

# Persistence of ZIKV-RNA in the cellular fraction of semen is accompanied by a surrogate-marker of viral replication. Diagnostic implications for sexual transmission

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## SUMMARY

As asymptomatic infections represent 80% of ZIKV-infected individuals, sexual transmission is a rising concern. Recent studies highlighted a preferential association of ZIKV with the cellular fraction (CF) of different specimen types. Our aim was to evaluate the presence of ZIKV-RNA in different body fluids, focusing on semen specimens to assess the ZIKV-RNA content in either the unfractionated sample, its CF or seminal plasma (SP). In addition, to establish if the presence of ZIKV genome was associated with active virus replication, we measured the levels of negative-strand ZIKV-RNA. ZIKV total-RNA was detected in blood, urine and unfractionated semen, and neg-RNA in semen CF and SP samples longitudinally collected from two ZIKV-positive men followed at the National Institute for Infectious Diseases "L. Spallanzani", Italy. In both patients, ZIKV total-RNA was detected in CF with ct values always lower than in the corresponding unfractionated samples, and was observed even in the CF from negative unfractionated semen samples. In Patient 2, neg-RNA was also detected in CF, suggesting ongoing viral replication. Our results demonstrate higher clinical sensitivity of CF as compared to whole semen testing, emphasizing the need to extend ZIKV-RNA testing to CF, to rule out virus presence and the possible risk of sexual transmission.

Received June 21, 2017

Accepted September 25, 2017

## INTRODUCTION

In 2015, the largest Zika virus (ZIKV) outbreak began in Brazil, spreading throughout the Americas and infecting nearly two million people. As the epidemic continued to expand, an increasing number of cases were imported to USA and Europe (CDC, 2017; ECDC, 2016), including Italy, with patients observed at the National Institute for Infectious Diseases "L. Spallanzani" (INMI), Rome, Italy. Although, ZIKV-associated illness was described as a self-limiting mild illness (Lanciotti *et al.*, 2008), it has generated a major challenge for the medical and scientific communities due to its severe clinical complications (such as neurological implications and adverse fetal outcomes) and sexual transmission (Petersen *et al.*, 2016). Moreover, in humans, persistent shedding of infectious ZIKV in immune-privileged sites (e.g. testes) has been reported

at times far beyond the acute viremic and symptomatic phases of infection (Uraki *et al.*, 2017). The finding that asymptomatic patients and women can transmit the disease sexually, even after a relatively long delay from symptoms onset, is very alarming (Moreira *et al.*, 2017), as asymptomatic infections represent 80% of ZIKV-infected individuals (Petersen *et al.*, 2016). To date, the longest reported duration of ZIKV-RNA shedding in semen is 188 days after symptoms onset (DSO), with viable ZIKV isolation up to 69 DSO (Nicastrì *et al.*, 2016). The implication of such a long persistence in ZIKV transmission is not fully acknowledged and, at the moment, the magnitude of person-to-person ZIKV transmission is underestimated and the role of sexual transmission is not yet clear. A major limitation for a better understanding of the extension of sexual transmission of ZIKV is the lack of diagnostic methods that could unequivocally identify the virus in semen samples. A recent study highlighted the differences in frequency with which ZIKV-RNA can be detected in various body fluids, with findings that may have implications for diagnostic recommendations (Paz-Bailey *et al.*, 2017). In addition, longer persistence of ZIKV-RNA in whole blood as compared to plasma in ZIKV-infected non-human primates has been reported in the rhesus macaque model, indicating a preferential association with the cellular com-

### Key words:

Zika virus, Semen, Replication markers, Cell-associated ZIKV-RNA, Sexual transmission.

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partment of blood (Coffey *et al.*, 2017). This preferential association with the cellular fraction has been observed for blood and other specimen types also in human infections, suggesting, on the whole, the increased sensitivity of cell-associated ZIKV-RNA detection (Murray *et al.*, 2017). This study evaluated ZIKV-RNA in semen specimens longitudinally collected from two ZIKV-infected patients observed at INMI. In particular, we analyzed the diagnostic value of ZIKV-RNA testing in different semen compartments, by comparing the ZIKV-RNA content in unfractionated semen, its cellular fraction (CF) and seminal plasma (SP). In parallel, the levels of negative-strand replicative ZIKV-RNA (neg-RNA) were measured to establish if the ZIKV genome was associated with active virus replication.

## MATERIALS AND METHODS

### *In vitro experiments*

Vero E6 cells (ATCC® Number CRL-1586™) were maintained in Modified Eagle Medium (MEM) supplemented with 10% heat inactivated Fetal Calf Serum (FCS) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Vero E6 cells were exposed to ZIKV MR766 strain for 1 hour at 37°C at a multiplicity of infection (MOI) of 1. At the end of the adsorption period, cells were washed and incubated at 37°C. At 0, 6, 24, 48, 72 and 96 hours post infection (hpi), cells and supernatants were harvested and assayed for ZIKV total and neg-RNA content.

### *Follow-up evaluation*

Two Caucasian men (Patient 1 and 2) aged 38 and 25, respectively, were enrolled in this study. Patient 1 developed fever and pruritic maculopapular rash associated with retrobulbar pain after returning to Italy from a two-week stay in Mexico. Patient 2 developed fever, maculopapular rash and cough, during the last few days of his two-month stay in Costa Rica. Patients did not show signs of prostatitis and did not report haematospermia. After returning to Italy, laboratory analyses for ZIKV diagnosis were performed at days 4 and 12 after symptoms onset in Patients 1 and 2, respectively. At clinical presentation, the diagnosis was based on the detection of ZIKV-RNA in plasma and/or urine and specific anti-ZIKV IgG and IgM testing. Dengue and Chikungunya infections were ruled out, while testing for ZIKV resulted positive. Both patients were periodically sampled for more than 70 DSO. Plasma, whole blood, urine and unfractionated semen samples were evaluated for ZIKV total-RNA, as described below.

### *Viral RNA amplification*

Total-RNA was extracted from Vero E6 cells and CF using Trizol (Life Technologies, NY, USA), and from Vero E6 supernatants, blood, urine, SP and unfractionated semen using QIAamp® Viral RNA Mini Kit (Qiagen), according to the manufacturer's instructions. ZIKV total-RNA was amplified by real-time quantitative RT-PCR (qRT-PCR) in a Rotor-GeneQ Real-Time cycler (Qiagen, Hilden, Germany). The SuperScript® III One-Step RT-PCR System kit (Invitrogen, Karlsruhe, Germany) was used with a 25 µl reaction mixture under the following conditions: 0.5 µl of kit enzyme mixture, 12.5 µl of 2X Reaction Mix, 0.5 µl of 25 µM primer mix, 0.5 µl of 20 µM of probe, 4.5 µl of nuclease free water (Mol Biograde, Hamburg, Germany) and 5 µl of the extracted sample. The following thermal profile was used: a cycle of reverse transcription of 30 min at 50°C,

2 min at 95°C for reverse transcriptase inactivation and DNA polymerase activation followed by 45 amplification cycles of 15 sec at 95°C and 1 min 60°C. Primers and probe sequences are described elsewhere (Faye *et al.*, 2013). To measure neg-RNA, the reverse transcription step was minus strand-specific, based on the use of the forward primer only, as described for the detection of antigenomic RNA of EBOV (Biava *et al.*, 2017). cDNAs were treated with 1µL of RNase H (20U/ µL) for 20' at 37°C, to remove remaining viral RNAs, and cDNA was amplified with SuperScript® III One-Step RT-PCR System kit, with a modified thermal profile, which omitted the reverse transcription step. To measure ZIKV total and neg-RNA, unfractionated semen samples were centrifuged at 800 rcf for 10 minutes to obtain CF and SP. Standards, kindly provided within the EVAg (Grant n. 653316) consortium, have been used to perform the standard curve and determine the concentration of the virus. In our experimental conditions, the limit of quantification (LOQ) was 10<sup>3</sup> cp/mL, and the assay was linear up to 10<sup>9</sup> cp/mL.

### *Ethics statement*

The Institutional Ethic Board (IEB) of the National Institute for Infectious Diseases "L. Spallanzani" approved the use of residual clinical samples for research purposes. The patients signed a written informed consent form.

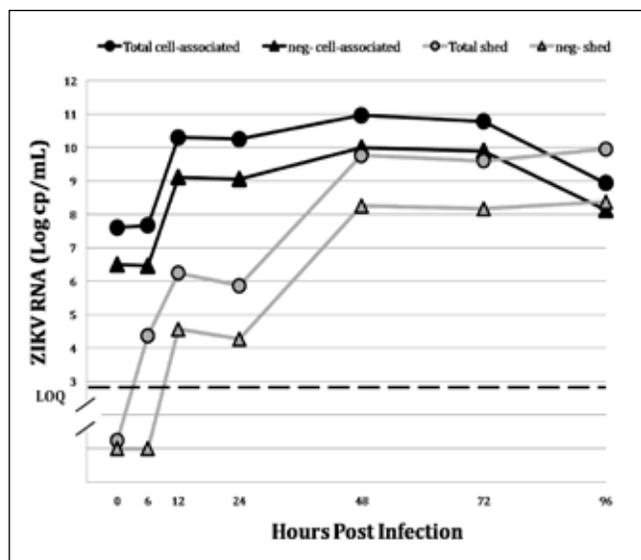
## RESULTS

### *In vitro experiments*

The value of neg-RNA levels as a surrogate marker of ongoing ZIKV replication was preliminarily established in Vero E6 cells infected in vitro. As shown in *Figure 1*, cell-associated ZIKV total-RNA and neg-RNA started to increase after 6 hpi and peaked at 48 hpi, whereas ZIKV total-RNA and neg-RNA were below the limit of quantification (LOQ) in supernatants at 0 and 6 hpi, increased at 24 hpi and peaked at 48 hpi. The neg-RNA was mostly cell-associated until 24 hpi, and the cells/supernatants ratio reversed after 72 hpi when cytopathic effect was maximal (*Figure 1*).

### *Clinical specimens*

Semen samples and their fractions (CF and SP) were analyzed for both ZIKV total-RNA and neg-RNA. The results for Patient 1 are shown in *Table 1-A*: ZIKV total-RNA in unfractionated semen samples at 51 DSO was detectable with a Ct value of 39.4, below the LOQ, and was undetectable at 94 and 147 DSO; in SP ZIKV total-RNA was undetectable. ZIKV total-RNA in CF was detected, with a Ct value of 34.97 (<LOQ), 29.81 (4 Log cp/mL) and 38.5 (<LOQ), at 51, 94 and 147 DSO, respectively; neg-RNA was never detected in all fractions of semen from this patient. The results for Patient 2 are shown in *Table 1-B*: ZIKV total-RNA in unfractionated semen samples had Ct values of 32.74 (2.2 Log cp/mL) and 20.95 (5.5 Log cp/mL) at 16 and 30 DSO, respectively, and was undetectable at 73 DSO. ZIKV total-RNA in SP had a Ct value of 25.53 (4.2 Log cp/mL) at 16 DSO and was undetectable at 30 and 73 DSO; neg-RNA was undetectable in SP throughout all DSO. ZIKV total-RNA in CF was always detectable with Ct values of 26.51 (5 Log cp/mL), 14.94 (8.3 Log cp/mL) and 33.72 (2.9 Log cp/mL), at 16, 30 and 73 DSO, respectively. Neg-RNA in CF, undetectable at 16 DSO, was detected at high titer (5.89 Log cp/mL) at 30 DSO, and was still detected, although with high Ct value (38.73, <LOQ), at 73 DSO.



**Figure 1** - Distribution of ZIKV RNA classes in infected cells: Vero E6 cells were exposed to ZIKV MR766 for 1 hour at 37°C at a MOI of 1; at 0, 6, 24, 48, 72 and 96 hpi, cell pellets and supernatants were collected and the levels of ZIKV total and negative strand RNA, either cell-associated or shed in the culture supernatant, were measured.

Overall, the results indicate that the cell-associated ZIKV total-RNA in semen specimens is detected with higher sensitivity (5-6 Ct lower values) than in the corresponding unfractionated samples.

In Patients 1 and 2, ZIKV total-RNA was always undetectable in blood samples collected at the same DSO. In Patient 1, ZIKV total-RNA was always undetectable in urine samples, whereas in Patient 2, it had a Ct value of 28.52 (3.35 Log cp/mL) at 16 DSO and was undetectable at the other DSO.

## DISCUSSION

Zika virus is a single-stranded RNA virus mainly transmitted by Aedes mosquito. Among the modes of transmission described to date (Petersen *et al.*, 2016; Musso *et al.*, 2014), sexual transmission is the one of the major concerns due to the prolonged persistence of ZIKV in semen (Nicastri *et al.*, 2016).

This study detected ZIKV total-RNA and neg-RNA, as a marker of ongoing viral replication, in unfractionated semen samples longitudinally collected after recovery from two men followed at INMI, who resulted positive to ZIKV diagnosis.

Although different extraction methods were used to isolate nucleic acids from the CF and the corresponding unfractionated samples, preventing direct comparison of viral RNA concentration in the two sample types, a higher ZIKV RNA concentration in the CF samples was suggested by the Ct values 5-6 times lower. This is consistent with recent findings by Barzon *et al.*, who observed ZIKV-RNA in the CF extracted from positive unfractionated semen samples (Barzon *et al.*, 2016), and adds strong support to the hypothesis of the association of ZIKV-RNA with CF. This is further supported by the detection of cell-associated viral proteins by indirect immunofluorescence in the CF of Patient 2 at 30 DSO, at the time of the peak of ZIKV-RNA in semen (not shown).

More interestingly, our results add novel findings to these observations, since we could detect cell-associated total ZIKV-RNA even in negative unfractionated semen samples up to 147 DSO and 73 DSO in Patients 1 and 2, respectively.

Overall, although the higher sensitivity of CF may in part be due to the different extraction methods, these data indicate that the test performed on the CF may have higher clinical sensitivity. Hence, extending testing to the CF and/or using more efficient extraction methods may be considered to rule out ZIKV in semen.

**Table 1**

| A - Patient 1             |                |                          |                        |                          |                        |                          |                        |
|---------------------------|----------------|--------------------------|------------------------|--------------------------|------------------------|--------------------------|------------------------|
| Days since symptoms onset |                | 51                       |                        | 94                       |                        | 147                      |                        |
|                           |                | Total-RNA Log cp/mL (Ct) | Neg-RNA Log cp/mL (Ct) | Total-RNA Log cp/mL (Ct) | Neg-RNA Log cp/mL (Ct) | Total-RNA Log cp/mL (Ct) | Neg-RNA Log cp/mL (Ct) |
| Semen                     | Unfractionated | <LOQ (39.74)             | ND                     | Und                      | ND                     | Und                      | ND                     |
|                           | SP             | Und                      | Und                    | Und                      | Und                    | Und                      | Und                    |
|                           | CF             | <LOQ (34.97)             | Und                    | 4 (29.81)                | Und                    | <LOQ (38.5)              | Und                    |
| B - Patient 2             |                |                          |                        |                          |                        |                          |                        |
| Days since symptoms onset |                | 16                       |                        | 30                       |                        | 73                       |                        |
|                           |                | Total-RNA Log cp/mL (Ct) | Neg-RNA Log cp/mL (Ct) | Total-RNA Log cp/mL (Ct) | Neg-RNA Log cp/mL (Ct) | Total-RNA Log cp/mL (Ct) | Neg-RNA Log cp/mL (Ct) |
| Semen                     | Unfractionated | 2.2 (32.64)              | ND                     | 5.5 (20.95)              | ND                     | Und                      | ND                     |
|                           | SP             | 4.2 (25.53)              | Und                    | Und                      | Und                    | Und                      | Und                    |
|                           | CF             | 5 (26.51)                | Und                    | 8.3 (14.94)              | 5.89 (23.01)           | 2.9 (33.72)              | <LOQ (38.73)           |

Und: undetectable; ND: not done.

Our results also suggest that the viral genome detected in CF is the result of replicative viral activity, since, at least in Patient 2, neg-RNA was also detected. Although we were not able to isolate viable virus from these samples despite several attempts, the evidence of neg-RNA as a surrogate marker of ongoing viral replication in the CF strongly argues in favour of the presence of infectious particles, and is compatible with the transmissibility of infection through this body fluid.

As previously described for other viruses, even if viral isolation in cell culture is the gold standard, it may not be sufficiently sensitive to rule out virus viability and infectivity, especially in semen (Crozier *et al.*, 2016).

Taken together, our observations indicate that a negative qRT-PCR result in unfractionated semen samples is not always sufficient to rule out the presence of ZIKV-RNA in semen.

Although the limit of this study is the small number of patients, our findings demonstrate that a more careful diagnosis of semen samples is mandatory to rule out ZIKV in this body fluid.

Further investigations are needed to better understand the persistence of ZIKV in semen, the cell types actually replicating the virus and their role in infection transmission.

### Acknowledgements

This work was supported by grants of Italian Ministry of Health, for “Ricerca Corrente” and “Ricerca Finalizzata”, by European Seventh Framework Programme [Grant n. 278433-PREDEMICS]; European Union Horizon 2020 Programme [Grant n. 653316-EVAg].

The contribution of Laboratory of Microbiology and Infectious Disease Biorepository from National Institute for Infectious Diseases “L. Spallanzani”, IRCCS, Rome, Italy, is warmly acknowledged.

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