Clinical aspects and detection of Zika virus RNA in several tissues of experimentally infected BALB/c mice

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Abstract
The aim of the present study was to infect BALB/c mice with ZIKV to evaluate clinical signs and to quantify the viral load in different tissues at three different kinetic points. For this purpose, fifteen mice were inoculated with a 100µl of a viral solution and five other mice were used as negative controls. After euthanasia, nine different tissues were collected and macerated for RNA extraction and quantification. The infections were not lethal. Some of them, however, showed great agitation, hair bristling and itchy skin. Viral RNA was detected in one heart sample, eight samples of spleen and two of skeletal muscle. Seven out of eleven positive samples were from mice euthanized on the third day after infection. Only spleen yielded positive results at a later time of infection. These results contribute to a better understanding of clinical signs and replication dynamics of Zika virus in different organs of BALB/c mice, which are still scarce data in the scientific literature.

Main Text
Zika virus (ZIKV), an arbovirus that belongs to the Flaviviridae family, is transmitted to humans mainly through the bite of Aedes mosquitoes [1, 2]. It was discovered in 1947 [3], but only after the epidemic occurred in Brazil in 2015, global scientific community got concerned about this pathogen [4], especially due to the association of this infection with severe clinical conditions such as microcephaly [5–7] and the Guillain-Barré syndrome [7, 8].

As most publications on this topic are extremely recent, many questions remain unanswered. Among them, a current important field of research is the ZIKV tropism, its capability of replication in different organs and possible clinical manifestations generated as a result of the infection of an immunocompetent organism.

In this perspective, the aim of this study was to experimentally infect BALB/c mice (immunocompetent animals) in order to verify their clinical signs and detect and quantify ZIKV RNA in several organs at three, seven, and fourteen days post infection.

The virus used in the experiments was isolated from a human sample during the epidemic that occurred in Brazil in 2015 and provided by the Laboratório de Flavivírus, Instituto Oswaldo Cruz. The sample was tested by real time RT-PCR, using specific primers and the complete genome sequence
was deposited in the GenBank (KX197205).

For viral stock production, one hundred microliters of the serum sample were inoculated into a monolayer of *Aedes albopictus* mosquito lineage cells, which was incubated at 28°C for 1h for viral adsorption. Cells were maintained in Leibovitz medium (Cultilab) supplemented with 1% nonessential amino acids, 2% fetal bovine serum (Cultilab) and 10% tryptose phosphate broth. The cell culture supernatant was collected after 72h and viral titration was performed in Vero cell culture by plaque assay. Viral titer was $2.8 \times 10^8$ PFU/ml and the viral stock was stored at –70°C until use.

In this experiment, we used 20 two-month-old male BALB/c mice, whose initial weights ranged from 20 to 25 grams. The animals were obtained from the Instituto de Ciência e Tecnologia em Biomodelos (ICTB), Fundação Oswaldo Cruz (FIOCRUZ), and kept in transparent and ventilated cages in the vivarium of the Hélio and Peggy Pereira Pavilion, where the mice were kept under controlled temperature, photoperiod, nutrition and hydration conditions during the experiment. Mice were divided into four groups of five animals each, according to the experimental kinetics: negative control, three, seven and fourteen days after infection.

Negative control animals were inoculated with 100μL of Leibovitz medium (Sigma, Germany). The remaining animals were inoculated with 100μL of a Zika virus solution diluted in Leibovitz medium (viral load: $10^4$ particles per microliter). In both situations, the inoculation was done through the animals’ caudal vein. The entire procedure was performed in a biological safety cabinet.

After the specific time of infection kinetics for each group, animals were anesthetized and euthanized by cervical dislocation. Then, they were surgically opened for the removal of brain, cerebellum, lung, heart, liver, spleen, kidney, testis and skeletal muscle. The tissues were individually placed in a 1.5ml plastic tube containing 500μl of Leibovitz medium. After maceration and centrifugation (10.000 rpm, 15 minutes), the supernatant of each sample was collected, transferred to new 1.5mL tubes and frozen at –70°C until the day of RNA extraction.

For RNA extraction, we used the QiaAmp viral RNA minikit (Qiagen), following manufacturer’s standards. From 140 microliters of macerate supernatant, 60 microliters of RNA were recovered in
each sample. This material was placed in identified 1.5mL plastic tubes and stored at -70°C.

For qRT-PCR procedure, we used Taqman Fast Virus 1 step kit (Applied Biosystems). Forward primer (5’- TTG GTC ATG ATA CTG CTG ATT GC -3’), reverse primer (5’- CCT TCC ACA AAG TCC CTA TTG C -3’) and probe (5’- FAM-CGG CAT ACA GCA TCA GGT GCA TAG GAG -NFQ -3)’. All of these sequences are related to the prM/E viral genomic region.

Thermal cycling protocol was: one cycle of five minutes at 50°C for reverse transcription, followed by one cycle of twenty seconds at 95°C for enzyme activation and, finally, forty cycles of three seconds at 95°C followed by thirty-three seconds at 60°C for the denaturation and amplification steps, respectively. We considered positive all samples whose RNA quantification was higher than twenty copies per reaction.

In the present study, experimental infection did not cause the death of any animal. Moreover, weight and temperature did not differ significantly from the control group. Some animals, however, showed great agitation, hair bristling and itchy skin. An additional movie file shows this in more detail [see Additional file 1].

Formerly, a study compared several murine models of Zika virus infection and BALB/c mice were the only ones which presented 100% survival rate after twenty-five days post-infection and presented the lowest clinical score [9]. However, another experiment showed that Zika virus infected BALB/c mice, when immunosuppressed by dexamethasone, have a high clinical score, and the onset of symptoms is slightly earlier and more intense in males, when compared to females [10].

Among the infected animals, it was possible to detect viral RNA in one heart sample, two muscle samples and eight spleen samples, as shown in Table 1. The third day of infection was the time of experimental kinetics that yielded more positive results and, at later times, RNA could only be detected in spleen samples. Spleen was also the organ with the highest viral load among all other samples analyzed (Table 2). Detection of viral RNA in cardiac tissue at the beginning of infection is noteworthy because, although studies in this area are very preliminary, zika virus has been associated to transient myocarditis in adults [11], heart failure in the elderly [12] and with defects in heart formation, especially when intrauterine infection occurred in the second gestational trimester.
Viral replication in muscle tissue has already been described in infections with other Flavivirus, such as dengue virus [14, 15] and yellow fever virus [16]. Data concerning ZIKV are very scarce, but tests using Rhabdomyosarcoma cell cultures have shown the susceptibility of muscle cell to the virus [17] and an experiment with ZIKV-infected rhesus macaques showed viral RNA detection up to 35 days post-infection in different skeletal muscles of these animals [18]. In spleen, high ZIKV titers were frequently reported [10, 18-21], with a high viral load in the first days of infection and subsequent reduction [10, 19]. However, data about the histopathological effects of the virus interaction with spleen cells in an immunocompetent organism is still lacking. Our results show that BALB/c mice are susceptible to ZIKV infection even in the absence of immunosuppressants. However, the immunological efficiency of these animals seems to slow down the clinical signs as well as reduce the success of virus replication in several organs.

| Table 1 - Detection of Zika virus RNA by qRT-PCR according to organ/tissue and experimental kinetic point |
|---------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| ? | BRAIN | CEREBELLUM | LUNGS | HEART | LIVER | SPLEEN | KIDNEY | TESTIS | MUSCLE |
| 3DPI | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) | 1/5 (20%) | 0/5 (0%) | 4/5 (80%) | 0/5 (0%) | 0/5 (0%) | 2/5 (40%) |
| 7DPI | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) | 2/5 (40%) | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) |
| 14DPI | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) | 2/5 (40%) | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) |

Legend: DPI: Days post-infection

| Table 2 - Description of the mean of RNA copies per reaction quantified in each positive sample |
|---------------------------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| ORGAN/TISSUE | KINETIC TIME | ANIMAL ID | RNA COPIES/ML |
| Heart | 3DPI | 2 | 6759.29 |
| Muscle | 3DPI | 3 | 14664.98 |
| Spleen | 3DPI | 5 | 133642.80 |
| 7DPI | 2 | 3143.59 |
| 14DPI | 1 | 5958.18 |

Legend: DPI – Days post-infection; ID – Identification.

In a study by Chan et al. (2016), viral load of $10^4$–$10^5$ was detected in several organs (brain, testis, prostate, kidney, urinary bladder, spleen, liver, intestine, pancreas, heart, lung and salivary gland) of mice at five days post-infection. These values dropped considerably at twelve and fourteen days after infection. Detection was also higher in males than in females [10]. Our data showed a larger number of positive samples at three days post-infection when compared to later moments of the experimental
kinetics. In positive samples, values did not exceed the mean of $10^3$ copies per reaction (Table 2).

**Conclusion**

We used BALB/c mice for experimental ZIKV infection, resulting in virus RNA detection in heart, skeletal muscle, and spleen of these animals. These findings contrast to scientific literature data on the large diversity of organs and tissues where the virus can be detected in mice that are immunodeficient or immunosuppressed by drugs. Viral load is higher at the beginning of infection, decreasing or becoming undetectable later in the experimental kinetics, yet the titers obtained in our study are lower than those found in researches with murine models with impaired immune functions. From the three organs with ZIKV RNA detection, only spleen samples had positive results at all three kinetic points we investigated. Moreover, further studies are needed to understand the impact of the infection on normal functioning of this organ.

**Abbreviations**

FIOCRUZ: Fundação Oswaldo Cruz; ICTB: Instituto de Ciência e Tecnologia em Biomodelos; RNA: Ribonucleic acid; qRT-PCR: quantitative retro-transcription polymerase chain reaction; ZIKV: Zika virus.

**Declarations**

**Ethics approval and consent to participate**

All procedures performed during this study were approved by the Animal Ethics Committee (protocol L-010/2017) and the Human Research Ethics Committee (protocol 59254116.0.1001.526) of Fundação Oswaldo Cruz (FIOCRUZ).

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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Authors' contributions

DMB performed animal experimental infection, sample collection, RNA extraction and wrote the manuscript. GCC, FCJ, ACR and ALT performed animal experimental infection and sample collection and its preparation for RNA extraction. RST, JFSA and GFT performed the qRT-PCR procedures. OMB - resources; DFB-V - conceptualization, formal analysis, methodology and resources.

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Supplementary Material
File name: Additional file 1

File format: .mp4

Title of data: Clinical manifestations of BALB/c mice infected with Zika virus

Description of data: Video showing control BALB/c mice not expressing any clinical sign and BALB/c mice infected with Zika virus after seven days of infection presenting great agitation, hair bristling and itchy skin.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.
Additional File 1.mp4
