes of the only 3 other complete Keystone virus L sequences available in Genbank (KT630288.1, KX817321.1, and KT630291.1). Similarly, the complete M genome has 98 to 99% in common with the corresponding M sequences (AF123489.1, KT630289.1, and KX817322.1) and 99% in common with the corresponding S genome sequences (KT630293.1, U12801.1, and KT630290.1).

Isolation and Visualization of Keystone Virus in Cultured Cells

Since Keystone virus vRNA was detected by unbiased sequencing (and later on was fully sequenced), attempts were made to isolate the virus in a cell line known to support its growth (Vero cells) and in mouse neuroblastoma cell line Neuro-2A; a description of the complete methods is provided in the Supplementary Data. Virus-induced mixed cytopathic effects (CPE) were evident in Neuro-2A cells by 2 days post-inoculation of the cells with urine, but not with saliva, and the same was observed 1 day later in Vero E6 cells. Upon subculture, CPE were evident 2 days post-inoculation of Vero E6 cells with spent media from urine-inoculated Neuro-2A. The KEYV-induced CPE formed in Vero E6 cells are depicted in Supplementary Figures S1A and S1B; both original urine-inoculated Vero E6 and Vero E6 cells inoculated with virus-infected Neuro-2A cells formed the same type of CPE. Supernatants of the virus-infected cells were positive for KEYV vRNA by RT-PCR, whereas those from mock-inoculated controls were negative for KEYV vRNA.

To demonstrate active Keystone virus replication, Vero E6 cells displaying significant CPE (>70% of the infected cell monolayer) 40 hours post-infection were analyzed by transmission electron microscopy, and were shown to display typical bunyavirus-induced CPE and production of progeny virions. Methods and images (Supplementary Figures S2A–F) are included in the Supplementary Data.

COMMENT

KEYV, a negative-sense single-strand RNA virus, is generally placed into what has been termed the “California Serogroup” of the genus Orthobunyavirus, which includes, among others, California encephalitis virus, Jamestown Canyon virus, and La Crosse encephalitis virus. KEYV, based on studies conducted in the 1960s and 1970s, appears to be widespread in coastal areas of the southeastern United States, with isolates identified (in mosquito and animal samples) from the Chesapeake Bay to

Figure 1. Rash observed with 16-year-old case patient.
Texas. The organism appears to have a number of vertebrate hosts, including, as previously noted, the gray squirrel (30% seropositivity in a study in the Pokomoke Cypress Swamp in Maryland [2]), raccoons (18% seropositive in the same study), and whitetailed deer (10% seropositive in the Maryland study; 2% seropositive in a study in Texas [4]); it appears to be rare in bird or reptile populations. The virus can be transmitted transovarially in mosquitoes, with *A. atlanticus* appearing to be a primary vector (although KEYV has been identified in other *Aedes* and *Culex* species [3]). The picture which emerges is that of a virus endemic to mosquitoes and woodland vertebrates, albeit of uncertain clinical significance in animal populations.

There is, then, the question of the relevance of KEYV to humans. Seroprevalence studies conducted 50 years ago using viral neutralization assays suggested that seropositivity in healthy human populations was in the range of 20% [7]. Our data demonstrated that human infections can (and still do) occur, with viable virus detectable in urine. While we cannot say definitely that the virus was responsible for the rash and reported fever, our data are clearly suggestive, and raise the possibility that a proportion of what are otherwise unremarkable rash and fever cases seen in primary care settings in coastal areas of the southeastern United States actually reflect KEYV infections. Many of the viruses in the California group have been linked with encephalitis and, as demonstrated in this report, the virus grows well in neuroblastoma cell lines; in viral encephalitis cases in which no etiology is determined, it may also be worthwhile to look for KEYV infections. In the current case, in the setting of concerns about a ZIKV infection, a comprehensive virologic evaluation resulted in isolation of the virus; it is highly unlikely that, outside of a similar research setting, it would be ever have been identified. Our findings underscore the diversity of potential arboviral pathogens in this region, and suggest that a comprehensive analysis of possible viral pathogens should include diagnostics for KEYV.

**Supplementary Data**

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

**Notes**

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**References**


