Yellow Fever Virus RNA in Urine and Semen of Convalescent Patient, Brazil

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Yellow fever virus RNA is usually detected in blood of infected humans. We detected virus RNA in urine and semen samples from a convalescent patient. A complete virus genome was sequenced for an isolate from a urine sample. This virus had a South American I genotype and unique synapomorphic changes.

Yellow fever virus (YFV) is a member of the genus Flavivirus and causes yellow fever in humans, characterized by fever, prostration, and hepatic, renal, and myocardial complications that lead to death in 20%–50% of cases (1). Clinical confirmation of YFV infections is based on detection of virus RNA in blood by reverse transcription PCR or antigen-based ELISAs. Detection of virus in urine samples has been used for confirming infections with flaviviruses, including West Nile virus (2), Zika virus (3), dengue virus (4), and YFV (5).

Despite availability of an effective vaccine, >200,000 cases of yellow fever and >30,000 deaths occur per year (6). A large epidemic of yellow fever with high death rates recently occurred in Brazil. In December 2016, the first cases of yellow fever during this epidemic were reported in Minas Gerais; cases were later identified in Espírito Santo, Goiás, Mato Grosso, Pará, Rio de Janeiro, São Paulo, Tocantins, and the Federal District. There were 792 confirmed cases and 274 deaths (case-fatality rate 35%) as of July 10, 2017 (7). We report a case of yellow fever in a 65-year-old man who was a native of São Paulo and had not been vaccinated against yellow fever. The study protocol was approved by the Ethics Committee on Research with Human Beings at the University of São Paulo. The patient provided informed consent for use of the samples during the study.

The patient had traveled to Januária, Minas Gerais, Brazil, on December 28, 2016, and to a rural area north of São Paulo on January 3, 2017. On January 6, he had fever, chills, body pain, and nausea. During days 1–3 after symptom onset, more severe symptoms developed: persistent fever (temperature 39.5°C–40°C), headache, body pain, prostration, vomiting, dizziness, anorexia, dark stools, dark yellow urine, and bitterness in the mouth.

The patient was admitted to a public hospital in Januária on January 9. An ELISA for nonstructural protein 1 (NS1) of dengue virus showed a negative result. The patient also had severe thrombocytopenia (platelet count 77,000/mm³ [reference range 140,000–450,000/mm³]).

On January 13, the patient returned to São Paulo and was admitted to a public hospital. Another ELISA for dengue virus NS1 was performed and showed a negative result. His platelet count decreased to 57,000/mm³. On January 16, the patient was admitted to a reference hospital for infectious diseases in São Paulo. He showed a moderate clinical presentation: anicteric form and mild spontaneous hemorrhage (ecchymosis in the right eye). High fever, gastrointestinal symptoms (vomiting and diarrhea), weakness, adynamia, and generalized myalgia were also observed. The patient had a weight loss of 4 kg over 8 days. Serum and urine samples were obtained (Figure, panel A; online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/1/17-1310-Techapp1.pdf).

We extracted virus RNA by using the NucliSens EasyMag Kit (bioMérieux, Marcy l’Étoile, France). We tested samples for YFV by using a real-time quantitative reverse transcription PCR (qRT-PCR) and primers specific for YFV (8) and a conventional PCR and pan flavivirus primers (9). Serum samples showed negative results for both PCRs. However, a urine sample obtained 10 days after initial symptoms was positive for YFV RNA (cycle threshold [C] 17.42, 9.3 × 10⁴ RNA copies/mL) by qRT-PCR. We also performed a qualitative IgM-capture ELISA with a specific virus antigen and obtained positive results (optical density 1.19) (10).

On January 27, we obtained serum, urine, and semen samples and tested them by using qRT-PCR. Urine (C, 28.57, 3.3 × 10⁷ RNA copies/mL) and semen (C, 31.00, 5 × 10⁷ RNA copies/mL) samples were positive for YFV RNA. To evaluate infectivity, we tested a urine sample obtained on this date (online Technical Appendix). We isolated YFV in cell culture, which confirmed virus integrity. We also confirmed infectivity after a second virus passage (C, 24.35, 6.7 × 10⁴ RNA copies/mL).
For the urine sample that was positive for YFV RNA by qRT-PCR, we directly characterized viral diversity by using next-generation sequencing (online Technical Appendix). In South America, phylogenetic studies have inferred 2 circulating YFV genotypes. The isolate from our patient (BRMG-2017) clustered with South America I isolates, including 2 viruses isolated in 2017 in Espirito Santo, a state bordering Minas Gerais, and other viruses isolated previously in Brazil (Figure, panel B).

We did not observe any insertions or deletions in BRMG-2017 nucleotide sequences when compared with sequences of other South America I strains. However, several synapomorphic changes were detected (V108I [capsid], E1572D [NS3], R1605K [NS3], K2608R [NS5], V2645I [NS5], G2680S [NS5], N2804S [NS5], and N3216S [NS5]). Most of these changes were located in the NS5 (RNA-dependent RNA polymerase) gene, which plays a major role in virus replication. Changes in the NS5 gene have been associated with differences in viral replication, immune response, and protein–protein interactions during virus replication.

Our results suggest that semen can be a useful clinical material for diagnosis of yellow fever and indicate the need for testing urine and semen samples from patients...
with advanced disease. Such testing could improve diagnostics, reduce false-negative results, and strengthen the reliability of epidemiologic data during ongoing and future outbreaks.

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Molecular Characterization of Autochthonous Chikungunya Cluster in Latium Region, Italy
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Technical Appendix

Clinical Findings

The case-patient showed a moderate clinical presentation of yellow fever. He had fever, headache, body pain, anorexia, dizziness, bitterness in the mouth, and dark yellow urine. The patient was no longer able to eat solid food during a 3-day period. Initial laboratory test results showed a platelet count 55,000/mm$^3$; basophil count 100 cells/mm$^3$ (reference range 0.0–0.2 cells/mm$^3$); alanine aminotransferase 343 U/L (reference range 0–55 U/L); aspartate aminotransferase 156 U/L (reference range 5–34 U/L); total bilirubin 1.11 mg/dL (reference range 0.2–1.2 mg/dL); creatine phosphokinase 157 U/L (reference value ≤170 U/L), creatinine 1 mg/dL (reference range 15–55 mg/dL); prothrombin time 13.4 s (reference range 10.7–15.0 s); urea 37 mg/dL (reference range 0.72–1.25 mg/dL); prothrombin activity 101% (reference value >70%); and C-reactive protein 0.90 mg/L (reference range 0.00–5.00 mg/dL).

Liver ultrasonography showed a moderate degree of fatty liver infiltration. Doppler imaging showed normal portal vein flow. On day 11 post-onset of symptoms (dps 11) he started eating solid food again and his urine showed a standard color. Normalization of his platelet count was observe on dps 12, and aminotransferase levels within reference ranges were observed on dps 39. During dps 13–dps 76, the patient reported having episodes of fever, dizziness, headaches, and bitterness in the mouth, especially during physical excursion. He also reported a loss of taste.

Next-Generation Sequencing

To obtain the complete genome sequence, virus RNA was reextracted from the urine sample by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA), purified with DNase I, and concentrated by using the RNA Clean and Concentrator-5 Kit (Zymo Research; Irvine, CA, USA). Paired-end RNA libraries were constructed and validated by using the TruSeq Stranded Total RNA HT Sample Prep Kit (Illumina; San Diego, CA). Sequencing was
performed on an Illumina NextSeq platform at Core Facility for Scientific Research at the University of São Paulo. Low-quality and unpaired reads/bases were filtered by using Trimmomatic version 0.36 (1). Paired-end reads (Qphred>33) were aligned to the reference sequence (Genbank accession no. KY885001) by using Bowtie2 (2). Using Samtools version 0.1.18 (http://samtools.sourceforge.net/), we extracted a consensus sequence of 10,948 nt and a 112.6× average depth and obtained a 99.5% breadth of coverage of the reference genome, with bases lacking only at the extremes of the untranslated region.

To infer the phylogenetic relationships of the yellow fever virus (YFV) sequence (GenBank accession no. MF465805) with other YFV sequences, we downloaded all available complete genome sequences from GenBank. We excluded all vaccine-derived strains except for the 17D viscerotropic strain isolated in 1975 (3). Sequences were aligned by using Clustal-Omega version 1.2.1 (http://www.clustal.org/omega/) and manually curated by using JalView version 1.18-β8 (http://www.jalview.org/download). A maximum-likelihood tree was estimated in the nucleotide substitution general time-reversible with gamma-distributed rate variation and invariant sites model. Support for the tree was accessed after 10,000 nonparametric bootstrap replicates with FastTree version 2.1.8 (https://bioweb.pasteur.fr/packages/pack@FastTree@2.1.8).

**Virus isolation in Cell Culture**

We attempted to isolate YFV from the positive urine sample on day 21 after onset of patient symptoms to confirm the presence of infectious virus. The sample was initially treated with 100× Antibiotic-Antimycotic (Gibco, Gaithersburg, MD, USA) for 1 h on ice. For virus isolation, a Vero-E6 cell culture was maintained in 25-cm² flakes by using high-glycoside Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gibco), 1% nonessential amino acids (Gibco), and 1% sodium pyruvate (Gibco) and maintained at 37°C in an atmosphere of 5% CO₂. After reaching ≈90% confluence in the monolayer, we prepared a 1:3 mixture with pure Dulbecco’s modified Eagle’s medium (pH 8.2), inoculated the mixture into Vero-E6 cells, and incubated the mixture for 1 h with gentle shaking every 10 min to enable homogeneous adsorption of viruses. At the end of the adsorption period, 5 mL of the culture medium (pH 7.2), plus 2% fetal bovine serum, 1% nonessential amino acids, and 1% sodium pyruvate were added. The sample was passed 2 times in cell culture and after 7 days of
incubation, cells were collected and tested for YFV by using a real-time quantitative reverse transcription PCR and a standard PCR.

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