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BRIEF REPORT

Detection of Zika Virus Replication in Human Semen by Reverse-Transcription Polymerase Chain Reaction Targeting of Antisense Ribonucleic Acid

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Background. Persistence of Zika virus (ZIKV) ribonucleic acid (RNA) in semen is common after infection.

Methods. We designed a reverse-transcription polymerase chain reaction assay that targets antisense ZIKV RNA (asRNA) to assess ZIKV replication competence in ZIKV RNA-positive semen samples.

Results. We detected ZIKV asRNA in semen of 9 of 19 men (47.4%) diagnosed with ZIKV infection. All asRNA-positive samples had high ZIKV loads (cycle threshold values <26) and were obtained within 21 days of symptom onset.

Conclusions. The sensitivity of the asRNA assay for detection of ZIKV replication was higher than that of conventional virus isolation methods (47.4% vs 21.1%, $P = .032$).

Keywords. antisense RNA; reverse-transcription polymerase chain reaction; semen; sexual transmission; Zika virus.

During the 2015–2016 Zika virus (ZIKV) outbreak in Latin America, ZIKV was identified as a cause of adverse fetal outcomes [1]. In addition to the dominant mode of transmission by *Aedes* spp mosquitoes, nonvector transmission of ZIKV via sexual intercourse became a global concern [2]. Sexual transmission is associated with shedding of ZIKV in genital fluids, particularly in semen [3].

A wide range of viruses can be detected in human semen [4]. Zika virus ribonucleic acid (RNA) is detected by reverse-transcription polymerase chain reaction (RT-PCR) in semen of approximately 60% of symptomatic men in the first month after ZIKV infection, and although viral shedding decreases gradually in many cases, viral RNA remains detectable in others for more than 6 months [5, 6]. The risk of sexual transmission by asymptomatic men is less well studied and may be underestimated [3, 7].

Routinely used diagnostic PCR methods do not distinguish between viral genome and the presence of infectious virus particles. Infectivity or transmissibility of pathogens are determined by multiple factors such as virulence, infectious dose, route of transmission and environmental conditions, and host defense mechanisms. A prerequisite for infectivity of virus is replication competence.

Under laboratory conditions, this is generally assessed by isolation of virus in cell culture or laboratory animals. This approach is costly, slow, and demands stringent biosecurity requirements, and sensitivity to prove infectivity may be low. Failure to isolate ZIKV from semen may be attributed to technological issues, such as degradation of the virus after sampling or toxicity of the inoculate to cell lines used for propagation. Therefore, the low success rate of attempts at isolating ZIKV from semen (3 of 78 samples with detectable ZIKV RNA) in the largest series to date may underestimate the presence of infectious virus [6].

Zika virus belongs to the genus *Flavivirus*, family *Flaviviridae*. The members of this genus encapsulate a positive-sense RNA genome that encodes a single polyprotein in a single open reading frame. After endocytosis of virus particles by the host cell, replication begins with the synthesis of full-length, uncapped, negative-sense RNA (Figure 1A). This antisense RNA (asRNA), detectable within hours after infection, is used as a template for the synthesis of multiple positive-sense RNA strands. The accumulation of positive-sense RNA exceeds the quantity of asRNA by approximately 10-fold. For ZIKV, Biava et al [8] found asRNA in the cellular fraction of semen (but not in seminal plasma) in semen of a single patient, at 2.5 log copies/mL or lower than total RNA.

We designed an RT-PCR assay that targets asRNA to demonstrate replication of ZIKV, based on methods previously described for Ebola [9], chikungunya [10], dengue [11], o'nyong-nyong [12], and ZIKVs [8]. To avoid false priming, we used the tagged-primer approach [11, 12]. This assay was tested against stored ZIKV RNA-positive semen samples from patients that were monitored for ZIKV persistence in semen after acute infection during the 2016 outbreak in the Americas.

METHODS

Test Panel

We used a panel of semen samples in which the presence of ZIKV RNA was detected using the RealStar Zika Virus RT-PCR

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Kit (Altona Diagnostics, Hamburg, Germany) in 2016. This assay was performed on unfractionated semen, which was transferred into sterile Eppendorf tubes after liquefaction of the ejaculate. We extracted RNA from 140 µL semen, using the QIAamp RNA viral kit. The samples were stored at −80°C. The asRNA assay was performed on the same RNA extracts in 2019. The samples were obtained from 2 cohorts of men and 1 individual, who were monitored longitudinally for ZIKV shedding in semen. The subjects lived in an area with active transmission of ZIKV (Trinidad cohort, n = 23 [13]) or had traveled to a country (Belgian cohort, n = 15 [5]) affected by the 2016 ZIKV outbreak. In addition, we studied sequential semen samples obtained from a symptomatic husband with confirmed ZIKV infection for whom male-to-female sexual transmission was demonstrated after returning from Martinique to France; identical ZIKV sequences were recovered from his semen and his wife's plasma, using next-generation sequencing [14]. Isolation of ZIKV by culture was done as previously described [5] for the Belgian and Trinidad cohort on the first RT-PCR-positive samples if cycle threshold (Ct) values were 30 or lower. For the French case, ZIKV isolation was attempted on all RT-PCR-positive semen samples.

Zika Virus Two-Step Antisense Reverse-Transcription Polymerase Chain Reaction Assay

To detect the presence of ZIKV antisense replicative RNA, we developed a ZIKV 2-step antisense RT-PCR assay. Viral replication of ZIKV (Figure 1A), the principle of the ZIKV asRNA assay (Figure 1B), and the primers and probe we used (Figure 1C) are presented. In the first reverse-transcriptase (RT) step of the asRNA assay, we only used a forward (FW) primer with a tag sequence for reverse transcription of asRNA (Figure 1B(a)). In the next step, we used a tag-primer, virus-specific reverse (RV) primer and a probe for PCR-amplification and detection of strands that carry the tag sequence (from the RT step). To confirm exclusive amplification of the antisense strand (Figure 1B(a)), we ran 2 additional reactions: one with only the RV primer that did not result in PCR amplification (Figure 1B(b)) and another that used the FW-tag and RV primers for detection of both the sense and asRNA strands (Figure 1B(c)). Negative controls (PCR grade water), sense, and antisense-positive controls that contain the NS1 amplicon sequence (ssRNA ultramer oligo obtained from Integrated DNA Technologies, Leuven, Belgium) were used in each run.

The Bonn NS1 primers and probe used (Figure 1C) target the ZIKV-specific NS1 region and produce an amplicon of 107 base pairs (bp) (ie, 91 bp plus the newly designed 16-bp tag sequence). The tag sequence did not show in silico dimerization with the Bonn NS1 primers and probe and did not recognize any ZIKV sequences in a BLAST search (NCBI).

The RNA extracted by the Maxwell automate (Promega) was reverse transcribed in a 20-µL reaction containing 5 µL RNA and 15 µL mix (2 pmol of gene-specific primer [NS1 FW-Tag primer and/or NS1 RV primer]), 0.4 µL dNTPs mix (Eurogentec, Liège, Belgium), 1 µL DDT 0.1 M (Invitrogen), 1 µL RNaseOut recombinant RNase inhibitor, 1 µL SuperScript III Reverse Tanscriptase (200 U/µL), and 4 µL of 5× First-Strand buffer (Fisher Scientific, Brussels, Belgium) according to the manufacturer's instructions. The RT reaction was run on the Quantstudio5 cycler (Applied Biosytems) for 30 minutes at 55°C and 15 minutes at 70°C for complementary deoxyribonucleic acid (cDNA) synthesis.

For amplification, a real-time PCR was set with 5 µL cDNA in a 20-µL PCR mix containing 0.6 µM of each primer (Tag primer and NS1 RV primer) and 0.3 µM NS1 probe and 12.5 µL of 2× iTaq universal probe one-step mix without RT enzyme (Bio-Rad). The PCR was run on the Quantstudio5 cycler at 95°C for 5 minutes, followed by 50 cycles at 95°C for 10 seconds and 56°C for 30 seconds.

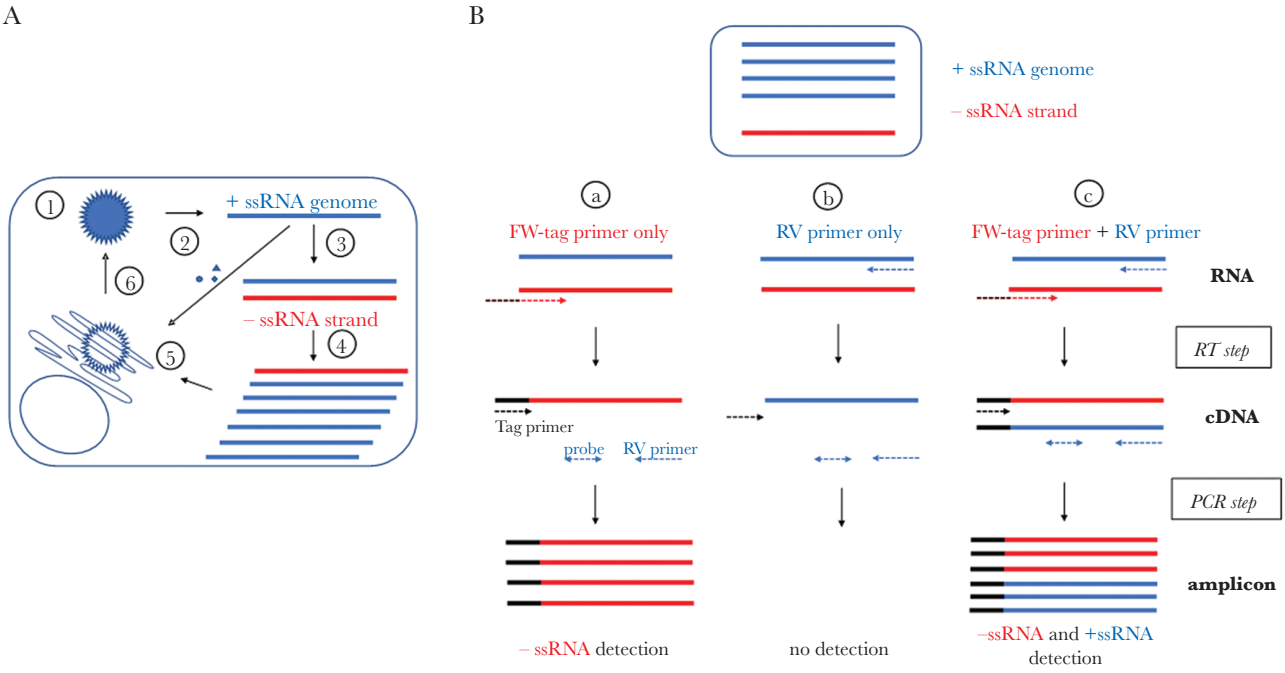
Statistics

We compared the sensitivity for detection of ZIKV replication in the first semen samples of the asRNA assay to that of viral isolation, using the one-sided exact McNemar test (95% significance level). Ethical approval was obtained from the Institutional Review Board (IRB) at the Institute of Tropical Medicine, the Ethics Committee of the University Hospital in Antwerp (Belgium), and the IRB of the University Hospital Center in Bordeaux (France). The protocol was registered with ClinicalTrials.gov (NCT 02733796).

RESULTS

We obtained 104 semen samples from 19 men who had ZIKV RT-PCR-positive semen at least once after symptomatic infection (ie, 9 of 15 men from the Belgian cohort) (Figure 2, cases A, E, F, G, K, L, M, N, and O), 9 of 23 individuals from the Trinidad cohort (Figure 2, cases B, C, D, H, I, P, Q, R, and S), and 1 individual from France (Figure 2, case J). The median number of samples per case was 5 (range, 1–18). Longitudinal monitoring was complete until loss of ZIKV RNA detection for 9 of 19 cases (47.4%) (Belgian cohort, mean 7.9 samples per case). The time until the first negative RT-PCR result was 94 days postsymptom onset (dpso) (median, interquartile range [IQR] = 55–103). For men whose last sample tested positive, the duration of follow-up was 61 dpso (median, IQR = 20–81) (Trinidad cohort and French case, mean 3.3 samples per case). The initial semen samples were obtained at 16 dpso (median, IQR = 2–49).

Zika virus isolation was successful in 4 individuals (21.1%) that had Ct values of 19, 20, 21, and 18 and were collected at 6, 11, 12, and 17 dpso (cases A, B, C, and D), respectively. Attempts to isolate ZIKV failed for all samples obtained from case J, whose first semen sample was collected at 27 dpso, that is, 14 to 27 days after confirmed sexual transmission of ZIKV to his spouse [14].



C

Oligo name	Used for	Sequence (5'-3')
NS1 FW-tag primer	RT	CATACGCACGGATAAACRACYACTGCAAGYGGAAGG
Tag primer	PCR	CATACGCACGGATAAA
NS1 RV primer	RT and PCR	GCCTTATCTCCATTCCATACC
NS1 probe	PCR	ATGGTGCTGYAGRGARTGCACAATGC

Figure 1. Replication of Zika virus (ZIKV) (A), amplification of antisense ribonucleic acid (RNA) (B), and primers and probe used (C). (A) Replication of ZIKV: after infection, the virus enters the cell through receptor-mediated endocytosis (1). The positive sense single-stranded (ss) RNA genome is released into the cytoplasm (2). The viral genome is translated into viral structural and enzymatic proteins and into antisense ssRNA intermediate strands (3). The antisense ssRNA is used for synthesis of full-length genomic RNA transcripts (4). The newly synthesized proteins and positive sense genomic RNA strands are assembled and encapsulated on the endoplasmic reticulum membrane (5). The viral particles (6) are transported to the plasma membrane and released as mature infectious virions by exocytosis. (B) Amplification of antisense ZIKV RNA by a newly developed reverse-transcription polymerase chain reaction (RT-PCR) to detect replicating virus as measure for infectivity. Clinical samples infected with ZIKV contain both positive sense ssRNAs (genomic strand and transcripts) and antisense RNA (used as template for synthesis of $^{+}$ssRNA transcripts). To demonstrate the proof-of-principle during the optimization phase, RNA extracts were tested with a forward (FW)-tag primer only (a), a reverse (RV) primer only (b), or both the FW-tag and RV primer in the reverse-transcriptase (RT) step. This created complementary deoxyribonucleic acid (cDNA) containing the Tag-sequence for (a) and (c), but not in the (b) reaction. During the PCR step, Tag-primer, RV-primer, and probe on cDNAs obtained from (a), (b), or (c), amplification was detected only in (a) and (c) reactions. In (b), the Tag primer could not bind any sequence, resulting in no amplification. In (a), only the antisense RNA strands were amplified; in (c), both the positive sense and antisense RNA strands were amplified. (C) Primers and probe used in the ZIKV 2-step antisense RT-PCR assay.

Our asRNA RT-PCR assay was positive in samples from 9 of 19 men (47.4%) (Figure 2, cases A through I, vertical lines followed by dark gray blocks). Zika virus asRNA was detected in all 4 samples from which ZIKV was isolated. The sensitivity of the asRNA assay for detection of ZIKV replication was significantly higher than that of viral isolation (47.4% vs 21.1%, $P = .032$). These 9 samples had Ct values of 26 or lower for detection of genomic ZIKV RNA by RT-PCR. The asRNA assay was negative in samples from the remaining 10 cases, all of which had Ct values above 26. None of the semen samples obtained later than 21 dpso had a positive asRNA assay result.

DISCUSSION

We demonstrated replication competence of ZIKV in human semen by detecting antisense ZIKV RNA (1) in 9 of 19 (47.4%) men who had ZIKV RNA-positive semen samples after symptomatic ZIKV infection and (2) in 9 of 15 (60%) whose semen was studied within 21 dpso. Zika virus was isolated from 21.1% of men with ZIKV RT-PCR-positive semen. The positive asRNA assay results were obtained in samples with lower ZIKV loads than culture-positive samples (Ct values 26 vs 21), and all samples that were obtained after 21 dpso had negative asRNA results. These outcomes

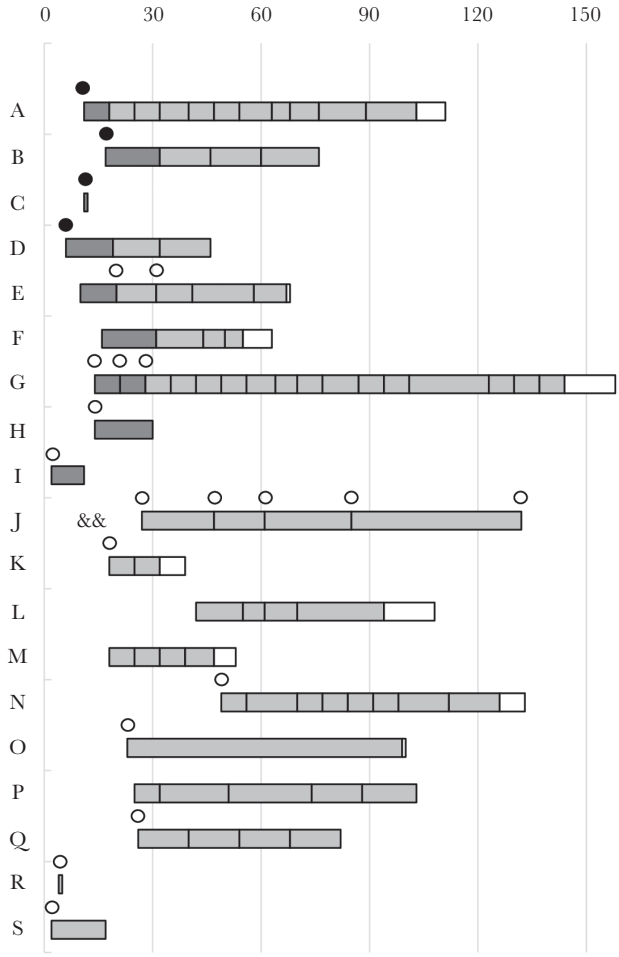


Figure 2. Sequential assessment of Zika virus (ZIKV) reverse-transcription polymerase chain reaction (RT-PCR)-positive semen samples, including isolation and antisense RNA assay results. (Horizontal Axis) Days postsymptom onset. (Vertical Axis) A through S indicate cases with sequential monitoring of ZIKV RNA shedding in semen. Bars indicate the timeline of sequential follow-up of semen samples per case (in days). Vertical lines between blocks represent instances of sampling. Dark gray blocks designate the time after a positive result in both ZIKV RT-PCR and antisense RT-PCR. Light gray blocks designate the time after a positive ZIKV RT-PCR result. White blocks designate the time after a negative RT-PCR result. ●, Zika virus isolation from semen successful. ○, Zika virus isolation from semen unsuccessful. **&&**, Semen samples from the index case (J) of confirmed sexual transmission to his pregnant spouse, which probably occurred 27 to 14 days before the first semen analysis [14].

support findings by many others that obtained positive ZIKV culture results only from semen samples that contain high numbers of RNA copies and that were obtained within 30 dpso, although a single case of ZIKV isolation from a postvasectomy sample with Ct value 30 obtained at 69 dpso was reported [2, 3, 6]. We believe that the negative ZIKV culture and asRNA results from case J's semen samples suggest that by the time of the first analysis (at 27 dpso), infectious virus was no longer present in his semen. However, the prolonged shedding of ZIKV RNA as detected by RT-PCR may result from ongoing ZIKV replication in the male genital tract [3].

Our results indicate that the sensitivity of the asRNA assay for the detection of ZIKV replication competence as a proxy determinant of infectivity was higher than that of virus isolation methods. In addition, our molecular approach has the advantage of being a faster, less cumbersome, cheaper, and potentially safer method than viral isolation for the assessment of infectivity. Therefore, antisense nucleic acid detection could be used to study the pathophysiology and epidemiological significance of RNA detection of ZIKV and other viruses in clinical samples, in the context of transmissibility.

Interpretation of our study results is limited by the use of samples after prolonged storage (up to 3 years) and repeated freeze-thaw cycles. Degradation of viral RNA may have occurred, and this could have led to underestimation of replication competence in affected samples. The low quantities of the targeted asRNA in the clinical samples may also have limited the sensitivity of the assay. Therefore, as with failed virus isolation attempts, negative ZIKV asRNA results do not exclude infectivity completely, but they do make the associated potential for sexual transmission less likely.

CONCLUSIONS

In a public health perspective, our findings support the current guidance issued by the Centers for Disease Control that a couple should use condoms or abstain from sex for at least 3 months after the male partner's symptom onset or last possible travel-associated ZIKV exposure to reduce the risk for sexual transmission of ZIKV [15].

Notes

Author contributions. R. H. and L. C. devised the conceptual ideas; R. H., B. D. S., K. E., C. M.-B., N. J., D. M., and L. C. contributed to data curation; R. H., I. L.-G., and L. C. contributed to formal analysis; R. H., D. M., and I. L.-G. contributed to funding acquisition; R. H., C. M.-B., D. M., I. L.-G., and L. C. contributed to investigations; R. H., B. D. S., K. E., and L. C. contributed to methodology; R. H. and L. C. contributed to project administration; R. H. and I. L.-G. contributed to resources; L. C. supervised the work; R. H. and L. C. contributed to visualization; R. H. and L. C. contributed to writing the original draft and preparation; R. H., B. D. S., K. E., C. M.-B., N. J., D. M., I. L.-G., and L. C. contributed to writing, review, and editing the manuscript.

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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