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

Aspectos clínicos e detecção de RNA do vírus Zika em diferentes tecidos de camundongos BALB/cAn infectados experimentalmente

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Derick Mendes Bandeira
Mestre em Ciências pela pós-graduação em Medicina Tropical
Laboratório de Morfologia e Morfogênese Viral – Instituto Oswaldo Cruz – Fundação Oswaldo Cruz. Avenida Brasil, 4365, Pavilhão Hélio e Peggy Pereira, térreo, sala B12
E-mail: derick_mendes@live.com

Gabriela Cardoso Caldas
Mestre em ciências pela pós-graduação em Biologia Parasitária
Laboratório de Morfologia e Morfogênese Viral – Instituto Oswaldo Cruz – Fundação Oswaldo Cruz. Avenida Brasil, 4365, Pavilhão Hélio e Peggy Pereira, térreo, sala B12
E-mail: gabrielacardosocaldas@gmail.com

Fernanda Cunha Jácome
Mestre em ciências pela pós-graduação em Biologia Parasitária
Laboratório de Morfologia e Morfogênese Viral – Instituto Oswaldo Cruz – Fundação Oswaldo Cruz. Avenida Brasil, 4365, Pavilhão Hélio e Peggy Pereira, térreo, sala B12
E-mail: fernandacunhajacome@gmail.com

Arthur da Costa Rasinhas
Mestre em ciências pela pós-graduação em Biologia Parasitária
Laboratório de Morfologia e Morfogênese Viral – Instituto Oswaldo Cruz – Fundação Oswaldo Cruz. Avenida Brasil, 4365, Pavilhão Hélio e Peggy Pereira, térreo, sala B12
E-mail: tukabr@gmail.com

Renata Tourinho Santos
Doutora em ciências pela pós-graduação em Biologia Parasitária
Laboratório de Tecnologia Virológica - Biomanguinhos - Fundação Oswaldo Cruz.
Av. Brasil, 4365 - Pavilhão Rocha Lima - 4º andar
E-mail: renata.tourinho@bio.fiocruz.br

Juliana Fernandes da Silva Amorim
Mestre em ciências pela pós-graduação em Biologia Celular e Molecular
Laboratório de Tecnologia Virológica - Biomanguinhos - Fundação Oswaldo Cruz.
Av. Brasil, 4365 - Pavilhão Rocha Lima - 4º andar
E-mail: juliana.silva@bio.fiocruz.br

Gisela Freitas Trindade
Doutora em ciências pela pós-graduação em Biologia Parasitária
Laboratório de Tecnologia Virológica - Biomanguinhos - Fundação Oswaldo Cruz.

Av. Brasil, 4365 - Pavilhão Rocha Lima - 4º andar
E-mail: gisela.freitas@bio.fiocruz.br

Debora Ferreira Barreto-Vieira
Doutora em ciências pela pós-graduação em Biologia Celular e Molecular
Laboratório de Morfologia e Morfogênese Viral – Instituto Oswaldo Cruz – Fundação Oswaldo Cruz. Avenida Brasil, 4365, Pavilhão Hélio e Peggy Pereira, térreo, sala B13
E-mail: barreto@ioc.fiocruz.br

ABSTRACT
Our group infected BALB/cAn mice with Zika virus to evaluate clinical signs and viral load in several tissues at three different kinetic points. We inoculated fifteen mice with a 100µl of a viral solution to collect nine different tissues, from each animal, for RNA extraction and quantification. Infections caused no lethality. Some of them, however, showed great agitation, hair bristling, and itchy skin. Viral RNA was detected in one sample of heart, eight of the spleen, and two of skeletal muscle. Seven positive detections were from the third day after infection. Only spleen yielded positive results at a later time.

Keywords: Zika virus, BALB/cAn mice, qRT-PCR.

RESUMO
Nosso grupo infectou camundongos BALB/cAn com o vírus Zika para avaliar sinais clínicos e a carga viral em diferentes tecidos em três pontos distintos de uma cinética. Inoculamos 15 camundongos com 100µl de uma solução viral para coletar, em cada animal, nove diferentes tecidos para a extração de RNA e quantificação. As infecções não foram letais. Alguns deles, no entanto, mostraram grande agitação, eriçamento de pelos e coceira intensa. O RNA viral foi detectado em uma amostra de coração, oito de baço e duas de músculo esquelético. Sete das detecções positivas ocorreram em três dias após a infecção. Apenas no baço resultados continuaram positivos em momentos mais tardios.

Palavras-chave: Vírus Zika, BALB/cAn, qRT-PCR.

1 INTRODUCTION

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 poorly detailed. Among them, a current important field of research is the ZIKV tropism in an organism of BALB/cAn mice (immunocompetent murine model), the viral

capability of replication in different organs, viral load along different kinetic points and possible clinical manifestations generated as a result of the infection.

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

2 MATERIAL AND METHODS

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 $2.8x10^8$ PFU/ml and the viral stock was stored at -70 °C until use.

In this experiment, we used 20 two-month-old male BALB/cAn 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 a Zika virus solution diluted in Leibovitz medium (10^4 $TCID_{50}$/100µL). In both situations, the inoculation was done through the animals' caudal vein. We performed the entire procedure 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 cerebrum, 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 1713.6 copies per milliliter. This value is equivalent to 20 RNA copies per reaction multiplied by 85.68, which is the conversion factor for RNA copies per milliliter, once the RNA quantification was performed from 140 microliters of extracted RNA.

3 RESULTS

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.

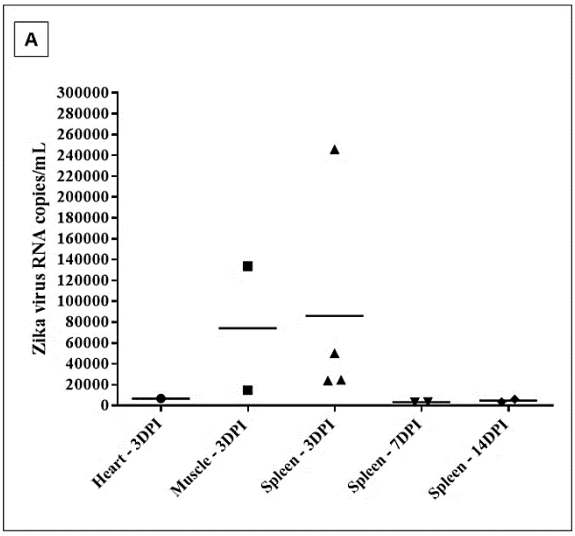
Among the infected animals, it was possible to detect viral RNA in one heart sample, two muscle samples and eight spleen samples, as shown in **Table 1**. The third day of infection was the time of experimental kinetics that yielded more positive results and, at later times, RNA could only be detected in spleen samples. Spleen was also the organ with the highest viral load among all other analyzed samples (**Figure 1**).

Table 1 - Detection of ZIKV RNA by qRT-PCR according to organ/tissue and experimental kinetic point.

	CEREBRUM	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

Figure 1 – Zika virus RNA quantification in positive samples from different tissues of BALB/cAn infected mice.



B

ORGAN/TISSUE	KINETIC TIME	ANIMAL ID	RNA COPIES/ML
Heart	3DPI	2	6759.29
Muscle	3DPI	3	14664.98
		5	133642.80
Spleen	3DPI	1	50152.78
		3	245975.28
		5	24030.66
		4	24700.68
	7DPI	2	3143.59
		5	3376.64
	14DPI	1	5958.18
		2	3600.27

Legend: DPI (Days post-infection). (A) Scatter plot chart of quantification from the positive samples, in copies per milliliter, organized by organ/tissue, and duration of experimental infection. (B) Individual viral load detection values, organized by organ/tissue and kinetic time.

4 DISCUSSION

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].

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 humans [11], heart failure in the elderly [12] and with defects in heart formation, especially when intrauterine infection occurred in the second gestational trimester [13].

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/cAn 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.

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, when positive samples values did not exceed the mean of 10^3 copies per milliliter (**Figure 1**).

5 CONCLUSION

We used BALB/cAn 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.

DECLARATIONS

List of 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.

Ethics approval

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).

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, and ACR performed animal experimental infection and sample collection and its preparation for RNA extraction. RST, JFSA and GFT performed the qRT-PCR procedures. DFB-V - conceptualization, formal analysis, methodology and resources.

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