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BRIEF REPORT

Shedding of Yellow Fever Virus From an Imported Case in the Netherlands After Travel to Brazil

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We report yellow fever infection in a Dutch traveler returning from Brazil. Yellow fever virus (YFV) was identified in serum and urine samples over a period of 1 month. Yellow fever virus genome sequences from the patient clustered with recent Brazilian YFV and showed with limited nucleotide changes during the resolving infection.

Keywords. genomics; vaccination; yellow fever virus.

Yellow fever (YF) is a severe mosquito-borne infection in tropical countries caused by infection with the YF virus (YFV), a positive-sense RNA virus in the *Flaviviridae* family. The disease causes 29 000–60 000 deaths annually in Africa and Central and South America [1]. Despite the availability of an effective YF vaccine since 1939 [2], vaccine uptake is variable and vaccine supply in some regions is insufficient [3]. This, combined with incomplete vector control and human mobility, has resulted in recent outbreaks in Brazil [4] and several areas in Africa (Angola, Nigeria, South Sudan, Democratic Republic of Congo) [5]. In addition to the local consequences, increased infections can also lead to YF in returning travelers, and this is important to monitor [6–9]. In this study, we present a case of YF in an unvaccinated Dutch traveler returning from Brazil in early 2018 and describe YFV genomic sequences obtained directly from multiple longitudinal clinical samples. (The YFV genomic sequences described here have been deposited in GenBank, accession numbers MK760660–MK760666.) To obtain insight into the kinetics of shedding of arboviral infections and to optimize diagnostic algorithms, serial samples were collected until the patient cleared the infection, allowing assessment of intrapatient variability in the virus as the infection was cleared. The patient consented to this study.

PATIENTS AND METHODS

A previously healthy 46-year-old traveler returned to the Netherlands on January 8, 2018 after a 3-week stay in Mairiporã, Brazil (Table 1). The patient began to show symptoms on January 7, 2018 and presented at the hospital on January 11 (day 5 of symptoms) with fever (38°C), myalgia, headache, nausea, vomiting, and lower back pain. Laboratory testing revealed increased transaminases with aspartate transaminase 2298 U/L (standard reference [SR] <35 U/L), alanine aminotransferase 3147 U/L (SR <45 U/L), gamma-glutamyl transferase 86 U/L (SR <55 U/L), and an increased lactate dehydrogenase (LDH) 1545 (SR <248 U/L) with a mild thrombocytopenia of 146 × 10^9/L (SR 150–370/L). Leukocytes were normal and C-reactive protein was not increased. The patient had never received a YFV vaccination.

Given the clinical presentation and the concurrent outbreak of YF in Brazil during the patient's traveling period, YF was suspected and the presence of YFV was confirmed by real-time reverse-transcription polymerase chain reaction (PCR) on blood and urine samples. Tests for other possible agents or diseases (human immunodeficiency virus, viral hepatitis viruses, dengue virus, chikungunya virus, Zika virus, leptospirosis, malaria, and typhus) were negative. Furthermore, YFV was successfully isolated, and a full genome sequence was obtained from Vero cell culture established from a urine sample (see below). The liver enzymes and LDH levels returned to the normal range, and the patient recovered and was discharged on January 15, 2018. Of interest, YFV nucleic acid was detected in blood, urine, and semen samples as described in Table 1, including full-genome sequences from multiple blood and urine samples. The declining YFV levels in the blood and urine, as well as the serum anti-YFV immunoglobulin (Ig)G and IgM values, were consistent with a resolving YFV infection (Table 1). Semen samples showed very low levels of YFV (high threshold concentration values of 33.5 and 34.8), and all attempts to sequence or culture YFV from semen failed. However, a full YFV genome was obtained from a urine sample 4 days after release of the patient from the hospital and approximately 2 weeks after symptom onset.

Due to the low levels of YFV in the later samples, sequencing primers to increase sensitivity were used to generate overlapping

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Table 1. Travel History, Clinical and Laboratory Sample Summary

Date	19 Dec 17	7 Jan 18	8 Jan 18	11 Jan 18	12 Jan 18	13 Jan 18	14 Jan 18	15 Jan 18	19 Jan 18	30 Jan 18	6 Feb 18	19 Feb 18	20 Feb 18
Patient timeline	Travel to Mairiporã, Brazil	Start of YF symptoms	Return to Holland	Day 5 of symptoms Presentation at Havenpolikliniek	Diagnostics	Diagnostics	Diagnostics	Day 9 of symptoms Patient released	Diagnostics	Diagnostics	Diagnostics	Diagnostics	Diagnostics
Whole blood PCR Ct				26.9	30.5			31.6	31.5	32	35.9		34.1
Virus quantitation				4.89E + 05	3.41E + 04			2.25E + 04	6.50E + 03	5.52E + 03	5.94E + 03		
EDTA blood PCR Ct				24.5	27.6			34.6	32.7	34	33.5		Negative
Serum NGS				NGS full genome									
Urine Ct				19[a]	18.9[b]	14.9	15		26.8	33.4	34.7		36.6
Urine NGS				NGS full genome	NGS full genome	NGS full genome	NGS full genome		NGS full genome	NGS partial genome	NGS no genome		
Semen Ct											33.5	34.8	
Semen NGS											NGS no genome		
Serum IgM[c]					<10/negative			400	1000	100	100		200
Serum Ig[c]					<100/negative			1000	8000	16 000	16 000		16 000

Abbreviations: Ct, threshold concentration; EDTA, ethylenediaminetetraacetic acid; Ig, immunoglobulin; NGS, next-generation sequencing; PCR, polymerase chain reaction; YF, yellow fever.

[a]Culture negative.

[b]Culture positive, NGS full genome.

[c]Dilution factor last positive sample.

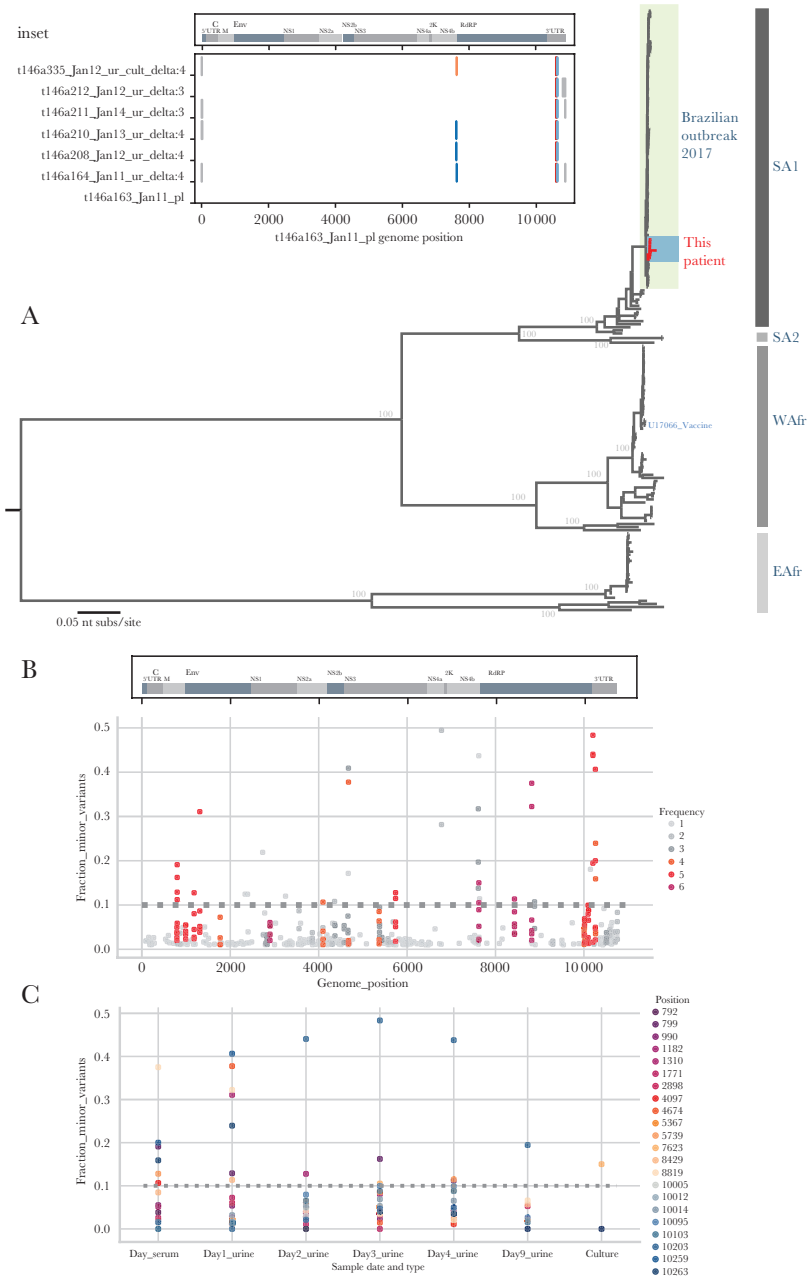

Figure 1. Genomic analyses of the reported yellow fever virus (YFV) genomes. (A) Maximum-likelihood phylogenetic tree. Yellow fever virus genomes from the case were aligned with complete YFV sequences available from GenBank, manually checked, and trimmed to complete open reading frame. A maximum-likelihood phylogenetic tree was constructed using the sequence alignment in RAxML [14], with 100 pseudoreplicates, under the GTR + Γ_4 model of evolution, which was determined as the best-fitted model using IQ-TREE [15] under the Akaike Information Criterion. The resultant tree was visualized in FigTree v1.4.3 [16]. The phylogenetic tree was mid-point rooted for clarity, and only bootstrap values for major clades were shown. The scale bar is given in units of number of nucleotide (nt) substitutions per site (subs/site). The Brazilian outbreak 2017 clade (light green box), YFV genomes from the case (blue box covering the red lineage), the YFV vaccine strain (blue node). Genotypes of YFV clades are indicated as follows: SA1 = South America I genotype, SA2 = South American II genotype, Wafr = West Africa genotype, and Eafr = East Africa genotype. (A_inset) Nucleotide differences across the reported YFV genomes. All assembled YFV genomes from this case were aligned and compared against the earliest genome obtained (t146a163; January 11, 2018; serum sample). Nucleotide differences were indicated by vertical lines, and gaps in the sequence were indicated by gray bars. Each row represents a YFV genome from the patient, and the panel above shows the positions of YFV coding regions. (B) Minor variant analysis. Positions in the YFV genome with minor (less than majority) variants in the short-read sequencing data. Quality-controlled and adapter and primer-trimmed data were mapped to the consensus genome. The number of reads with nonmajority nucleotides at each position were determined using BWA mapping followed by visualization of minor variant counts. Only positions with at least 30-fold read coverage and Phred values >30 were reported. The upper insert shows the positions of the YFV protein coding regions. The main panel indicated positions with minor variant content. Each marker represents a sample/genome, and the markers are colored by the number of the 7 samples that showed variation at that site, with orange, red, and dark red indicating 4, 5, or 6 of the 7 samples showing minor variants at that site. The dark red markers at 7623 indicate a site where 6 of the 7 samples showed variation, and the heights of the markers indicate the level of each sample's minor variant content. (C) Decline in minor variant content over the course of the infection. The minor variant content was presented by each sample organized by day of infection (except for the cell culture sample). Each marker (color-coded by position) represents the minor variant content at that position. In both B and C, fraction 0.1 (10% of the reads at that position, the significance cutoff) is marked with a gray dotted line.

1200–1500 nucleotide amplicons spanning the YFV genome. Yellow fever virus genome primers were designed from all publicly available YFV genome sequences (N = 72 genomes, March 2017), all potential primer targets in the sequences were identified (calculated melting temperature of 48°C, GC content ≤40% absence of homopolymers), and highly conserved targets were selected at the appropriate positions in the genome. Primer sequences and further details are available (see https://github.com/mlcotten/Yellow_fever_virus). Next-generation sequencing was performed directly on clinical samples (blood, urine, and semen) as follows. Nucleic acid was extracted with Roche MagNa Pure extraction kit (Roche, Mannheim, Germany) and subjected to reverse transcription and PCR amplified using YFV genome primers. Polymerase chain reaction products were pooled, enzymatically sheared to 400-base pair fragments, converted to Ion Torrent libraries, and sequenced (Ion Torrent S5XL) yielding 2–4 million reads/sample. Short reads were trimmed to remove read-terminal primers and trimmed from the 3' end to a median read Phred score <25 and de novo assembled using SPAdes v.3.11.0 [10]. Yellow fever virus contigs were identified by protein homology to a *Flaviviridae* protein database. Partial but overlapping genomic contigs were further assembled, and ambiguous nucleotide positions were resolved by counting sequence motifs in the short-read data.

Six full YFV genome sequences were obtained from patient samples over an 8-day period, and 1 genome sequence was obtained from passage 3 Vero cell culture (initiated from the 12-Jan-18 urine sample). All YFV genome sequences were in the South America I (SA1) clade of YFV (Figure 1A) and were most closely related to Brazilian YFV sequences from 2017 (Figure 1B), consistent with the patient's travel history. The Yellow Fever Virus Typing Tool [11] assigned the novel genomes to SA1, supporting the phylogenetic analysis.

RESULTS AND DISCUSSION

Few changes were observed in viral genome sequences during the infection (Figure 1 A, inset). Two nucleotide changes in the NS4B coding region did not alter the NS4B protein. Several positions in the 3' terminal repeat sequences [12] showed C to T changes (Figure 1 A, inset). The cell culture passaged virus genome t146a335 showed 1 nucleotide difference from the genome derived directly from the January 12, 2018 urine sample, resulting in a lysine to glutamic acid change in the NS4B protein. We examined levels of nonconsensus minor variant sequences across the course of infection (Figure 1 B). Multiple positions in the genome showed variation in at least 4 of the samples (Figure 1 B, in orange, red, or dark red; consistent with variant persistence during the infection); however, only 7 positions showed positions with greater 10% minor variant (Figure 1 B, above the gray dotted line). The day1_urine sample showed slightly higher variant levels than the day1_serum sample (Figure 1 C). The minor variant content declined as the infection was cleared with day4_urine showing only 3 positions with minor variants above 10% and day9_urine showing only 1 site with minor variants above 10%, and no consistently fixed changes. The culture-derived virus showed the lowest level of minor variants (Figure 1 C).

CONCLUSIONS

The YFV genomes from this case clustered with recent sequences from Brazil, consistent with the patient's travel history and ruling out other geographical sources of the infection. Yellow fever virus RNA was detectable for 14 days postsymptom onset. Serial samples from the patient clearing infection indicated that variant sites were present and persisted across multiple time points, but the total number of variants and variant sites declined as the infection was resolved. No changes were observed in the encoded viral proteins in vivo, and only a single amino change was observed after 3 passages of the virus in vitro. To our knowledge, viral nucleotide variants across a resolving YFV infection has not been examined in this detail before, and we conclude that the variant nucleotide differences were unlikely to provide an advantage for the virus and disappeared late in the infection. The functional impact of the single amino acid change observed in cell culture passage virus is unknown and should be investigated. In recent studies, YFV has been detected in urine and semen samples [12, 13]. The data reported here further demonstrate the utility of urine samples as a diagnostic sample and an easily obtained, noninvasive source of readily sequenced YFV. It is notable that at all time points in which both sample types were measured, the quantity of YFV in urine was greater than in blood supporting urine as a convenient and sensitive source for YFV diagnostics. The YFV amplicon primers and method provide a useful tool for generating YFV genomic sequences. Finally, the case provides an important message for vigilance for YF by infectious disease clinicians and alert for travelers to organize YFV vaccination before traveling to regions with endemic or ongoing YF infections.

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