RECOMMENDED CITATION

Huits RMHG, De Smet B, Ariën KK, Van Esbroeck M, de Jong BC, Bottieau E et al. Kinetics of Zika virus persistence in semen. [Submitted]. Bull World Health Organ. E-pub: 06 Jul 2016. doi: http://dx.doi.org/10.2471/BLT.16.181370.

Kinetics of Zika virus persistence in semen

Ralph MHG Huits,[a] Birgit De Smet,[a] Kevin K. Ariën,[a] Marjan Van Esbroeck,[a] Bouke C. de Jong, [a] Emmanuel Bottieau[a] & Lieselotte Cnops [a]

[a]Institute for Tropical Medicine, Kronenburgstraat 43/3, B-2000 Antwerpen, Belgium

Correspondence to Lieselotte Cnops (email: lcnops@itg.be)

(Submitted: 05 July 2016 – Published online: 06 July 2016)

Abstract

Objective

Interim guidelines to prevent sexual transmission of Zika virus (ZIKV) do not recommend diagnostic testing, because the knowledge of viral kinetics in semen after acute infection is limited. We studied the presence and duration of ZIKV in semen after onset of symptoms.

Methods

A prospective observational cohort study (NCT 02733796) is ongoing to determine the presence, persistence and kinetics of ZIKV in semen of men with confirmed ZIKV infection after returning from areas with vector-borne transmission. ZIKV RT-PCR is performed in weekly semen samples until ZIKV is no longer detectable. The main endpoint is the proportion of ZIKV positive semen samples over time after acute ZIKV infection. We report preliminary findings after enrolment of the first 4 patients.

Findings

Semen of two out of four immunocompetent men with symptomatic ZIKV infection tested positive for ZIKV-RNA by RT-PCR. ZIKV levels in semen declined to undetectable after 68 and 56 days respectively, suggesting elimination of the virus.

Conclusion

Our findings indicate that presence and persistence of ZIKV in semen after acute infection appear common. Algorithms can be developed that incorporate ZIKV-specific molecular and antibody detection assays and subsequent testing for ZIKV in semen of selected men. We suggest that future guidelines to prevent sexual transmission of ZIKV, adopt diagnostic testing of both symptomatic and asymptomatic individuals returning from endemic areas.

Introduction

The World Health Organization (WHO) declared the Zika virus (ZIKV) epidemic in the Americas a Public Health Emergency of International Concern because of its association with microcephaly in newborns and Guillain-Barré syndrome[1]. ZIKV is primarily transmitted by *Aedes* mosquitoes in endemic areas, but person-to-person transmission via infected semen has been undisputedly demonstrated. To date, ten countries have reported sexual transmission of ZIKV[2]. Partners of either sex, who had not traveled and lived in areas without arthropod-borne transmission, developed Zika virus disease (ZVD) after oral, vaginal or anal sex with infected men[3–5]. Infection of the partner occurred for up to 41 days after the index infection[6]. Sexual transmission was also demonstrated in an asymptomatic couple seeking Assisted Reproduction Treatment (ART)[7]. Up to 80% of ZIKV infections may be asymptomatic[17].

ZIKV RNA has been detected in semen by reverse-transcriptase polymerase chain reaction (RT-PCR) for as long as 62 days after infection[8–10]. A case report documenting longitudinal follow-up of semen with ZIKV RNA levels declining to undetectable at day 62 post infection, was recently published[11]. ZIKV replication competence was demonstrated by isolation of the virus from semen, more than two weeks after the onset of ZVD[4].

Pending the emergence of new evidence, the WHO and the Centers for Disease Control (CDC) issued interim guidance to prevent sexual transmission of ZIKV[2,12]. For men and women returning from areas with known vector-borne ZIKV transmission, a distinction between symptomatic or asymptomatic infection is made. Current recommendations include safer sex practices or abstinence for at least 8 weeks after potential exposure and 6 months if the male partner had symptomatic ZVD. These timeframes are based on estimates of the maximum incubation period for ZIKV or related flaviviruses, and they allow for variability in individual's immune responses. Diagnostic testing for ZIKV infection or routine testing to

detect ZIKV in semen is currently not recommended[2,13] because the understanding of the duration and pattern of seminal shedding of ZIKV is limited.

To assess the incidence and kinetics of ZIKV infection of semen after acute ZVD, a prospective cohort study has started at the Institute of Tropical Medicine in Antwerp (ITM) (ClinicalTrials.gov, NCT 02733796). Here we report the preliminary findings after enrolment of the first 4 patients.

Methods

A prospective observational cohort study was designed to study the presence, persistence and kinetics of ZIKV in semen. After obtaining informed consent, men who were diagnosed with ZIKV infection at the ITM are consecutively recruited for sequential follow-up of ZIKV presence in semen. A confirmed ZIKV infection is defined as a positive RT-PCR for ZIKV in a serum or urine sample, or presence of ZIKV-specific IgG and/or IgM antibodies (Euroimmun Zika virus (ZIKV) ELISA (Lübeck, Germany)), confirmed by a ZIKV neutralization test. Inclusion criteria are age 18 years or older, and residence in an area without epidemiologically important arthropod-borne ZIKV transmission. Men with a recent (<2 years) history of urinary tract infection, or with previous urologic surgery are excluded.

Clinical and epidemiological data are recorded in a standardized Case Record Form (CRF). Baseline serum, blood and urine samples are collected as required for routine clinical evaluation; semen samples are collected for ZIKV RT-PCR weekly until tested negative.

Fresh semen samples are analyzed for sperm count, morphology, motility, leukocyte and erythrocyte counts.

Semen is collected in sterile cups and, after liquefaction for 30 to 60 minutes, transferred into sterile collection tubes. To facilitate shipment of the samples, we also validated RNA extraction from ZIKV-infected semen on filter paper (Whatman 903 Proteinsaver Card, GE

Healthcare, Cardiff, UK). We found that the sensitivity of ZIKV RNA detection in a 5 mm punch (~10µL) of semen blotted onto filter paper was lower than in the 140µL fraction of the same semen sample collected in the sterile tube, but the loss of sensitivity was acceptable for monitoring a trend of ZIKV persistence in sequential samples.

A ZIKV-specific, in-house duplex real-time RT-PCR is used, targeting a 102bp and 121bp sequence of the NS5 gene. Because a standardized viral load reference method is not available, viral RNA levels were expressed as threshold cycle values (Ct-values). Lower Ct-values correspond to higher viral RNA levels. From each available semen sample with a positive PCR result and Ct-values below 30, ZIKV isolation is attempted by inoculation in Vero cells.

Study endpoints of the prospective observational cohort study are the proportion of ZIKV RT-PCR positive semen samples over time after acute ZIKV infection and the proportion of successful ZIKV isolations from these samples. Because of the exploratory character of the primary study objective, a sample size is arbitrarily set at 20. We report preliminary findings after enrolment of the first 4 patients.

Ethical approval was obtained from the Institutional Review Board of the ITM and the Ethics Committee of the University Hospital in Antwerp.

Results

Patient 1 is a previously healthy 43-year old Belgian man who had returned from Venezuela. He presented with a 3-day history of headache, arthralgia and fever. He developed a generalized maculopapular rash that lasted for 3 days. He had no conjunctivitis and no symptoms of urinary tract infection. ZIKV-RNA was detected by RT-PCR in urine. The patient made an uneventful recovery after 7 days. Semen samples were obtained on days 10,

20, 31, 41 and 58. Semen samples on filter paper were collected on days 20, 31 and 41. Ct-values for ZIKV in semen obtained on day 10 were much lower than those found in urine on days 3 and 10. Over 68 days, ZIKV-RNA levels in semen gradually decreased to undetectable levels (Figure). Attempts to isolate ZIKV from semen were not successful. Microscopic hematospermia was observed in semen from day 31, 41, 59 and 68. Oligospermia with normal morphology was present on day 31. Sperm counts increased to normal in subsequent samples (Table).

Patient 2 is a healthy 33-year old man living in Port-au-Prince, Haiti, who consulted our clinic via Email because of painless hematospermia, 8 days after a flulike illness with fever and arthralgia. He developed an exanthema that lasted from 3 to 5 days after fever onset. Urine and semen were collected in Haiti and sent to our laboratory. ZIKV infection was confirmed by RT-PCR on urine collected on day 16. Semen samples in collection tubes were obtained on days 16, 44 and 50, and semen blotted on filter paper in Haiti on days 16, 31, 44, 50, 56 and 64. The Ct-value in semen on day 16 was lower than the Ct-value in the urine collected on the same day (Table). ZIKV-RNA levels in semen gradually declined to undetectable on day 56 (Figure). The samples collected in Haiti were not suitable for microscopic analysis, cell counts, and viral culture.

Patient 3 is a 46-year old man who had fever, exanthema and myalgia after a holiday in Martinique. He was evaluated at our clinic 3 days after onset of symptoms. Serum tested positive for ZIKV IgM; the diagnosis was confirmed by ZIKV Immunoglobulin G (IgG) seroconversion, and a ZIKV-specific neutralizing antibody titer of 1:190 after 18 days. RT-PCR did not detect ZIKV-RNA in a semen sample obtained on day 26. The sample showed a normal sperm count, 252 leucocytes and 4 erythrocytes (Table).

Patient 4 presented with fever and mild diarrhea after returning from Guadeloupe. ZVD was confirmed by RT-PCR on day 4 after symptom onset (Ct-value 31.77). No ZIKV-RNA was

detected in a semen sample obtained on day 10, but RT-PCR on urine was still positive on day 11 (Ct-value 32.88). The semen sample showed oligospermia, with 112 leucocytes and 12 erythrocytes (Table).

Discussion

We report on the first inclusions in a prospective observational cohort, designed to study the incidence and persistence of ZIKV infection in semen (NCT 02733796). Semen of two out of four consecutively included immunocompetent men with symptomatic ZIKV infection tested positive for ZIKV-RNA by RT-PCR. The other two had negative RT-PCR results in semen samples obtained on day 26 and day 10. Although we cannot exclude that infection of the male reproductive organs in patients 3 and 4 may have passed before sampling, ZIKV certainly did not persist as long as in Patients 1 and 2. Further studies are needed to determine the frequency and determinants of semen positivity in ZIKV infected men.

Sequential semen samples of patients 1 and 2 showed increasing Ct-values until ZIKV became undetectable, after 68 and 56 days respectively. Our findings confirm earlier reports[8–11]. Follow-up was too short to exclude the possibility of recurrence, but the decline of ZIKV-RNA levels in semen observed in our patients suggests elimination of the virus.

ZIKV-RNA was successfully recovered from semen blotted on filter paper by a patient at home. This technique facilitates the collection, storage and shipment of semen samples to reference laboratories.

In spite of low Ct-values, isolation of ZIKV from the semen of our patients was not successful. This could indicate degradation of the virus, although others have demonstrated ZIKV replication competence in semen up to 18 days after the onset of ZVD[3,8,10]. Therefore, we consider technical difficulties a better explanation for our failed attempt.

Because of the steady decline of ZIKV-RNA levels observed in sequential semen samples of our patients, we hypothesize that the testes are seeded during the viremic phase. Viral replication would then be contained in the immunoprivileged reservoir of the seminiferous epithelium. Interestingly, the kinetics of seminal shedding of ZIKV in our patients coincide with the duration of human spermatogenesis, which takes 69 to 80 days[14]. Host-pathogen relationships and possible lasting effects of ZIKV infection on reproductive function need clarification. An animal model is available for further study; in immunodeficient mice with experimental ZIKV infection, the highest levels of viral replication were found in the testes and brain[15].

Both patients with ZIKV-RNA positive semen had hematospermia. Patient 2 reported macroscopic hematospermia 8 days after onset of ZVD. In patient 1 it was detected by microscopy. Neither patients had symptoms of urinary tract infection. Hematospermia following ZVD has been reported[8,16]. The presence of erythrocytes, leucocytes and oligospermia with increasing sperm counts over time, suggests an inflammatory process in the male reproductive tract. Blood in semen samples of men with ZVD may be a valuable predictor for ZIKV presence of semen.

ZIKV in semen may become the major source of transmission in non-vector areas. Our preliminary results indicate that RT-PCR testing can detect the presence and persistence of ZIKV in semen.

We fear that the timeframes for adopting safer sex practices or abstinence to prevent sexual transmission of ZIKV as currently recommended by WHO and CDC [2,12], may either be too strict or too permissive. We suggest that updated guidelines adopt diagnostic testing for ZIKV infection. Given the high sensitivity of antibody detection assays for ZIKV or related flaviviruses, algorithms that incorporate serologic testing in convalescent sera can make

infection with ZIKV or related flavivirus very unlikely in case of negative results in individuals returning from endemic areas[18]. At one month after potential exposure to ZIKV, false positive rather than false negative antibody test results may complicate laboratory diagnosis. ZIKV RT-PCR could then be performed on semen of men with potential exposure who test positive for antibodies to ZIKV. Since sexual transmission of ZIKV can occur even if the index patient remains asymptomatic as was illustrated by Fréour et al.[7], both symptomatic and asymptomatic individuals returning from areas with ongoing vector-borne ZIKV transmission should be tested.

If future studies corroborate our findings, the data we present contribute to a more rational approach to reduce the risk of sexual transmission of ZIKV.

Funding

The National Reference Center of arboviruses (ITM) is partially supported by the Belgian Ministry of Social Affairs through a fund within the Health Insurance System. LC holds an innovation mandate (140779) from the Flanders Innovation & Entrepreneurship.

Competing interests

The authors declare no competing interests.

Acknowledgements

We thank both our patients, who gave informed consent for these studies and for publication of the results.

Figure: Kinetics ZIKV RNA levels in semen of patient 1 and patient 2.

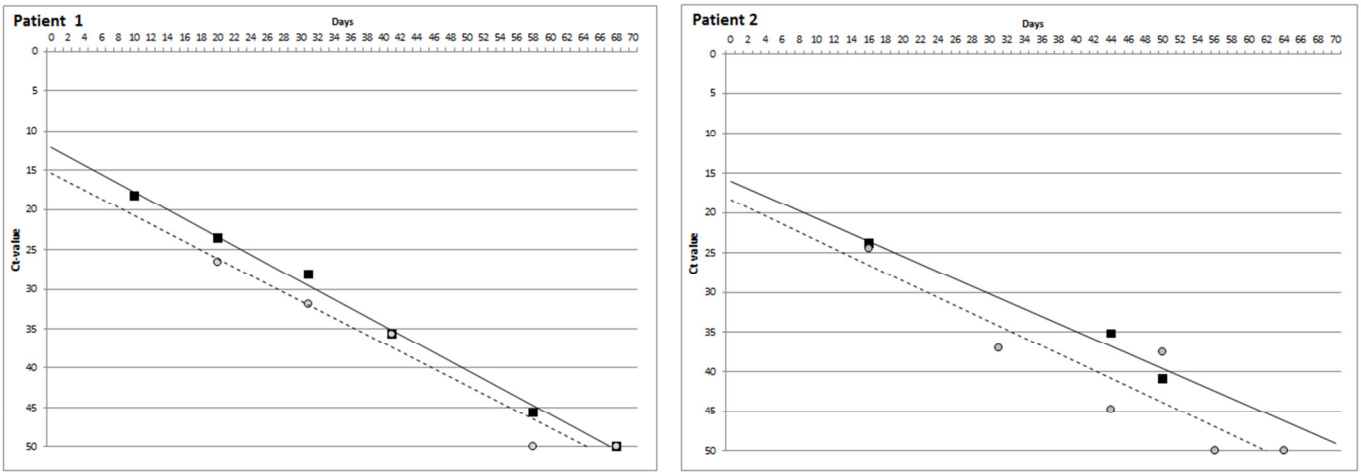

Table. Results of tests for Zika virus, by patient and day after onset of symptoms.

Patient 1

day	Ct (serum)	Ct (urine)	Ct (semen)	Ct (semen filter)	count/ml	WBC (semen)	RBC (semen)
3	negative	34,22	ND	ND	ND	ND	ND
4	negative	34,06	ND	ND	ND	ND	ND
10	ND	ND	18,22	ND	ND	ND	ND
11	ND	40,82	ND	ND	ND	ND	ND
20	ND	ND	23,56	26,55	ND	ND	ND
31	ND	ND	28,16	31,92	8.784.000	960	922
41	ND	ND	35,7	35,62	16.320.000	980	480
58	ND	ND	45,63	negative	20.368.000	900	122
68	ND	ND	negative	negative	23.664.000	640	12

Patient 2

day	Ct (saliva)	Ct (urine)	Ct (semen)	Ct (semen filter)	count/ml	WBC (semen)	RBC (semen)
ND	ND	ND	ND	ND	ND	ND	ND
ND	ND	ND	ND	ND	ND	ND	ND
ND	ND	ND	ND	ND	ND	ND	ND
16	negative	26,96	23,85	24,46	ND	ND	ND
31	negative	negative	ND	36,95	ND	ND	ND
44	ND	ND	35,15	44,71	ND	ND	ND
50	ND	ND	40,95	37,57	ND	ND	ND
56	ND	ND	ND	negative	ND	ND	ND
64	ND	ND	ND	negative	ND	ND	ND

Patient 3

day	Ct (serum)	Ct (urine)	Ct (semen)	Ct (semen filter)	count/ml	WBC (semen)	RBC (semen)
3	negative	ND	ND	ND	ND	ND	ND
18	ND	ND	ND	ND	ND	ND	ND
26	ND	negative	negative	ND	24.144.000	252	4
39	ND	negative	negative	ND	ND	ND	ND

Patient 4

day	Ct (serum)	Ct (urine)	Ct (semen)	Ct (semen filter)	count/ml	WBC (semen)	RBC (semen)
4	negative	31,77	ND	ND	ND	ND	ND
10	ND	ND	negative	ND	ND	ND	ND
11	ND	32,88	ND	ND	896.000	112	12
17	ND	negative	negative	ND	ND	ND	ND

ND: not determined, Ct: threshold cycle values, count/ml: sperm per ml semen, WBC: white blood cells, RBC: red blood cells

References

1. WHO Situation Report : Zika virus, Microcephaly, Guillain-Barré Syndrome. 2016;(16 June). http://www.who.int/emergencies/zika-virus/situation-report/16-june-2016/en/. (accessed June 29, 2016)
2. World Health Organization. Prevention of sexual transmission of Zika virus. WHO/ZIKV/MOC/16.1 Rev.2. 7 June 2016:1-4. (accessed June 7, 2016)
3. D'Ortenzio E, Matheron S, de Lamballerie X, et al. Evidence of Sexual Transmission of Zika Virus. *N Engl J Med*. 2016:1-3. doi:10.1056/NEJMc1604449.
4. Trew Deckard D, Chung WM, Brooks JT, et al. Male-to-Male Sexual Transmission of Zika Virus — Texas, January 2016. *MMWR Morb Mortal Wkly Rep*. 2016;65(14):372-374. doi:10.15585/mmwr.mm6514a3.
5. Hills SL, Russell K, Hennessey M, et al. Transmission of Zika Virus Through Sexual Contact with Travelers to Areas of Ongoing Transmission — Continental United States, 2016. *MMWR Morb Mortal Wkly Rep*. 2016;65(8):215-216. doi:10.15585/mmwr.mm6508e2.
6. Jean Marie Turmel PA, Bruno Hubert YMV, Maquart M, Guillou-Guillemette H Le, Art IL-G. Late sexual transmission of Zika virus related to persistence in the semen. *Lancet*. 2016;6736(16):30775. doi:doi.org/10.1016/S0140-6736(16)30775-9.
7. Fréour T, Mirallié S, Hubert B, Splingart C, Barrière P, Maquart M L-GI. Sexual transmission of Zika virus in an entirely asymptomatic couple returning from a Zika epidemic area, France, April 2016. *Euro Surveill*. 2016;21(23):pii=30254. http://dx.doi.org/10.2807/1560-7917.ES.2016.21.23.30254.
8. Musso D, Roche C, Robin E, Nhan T, Teissier A, Cao-Lormeau VM. Potential sexual transmission of zika virus. *Emerg Infect Dis*. 2015;21(2):359-361. doi:10.3201/eid2102.141363.
9. Atkinson B, Hearn P, Afrough B, et al. Detection of Zika Virus in Semen. *Emerg Infect Dis*. 2016;22(5):940-940. doi:10.3201/eid2205.160107.
10. Mansuy JM, Dutertre M, Mengelle C, et al. Zika virus: high infectious viral load in semen, a new sexually transmitted pathogen? *Lancet Infect Dis*. 2016;6736(16):6736. doi:10.1016/S1473-3099(16)00138-9.
11. Reusken C, Pas S, GeurtsvanKessel C, Mögling R, van Kampen J, Langerak T, Koopmans M, van der Eijk A van GE. Longitudinal follow-up of Zika virus RNA in semen of a traveller returning from Barbados to the Netherlands with Zika virus

disease, March 2016. *Euro Surveill*. 2016;21(23):pi=30251. http://dx.doi.org/10.2807/1560-7917.ES.2016.21.23.30251.

12. Oster AM, Brooks JT, Stryker JE, et al. Interim Guidelines for Prevention of Sexual Transmission of Zika Virus — United States, 2016. *MMWR Morb Mortal Wkly Rep*. 2016;65(5):120-121. doi:10.15585/mmwr.mm6505e1.
13. Oster AM, Brooks JT, Stryker JE, et al. Interim Guidelines for Prevention of Sexual Transmission of Zika Virus — United States , 2016. 2016;65.
14. Amann RP. The cycle of the seminiferous epithelium in humans: a need to revisit? *J Androl*. 2008;29(5):469-487. doi:10.2164/jandrol.107.004655.
15. Rossi SL, Tesh RB, Azar SR, et al. Characterization of a Novel Murine Model to Study Zika Virus. *Am J Trop Med Hyg*. 2016. doi:10.4269/ajtmh.16-0111.
16. Foy BD, Kobylinski KC, Foy JLC, et al. Probable Non–Vector-borne Transmission of Zika Virus, Colorado, USA. *Emerg Infect Dis*. 2011;17(5):880-882. doi:10.3201/eid1705.101939.
17. Duffy MR, Chen T-H, Hancock WT, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. *N Engl J Med*. 2009;360(24):2536-2543. doi:10.1056/NEJMoa0805715.
18. CDC. Revised diagnostic testing for Zika, chikungunya, and dengue viruses in US Public Health Laboratories. 2016.