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Persistence and infectivity of Zika virus in semen after returning from endemic areas: Report of 5 cases

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ABSTRACT

Background: There are limited data about the persistence and infectivity of Zika virus in semen of symptomatic travelers returning from endemic areas and even less data in asymptomatic cases.
Objective: We investigated the persistence and infectivity of ZIKA virus in semen in five patients with Zika virus infection returning to Spain from endemic areas.
Study design: We evaluated the epidemiological, clinical and virological characteristic of the five patients. In semen we detected ZIKA virus by PCR, partial sequencing and cell culture. We also performed phylogenetic analysis.
Results: We detected Zika virus RNA (Asian lineage) by PCR in semen samples from day 14th to day 96th since the day of illness onset. Semen viral culture was positive for Zika virus in two patients at days of illness 30 and 69 by virus propagation. Phylogenetic analysis strongly suggested male to female sexual transmission in a couple returning from Maldives.
Conclusion: This case series confirms that Zika virus RNA can be detected in semen up to three months after infection. Viral culture of semen samples shows prolonged infectivity that can lead to sexual transmission of Zika virus.

1. Background

Zika Virus (ZIKV) is an emerging mosquito-borne *Flavivirus* that since 2015 is producing a major outbreak in South America and the Caribbean regions. Zika virus can produce multiple congenital defects. Due to this congenital Zika virus syndrome, the World Health Organization declared that the Zika outbreak was a Global Public Health Emergency from February 2016 to November 2016. The unprecedented high number of Zika cases has been probably the consequence of climate changes that facilitated the rapid spread of *Aedes* mosquito species in the affected regions as well as the spread of ZIKV in an immunological naive population.

Zika virus RNA has been infrequently detected in vaginal secretions [1,2]. However, it has been detected often in semen [3–5]. During the ongoing outbreak sexual transmission of ZIKV virus has been repeatedly reported from symptomatic men [6–8], from an asymptomatic male [9], from a female [10] and probably from a symptomatic man to his pregnant partner [11]. The relative importance for the spread of ZIKV by sexual vs vector transmission is unclear. In endemic areas where both transmission routes are possible, it is complicated to establish the relative importance of these two routes of transmission. However, in non-endemic countries such as Spain, sexual transmission of ZIKV from imported cases could potentially be the most important way of ZIKV dissemination.

2. Objectives

In this study we have determined in five imported cases the evolution of ZIKV RNA detection in semen. We investigated the duration of ZIKV infectious particles in semen since the date of onset of illness (DOI) in symptomatic patients or since the departure of the endemic country (DEC) in patients with asymptomatic infection. This report describes the epidemiologic, virological, phylogenetic and clinical characteristics of these five cases.

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3. Study design

We have evaluated the clinical characteristics of five patients with ZIKV-infection acquired in endemic areas after returning to Spain. In these five cases we have also performed a detailed virologic and phylogenetic analysis of ZIKV in different body fluids. Serology testing was performed in serum and ZIKV RNA was detected by RT-PCR (reverse-transcriptase polymerase chain reaction) assay both in semen and urine. Viral replication in cell supernatant cultured in shell-vials was confirmed by quantification-PCR and virus propagation. Partial sequencing of the genomic regions of ZIKV was performed.

3.1. RNA extraction

Viral RNA was extracted using the NucliSENS easy MAG system (bioMérieux, France) from 500 µL of urine and from 500 µL of semen diluted in 500 µL of lysis buffer; all of them were eluted by 25 µL of elution buffer. An internal PCR control was added to the lysis buffer as described by the manufacturer.

3.2. Zika virus detection by RT-PCR

The real time amplification was performed in a m2000rt Instrument (Abbott Diagnostics, USA) using the RealStar® Zika Virus RT-PCR Kit 1.0; Altona Diagnostics GmbH, Hamburg, Germany). Viral loads were calculated using a standard curve generated from 4 standards (QS1-QS4 RealStar® Zika virus RT-PCR Kit 1.0) of known copies.

3.3. Zika virus culture of semen samples

Positive semen samples for ZIKV by RT-PCR were inoculated onto Vero cell lines from: patient 1 (on DEC 68), patient 2 (on DOI 14), patient 3 (on DOI 19 and DOI 49), patient 4 (on DOI 30) and patient 5 (on DOI 69 and DOI 96). For virus isolation [12,13] we followed the procedures described for shell-vials (Vircell S.L, Granada, Spain). We inoculated 200 µL of each semen sample diluted in 60 µL of 2% fetal bovine serum (FBS) minimum essential medium (MEM). Shell-vials were then centrifuged at 700 × *g* for 45 min and incubated for 60 min at 37 °C in 5% CO_2. The inoculum was removed and replaced by 1.5 mL of culture medium (MEM with 2% FBS). The Vero cells were incubated at 37 °C for 5–7 days. We also inoculated a negative control (semen samples negative RT-PCR for ZIKV RNA from patients tested for ZIKV infection) and a positive control (semen samples positive from patients tested for ZIKV by RT-PCR) for each patient. Viral replication in cell supernatant cultured in shell-vials was confirmed by RT-PCR to detect an increasing of ZIKV RNA load between the 5th and the 7th day. For a second viability assay, frozen semen samples at −80 °C were tested for subcultures: from patient 1 (DEC 38 and DEC 68), patient 2 (DOI 14), patient 4 (DOI 30) and patient 5 (DOI 69 and DOI 96) the culture fluids were again sub-cultured in Vero cell, 500 µL of supernatant was used for virus propagation in cell culture and incubated at 37 °C for 7 days as described above. The viral load was performed with the cell culture supernatant from the second passage.

3.4. Serological assays

For serological assays we used Anti-Zika Virus ELISA (IgM) and Anti-Zika Virus ELISA (IgG) Euroimmun, Medizinische Labor diagnostika AG, Dengue ELISA IgM capture and Dengue ELISA IgG (Vircell Microbiologist, Spain).

3.5. Sanger sequencing

Zika virus cultured from semen samples of five patients and an urine sample from the partner of patient 5 were sequenced by Sanger sequencing. We used the set of primers described by Oliveira et al. [11]. The RT-PCR was performed using SuperScript® III One-Step Platinum Taq DNA polymerase Kit (Invitrogen by Life technologies), according to manufacturer's instructions. The cycle conditions were as follows: 55 °C 30 min for reverse transcription, 94 °C 2 min, then 40 cycles of 95 °C 15 s/59 °C 30 s/68° 1 min, then 68° 5 min and hold forever 4 °C. The PCR product was purified using exo-SAP (PCR Cleanup Kit, Celera, Abbott) and diluted according the band intensity of electrophoresis gel. The PCR primers used for PCR amplification and sequencing by Sanger were: LEMB_SD1_fwd (5′- GACAGTTCGAGTTTGAAGCGAA- 3′) and LEMB_SD2_rev (5′-CTAGTGGAATGGGAGGGGAGC-3′). The BigDye® Terminator v3.1 Cycle sequencing kit was performed in a 3130xl Genetic Analyzer (Applied Biosystems) using the POP6 polymer. Sequences were edited and aligned using SeqScape v2.5 (Thermo Fisher Scientific).

3.6. Phylogenetic analysis

We constructed a phylogenetic tree using the neighbor-joining method and based on partial sequences of the ZIKA virus genome. Sequencing of the genomic position 29–781 encompassing the 5′UTR (107 nucleotides), Capsid (366 bp) and premembrane/membrane (PrM) (503 bp) regions of ZIKV were performed. We aligned seven PCR amplicon sequences obtained from patients 1 to 5 (patient 4, semen sample and culture, both tested), and one from the female partner of patient 5. The generated sequences were submitted to GeneBank (Accession numbers KY765304, KY765307, KY765303, KY765308, KY765309, KY765305 and KY765306).

4. Results

Epidemiological, clinical and virological characteristics of the patients included in this study appear in Table 1.

The recorded days of follow up for each patient were listed as follows: days out of endemic area 38, 68, 98 and 128 from patient 1, days of illness (DOI) 14 and 22 from patient 2, DOI 19, 49, 100, 131 from patient 3, DOI 30 and 84 from patient 4 and days of illness 47, 69, 96 and 124 from patient 5.

Fig. 1 shows the day the patients provided their semen sample to test when they were in the hospital. The day of first negative for ZIKV detection of each patient was different depending on when he was attended by the clinician.

4.1. ZIKV detection by RT-PCR

RT-PCR for ZIKV RNA of semen samples were first positive from day 14 to day 47. RT-PCR ZIKV in semen samples were negative on days 84, 98, 100, 124, 128 and 131, except for the patient 2 who was lost to follow up. Evolution of ZIKV detection in semen appears in Fig. 1, according to days after illness onset (between day of first positive ZIKV detection and day of last negative ZIKV detection by RT-PCR). The female partners of male patients 1, 4 and 5 were symptomatically infected for ZIKV. The female partner of patient 1 had positive serology for ZIKV, but we did not perform ZIKV RT-PCR in her serum. Detection of ZIKV RNA in serum and urine was analyzed from female partners of patient 4 and 5. Blood sample tested from female partner of patient 5 was negative. Only one blood sample was positive from pregnant-woman partner of patient 4. She maintained prolonged viruria for three months. Her newborn was born healthy.

4.2. ZIKV culture of semen samples

Zika viral replication in cell supernatant cultured in shell-vials was confirmed by the increasing of the viral load by RT-PCR on day of illness (DOI) 19 (patient 3, Ct 22.34 and 22) and day of illness (DOI) 69 (patient 5, Ct 31.9 and 31.8). The ZIKV propagation was confirmed in patient 4 (1.8×10^5 copies per mL in first passage and 2.9×10^4

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