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SCHOLARENA

Journal of Infectious Diseases and Pathogenesis

RESEARCH ARTICLE

ISSN: 2577-7939

Impact of Zika Virus Infection on Male Reproductive Health (Case Study)

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Citation: Melissa M Pérez Millan, Mayling Alvarez Vera, Dailyn Medero Díaz, Silvia Serrano Alvarez, Magile Fonseca et al. (2024) Impact of Zika Virus Infection on Male Reproductive Health (Case Study), J Infect Dis Pathog 7: 101

Abstract

Zika is an arthropod-borne flavivirus identified in 1947. It has stood out in the world health panorama since the increase in the report of cases of microcephaly and Guillain-Barré Syndrome. It is reported that after infection by this agent, severe damage to the epididymis and testes can cause infertility; studies in experimentally infected animals showed reduction of sex hormones, destruction of germ and somatic cells, with loss of mature spermatozoa. There are also reports of viral isolation in humans from semen samples. Taking into account this background, a study was carried out to determine the impact of Zika infection in a patient treated at the infertility clinic of the Maternal and Child Hospital ¨Ramón Gonzalez Coro¨, Cuba. For this purpose, RT-PCR of the patient's semen was performed at different periods in order to detect viral excretion. Clinical and seminal parameters were evaluated following the protocol of the clinic for these cases. The presence of viral genetic material in the samples was verified over time, with a positive RT-PCR result. The spermograms performed were in the normal range. During the phylogenetic analysis, the Asian genotype was found to be infecting. All this led to the conclusion that the Zika virus infection did not provoked the patient's infertility.

Keywords: Zika; Infertility; Cuba; Phylogenetic Analysis; Spermogram; Sexual Transmission; Next Generation Sequencing

Introduction

Zika virus (ZIKV) was identified in 1947 in the Zika forest in Uganda. Virus was first isolated in a rhesus monkey used as part of the sylvatic yellow fever monitoring program.Subsequently it was isolated in Aedes mosquitoes collected in Africa and Asia [1].

After a long period of silent transmission with few human cases, in 2007, an outbreak of Zika fever is reported in the Yap island followed by the outbreaks of French Polynesia in 2013 and 2014 [2]. ZIKV isolated in these outbreaks was classified as Asian lineage. In 2015, Brazil recognized the ZIKV transmission that lately spread to other countries of the American region. During the French Polynesia and later in the Brazil outbreaks, congenital malformations such as microcephaly were recognized in children born from ZIKV infected pregnant women [3,4].

Additionally, fragments of the ZIKV genome were detected in semen samples suggesting the sexual transmission of the virus [5,6]. Govero et al., 2016 proposed that damage to the epididymis and testes of animals experimentally infected with ZIKV produces a reduction of sex hormones, destruction of somatic and germ cells, with loss of mature spermatozoa. Additionally, infiltrating cells could extend the destruction to the testicular architecture [7]. Duggal et al., 2017, reported that in immunosuppressed AG129 mice, virus excretion in semen can be detected between 7 and 21 days post viral inoculation, being viral RNA detectable up to 58 days [8].

Considering the epidemiological situation in the American region, Cuba established the ZIKV molecular diagnostic and surveillance in mid-February 2016, accompanied by an intensive vector control campaign. The autochthonous ZIKV transmission was recognized in 2016, preceded by the detection of confirmed cases in travelers. Molecular surveillance was directed to confirm the infection in zika clinically suspected cases including pregnant women and patients with Gillain Barre symptoms (GBS). Also semen collected from individuals attended infertility program were studied as part of the national surveillance. Here we present the case of an individual attending the infertility consultation with a clinical confirmed ZIKV infection.

Materials and Methods

Description

Young couple, in reproductive age, with previous health history and unprotected sexual practices for more than 12 months without success. They attended the infertility consultation for study and treatment.

Male: 38 years old, fair-skinned, with no history of endocrine and non-endocrine diseases or exposure to physical agents. He was immunologically competent and reported no medication use or toxic habits. Prior to his marriage to his couple, he had no history of pregnancy. He was diagnosed with ZIKV infection during his visit to the clinic and because of this, the attending physician decided to perform a virological study of the semen following the protocol established in the country. Virus infection was confirmed by Real-time reverse transcriptase polymerase chain reaction (RT/PCR) in serum and urine collected at day 4 of symptoms.

At the ¨Ramon Gonzalez Coro¨ Hospital infertility consultation he was diagnosed as suffering a picture of epididymitis on the left side, developed as a result of physical efforts during his daily activity however, his sexual function was normal. He did not suffer from erectile dysfunction or premature ejaculation; he had no urinary infections, no sexually transmitted diseases or systemic infections.

His wife's referred a previous ectopic pregnancy, which caused the loss of one ovary and one fallopian tube, reducing by 50% her chances of becoming pregnant. She was classified as a tubal woman, for this reason, initial studies at the infertility consultation were directed to her.

Clinical Evaluation and Samples Collection

Clinical evaluation consisted of Interrogation, physical examination, samples collection and laboratory studies. Spermogram was indicated several times during consultation. Semen sample was collected after three to seven days of sexual abstinence to ensure a sperm concentration greater than 20 million per milliliter. For the evaluation of the spermograms, the criteria proposed by the World Health Organization in 2021 were used. (https://www.reproduccionasistida.org/analisis-de-la-concentracion-de-espermatozoides/valores-normales-concentracion-espermatozoides/).

After zika fever, semen (1ml) was periodically collected at different time points of the first symptoms (15 days and 3, 6, 15, 21 and 42 months) to determine the presence of the virus genome by RT/PCR. Spermograms were also developed in the same samples.

Procedures

Semen samples collected after infection were studied by RT/PCR to detect ZIKV genome as well as to the study of clinical and seminal parameters. Phylogenetic analysis to determine the virus lineage was performed after nucleotidic sequence.

Viral RNA Extraction and RT/PCR

The manual extraction method was used using the commercial RNeasy Mini Kit (Qiagen, Germany) for RNA extraction. Manufacturer's instructions were followed. Purified viral RNA was stored at -200 C until RT/PCR. Lanciotti et al., RT/PCR protocol was followed for ZIKV detection. Table 1 shows the primers used. The Super ScriptTMOne-Step RT-RCP System with Platinum® Taq High Fidelity commercial kit (InvitrogenTM, EU) was employed for the reaction mixture.

Table 1: Primers used for the detection of ZIKV RNA by RT-PCR (Melissa María Pérez Millan)

Primers	Position (genome)	Sequence (5'-3')
VZIK 1086	1086-1162	CCGCTGCCCCCAACACAAG
VZIK 1162 c	1162-1139	CCACTAACGTTCTTTTGCAGACAT
VZIK1107-FAM	1107-1137	AGCCTACCTTGACAAGCAGTCAGACACTCAA

Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The system used was one of those proposed by the PAHO/WHO Laboratory Network for Arbovirus Diagnosis (RELDA), with an analytical sensitivity of 25 genome copies. This system uses hydrolysis probes or TaqMan®. Amplification was performed using the primers: VZIK 1086, VZIK 1162c and VZIK 1107-FAM. These primers amplify a fragment of the E protein gene of the VZIK genome. Table 1.

The Super ScriptTMOne-Step RT-RCP System with Platinum® Taq High Fidelity commercial kit (InvitrogenTM, EU) was used for the real-time RT-PCR reaction mixture. The reaction mixture was prepared under the conditions shown in Table 2.

Table 2: Results of spermograms* performed to the patient after the first symptoms of ZIKV infection (Melissa María Pérez Millan)

Date of study	December 3, 2018 (15 months after first symptoms).	June 26, 2019 (21 months after first symptoms)	March 24, 2021 (42 months after first symptoms)
Seminal volume (ml)	2ml	3ml	3ml
Progressive movement	0.61	0.46	0.59
Viability (%)	0.74	0.6	0.81
Days of sexual abstinence	4	4	4

Concentration (x 10^6 ml)	95 x 10^6 ml	97 x 10^6 ml	82 x 10^6 ml
Non-progressive movement	0.07	0.07	0.11
Morphology (% normal)	normal	normal	normal

The prepared mixture was distributed in 96-well plates [Micro Amp Fast Optical 96-well reaction Plate with Barcode (0.1 mL)], from Applied Biosystems (ABI), USA. The plates were transported to the amplification area under refrigerated conditions and in the absence of light. Ten µL of RNA (previously purified) from each of the clinical samples were added to each well in the UVP Workbench (DBA Analytik Jena, California, USA) in the amplification area using nuclease-free barrier tips. Subsequently, 10 µL of purified RNA from the positive and negative controls were added. The plates were then sealed with cling film to prevent evaporation of the reaction mixture and placed in the ABI 7500 FAST DX thermal cycler from Applied Biosystems®, USA. The programmed cycle for the run, using the Sequence Detection Software (Version 1.4.1, IVD), is shown in Table 3. Once the real-time RT-PCR run was completed, the data were analyzed following the instrument manufacturer's instructions. Samples whose Threshold Cycle (Ct) was below and at cycle 38 were considered positive and those whose Ct was above cycle 38 were considered negative.

Table 3: Mutations and amino acid changes observed in the studied semen sample* (Melissa María Pérez Millan)

Mutation (Viral Protein location)	Amino acid change	Associated to male infertility
Y152H (C) -	Tyrosine for Histidine	NO
R435W (E) -	Arginine for Tryptophan	NO
*670Q (E) -	Glutamine	NO
S760G (E)-	Serine for Glycine	NO
S775P (E) +	Serine for Proline	NO
Y795H (E)-	Tyrosine for Histidine	NO
R889C (NS1)+	Arginine for Cysteine	NO
P891L (NS1)-	Proline for Leucine	NO
S912R (NS1)+	Serine for Arginine	NO
R973W (NS1)-	Arginine for Tryptophan	NO
W1047R (NS1)-	Tryptophan for Arginine	NO
K1189E (NS2A)+	Lysine for Glutamic Acid	NO
T1271A (NS2A)+	Threonine for Arginine	NO
T1578A (NS3)+	Threonine for Arginine	NO
T1715A (NS3)+	Threonine for Arginine	NO
H1733Y (NS3)-	Histidine for Tyrosine	NO
G1755S (NS3)+	Glycine for Serine	NO
*1930R (NS3)	Arginine	NO
C1971R (NSE)-	Cysteine for Arginine	NO
C2229R (NS4A)+	Cysteine for Arginine	NO
Y2286H (2K)-	Tyrosine for Histidine	NO
G2311S (NS4B)-	Glycine for Serine	NO
G2484D (NS4B)-	Glycine for Aspartic Acid	NO

P2819S (NS5)-	Proline for Serine	NO
E2983K (NS5)-	Glutamic Acid for Lysine	NO
L3063S (NS5)+	Leucine for Serine	NO
H3079Y (NS5)-	Histidine for Tyrosine	NO
G3087R (NS5)-	Glycine for Arginine	NO
*3417G (NS5)	Glycine	NO

*New amino acid changes detected only in the studied sample (-); amino acid changes, previously reported in GenBank samples (+).

The results were statistically analyzed using the Microsoft Office Excel platform, the statistical software RStudio (Version 1.1.456 - © 2009-2018) and the mathematical program MATLAB® (Version R2013a (8.1.0.604)).

Analysis and Interpretation of Results

Once the RT/PCR run was completed, the data were saved and analyzed according to the instrument manufacturer's instructions.

Next Generation Sequencing and Phylogenetic Analysis

Amplified DNA was studied at the Institute of Tropical Medicine, Belgium for sequencing by the next-generation sequencing method [9-11].

The ZIKV nucleotide sequence obtained from the patient's semen was aligned with other ZIKV sequences isolated from clinical samples of male patients, including semen samples, and sequences from the West and East African ZIKV genotypes. Asian genotype sequences available from GenBank were also included. The alignment was carried out using the MEGA 11 program (Molecular Evolutionary Genetics Analysis), to generate the phylogenetic tree by the Maximum Likelihood method in order to observe the grouping of the analyzed strains. The GTR +I (General Time Reversible + Has Invariant Sites) was used as a substitution model.- For this, we used a sequence of a virus strain as outgroup, aligned with samples of the Asian genotype, a result that coincides with the genotype that circulated in the American region in the period 2016-2019, at the same timethat Zika transmission was reported in Cuba (data unpublished, article in preparation between the National Arbovirus Reference Laboratory of the Pedro Kouri Institute of Tropical Medicine and the Antwerp Institute of Tropical Medicine in Belgium), it can also be seen that the ZIKV sequences taken from the GenBank database corresponding to Isolated semen samples from male patients were grouped into the Asian genotype.

Results

In September 2017, the patient reported rash and general malaise for a week with non-purulent conjunctivitis. ZIKV infection was confirmed by RT/PCR in serum and urine collected during the acute phase of illness (data not showed). After few days he totally recovered. His couple never reported clinical symptoms and consequently she was not studied by RT/PCR.

The semen samples collected at days 15 and 3 and 6 months after ZIKV initial symptoms were positive by RT/PCR performed at the National Reference Laboratory of Arbovirus of the ¨Pedro Kouri¨ Institute of Tropical Medicine. The CT values of the RT/PCR were 20, 26 and 33 respectively showing a reduction in time and suggesting less virus concentration.

The rest of the collected semen samples were negatives. These results confirmed the virus excretion in semen and its tropism throughout the male urogenital tract.

Spermograms performed to the same samples (Table 4) showed values within normal parameters.

*Spermatozoa are classified into four groups (a to d) according to the direction and speed of their movement (12). Group (a): movement in a straight line in a single direction without deviation and in a rapid manner. Group (b): movement in various directions in a rapid manner. Group (c): movement in the form of circles in place, not moving in any direction. Group (d): they are immobile and remain in place.

Spermograms studies after ZIKV infection showed that the patient's spermatozoa were grouped within group (a), since they showed a rapid movement in a single direction (rectilinear fashion). In the seminal study, spermatozoa with a percentage of progressive motility higher than 40% are considered normal and those with a percentage of progressive motility lower than 40% are considered pathological.

As shown in Table 2, the values of progressive movement were 61%, 46% and 59% in 2018 (15 months after first symptoms), 2019 (21 months after first symptoms) and 2021 (42 months after first symptoms) respectively. Additionally, the percentage of viability in the three spermograms was higher than 50% and the movement was rapid and in one direction only, which confirms that the results of the seminal study carried out in the three occasions were normal, without apparent damage after three years post ZIKV infection, time enough to detect some damage in the male reproductive system as well as changes in the parameters previously mentioned. Reduction of fertilitywould be noticed).

The ZIKV genome obtained from one semen sample grouped it in the Asian genotype. This result coincides with the genotype circulating in the American region in the period of 2015-2019 and the period of ZIKV circulation in Cuba 2016-2019. Figure 1 shows the results of the phylogenetic analysis.

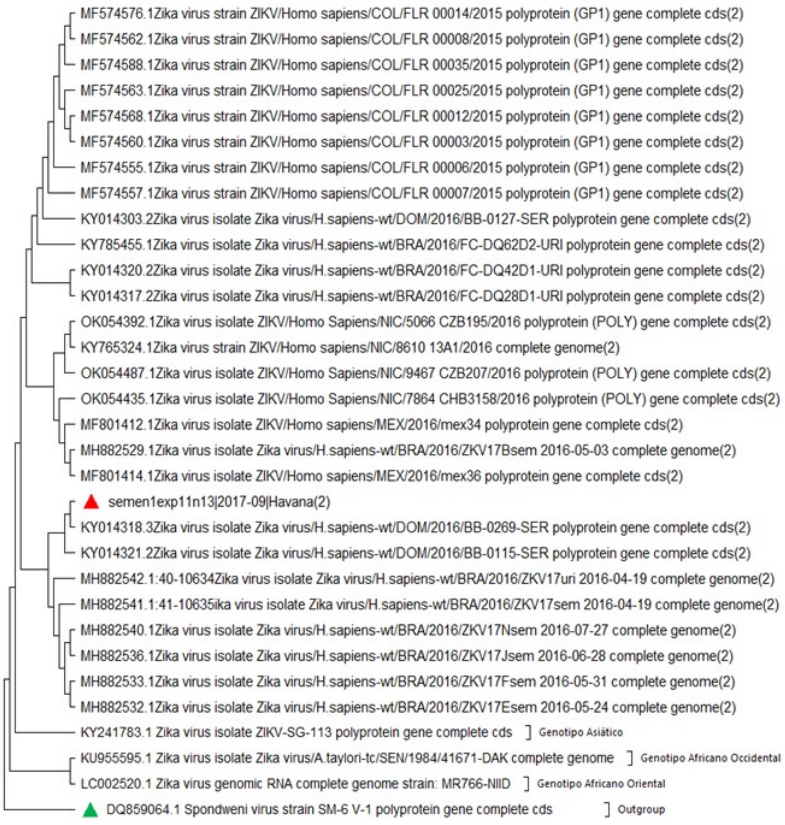

Figure 1: Phylogenetic tree showing Zika (Melissa María Pérez Millan)

Figure 1- Phylogenetic tree showing Zika virus sequences taken from the GenBank database and aligned with a Zika virus sequence isolated from the semen of a Cuban patient treated for infertility at the Ramón Gonzales Coro Materno Infantil Hospital; In the tree it is observed that the Zika sequence isolated from semen in Cuba was grouped in the Asian genotype, this was the one that was circulating in the region of the Americas at the time of confirmation of the case, they also aligned in this genotype other sequences obtained from semen samples in Brazil. To root the tree, the sequence of a Strain virus was used as Outgroup.

The analysis of the amino acid sequence of the virus showed no mutations associated to infertility as have been previously reported. However we do observed some others mutations such as S775P (E), R889C (NS1), S912R (NS1), K1189E (NS2A), T1271A (NS2A), T1578A (NS3), T1715A (NS3), G1755S (NS3), C2229R (NS4A), L3063S (NS5) commonly observed in ZIKV positive semen samples but not associated with male fertility disorders.

Of interests is the observation of some amino acid changes no previously reported. On consequence, we cannot exclude if they are associated to fertility disorders or not. Table 5 shows the amino acid changes found in the studied sample.

Discussion

Taking into account that several reports support the capacity of the virus to infect the testes and epididymis, as well as Sertoli cells, Leydig cells, spermatogonia, and even mature sperm and due to the transmission of ZIKV in Cuba in the period 2016-2019, it was decided to study male patients with confirmed ZIKV infection who attended infertility consultation [13].

It has been suggested that testicular macrophages may constitute the main target cell of early ZIKV infection in the testicular interstitium.This is supported by studies in mice, in which interferon receptor deficiency (IFNAR) and human testicular explants were macrophages viral infection has been demonstrated [13].

Additionally acute and chronic prostatitis have been reported during ZIKV infection. This has generated a controversy regarding the establishment of seminal vesicle and prostate infection. On the other hand, ZIKV replication has been reported in different types of testicular cells, suggesting they are potential reservoirs for the virus [13].

Viral persistence in testicular tissue has been associated with changes in sperm morphology and motility, in addition to hormonal alterations that have a negative impact on fertility. In patients with demonstrated ZIKV presence in the semen, alterations in the production pattern of proinflammatory cytokines, which can persist even after virus clearance, have been observed since the early stages of the infection. On the other hand, the integrity of the mucosa of the female reproductive tract can be altered due to the high levels of inflammatory factors, which in turn, increases the susceptibility to viral infection, as well as altering the function of spermatozoa and, therefore, the fertilization process [13].

The presence of the viral genome in semen could be associated with male infertility, In mice the virus has affinity for testicular macrophages, being able to replicate actively in the different types of testicular cells and acting on the Sertoli cells, which would reduce the size, affect the nutrition of the spermatozoa and reduce the level of the testosterone hormone [7, 14].

Here we studied a patient with a previous health history who was attending the infertility consultation. The previous patient's antecedents only reflects epididymitis, while his couple, classified as tubal, was considered the possible cause of the couple's infertility. During his follow-up he developed a confirmed clinical picture of ZIKV infection. For that reason it was decided to carry out this study.

Here, we demonstrated the virus excretion in semen during 6 months after illness, however we could not demonstrate spermograms affectation after the infection. We should remark that these were the first spermograms done to the patient, as he was considered fertile according to his previous clinical history. Even more, we could not demonstrate amino acid mutations associated to

infertility. However we do observe some new mutations not previously reported so we cannot exclude they are associated or not with this disorder.

As the spermogram parameters of our patient were within normal values, some other investigations (follicle stimulating hormone (FSH), luteinizing hormone (LH), free testosterone, Inhibin B and sex hormone binding globulin, that are obligated in cases of azoopermia, oligospermia or when there is a degradation of the parameters) were not indicated. The national study protocol for this type of patients supports this decision.

Here we demonstrated the excretion of the virus in semen, as the viral genome was observed in the first three collected samples covering a period of 6 months after symptoms, however, in spite of the viral excretion, no changes in the spermograms were demonstrated even after 42 months of illness. Additionally, genetic analysis did not demonstrated amino acid changes associated to fertility disorders although we do report some new amino acid changes with meaning deserves further study.

In conclusion, our results do not confirm in this particular case, the association of ZIKV infection to a fertility disorder. We recommended the follow-up of the patient's couple, as apparently she has a fertility disorder rather him as has been mentioned previously.

It´s important to continue the studies on the influence of male ZIKV infection and its relationship with fertile disorders being necessary to study a large number of patients with confirmed ZIKV in semen as well as the study of the virus nucleotide mutations compared to the ZIKV sequences obtained from other clinical samples such as serum and urine. These results, together with those from spermograms and other studies on fertility disorders, are important to more precisely determine the true impact of Zika virus infection on the human male reproductive tract.

Limitations

Among the limitations of the study, the fact that it was only possible to obtain the genome sequence of a patient treated in an infertility consultation stands out, as well as the lack of carrying out other studies on the patient such as hormonal testing, karyotype or FISH, as well as the study of pro-inflammatory cytokines and other studies in the patient's partner to rule out all possible causes of infertility in the couple, it must also be taken into account that for the evaluation of the implication of the amino acid changes found in the genome sequence . In male fertility, it is necessary to use animal models such as experimental mice in which tissue damage in the testicles after infection with this virus has been demonstrated.

Acknowledgments

We would like to thank Drs. Kevin Arien and Phillipe Selhorst from the Institute of Tropical Medicine in Antwerp, Belgium for sequencing the semen sample of the patient studied.

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