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Running title: Ultrastructure of Zika virus in cell cultures

Ultrastructure of Zika virus particles in cell cultures

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Abstract

Since 2015 Zika virus (ZIKV) infected thousands of Brazilian people and spread to other American countries. Beside real time reverse transcriptase polimerase chain reaction (RT-PCR) diagnosis detecting the viral genome, virus isolation in cell lines was useful also and to study the viral particle structure and location inside the cells. This tool was achieved using transmission electron microscopy (TEM) of ZIKV infected cell lines. Human blood, obtained from a Brazilian patient during the first days of symptoms, was isolated in C6/36 mosquito cells and inoculated in sequence in Vero cells, fixed after six days, embedded in polymers, and ultrathin cut. This flavivirus, like dengue viruses, showed numerous virus particles after replication occurring inside vesicles of the endoplasmic reticulum confirming the susceptibility of the Vero cell line to ZIKV. TEM is the unique technique disposable to make the virus really visible.

Key words: Zika virus, virus particles, cell culture, transmission electron microscopy

Introduction

Zika virus (ZIKV), so-called because it was originally isolated from a rhesus monkey in the Zika Forest, Uganda, in 1947 (Dick et al. 1952), is a flavivirus that is transmitted to people mainly through the bite of an infected *Aedes* sp. mosquito (*A. aegypti* and *A. albopictus*). The *Aedes* species mosquito (that also transmits dengue, chikungunya, and yellow fever) occurs worldwide, posing a high risk for ZIKV global transmission. ZIKV infection usually is asymptomatic or causes mild illness, such as fever, rash, muscle/joint pain, and conjunctivitis; severe disease and fatalities are uncommon (Lucey & Gostin, 2016). Infections in humans have occurred in several African and Asian countries, but in 2007 ZIKV caused an outbreak of relatively mild disease characterized by rash, arthralgia, and conjunctivitis on Yap Island in the southwestern Pacific Ocean. This was the first time that ZIKV was detected outside of Africa or Asia (Hayes 2009, Duffy et al. 2009). French Polynesia has recorded a large outbreak since October 2013 with a great number of cases and some with neurological and autoimmune complications (Guillain-Barre syndrome); the clinical presentation is a “dengue-like syndrome” (Loos et al. 2014).

In Brazil (Northeastern region), in the early 2015, ZIKV was detected in sera of eight patients by reverse transcription-polymerase chain reaction; these patients presented symptoms of mild fever, rash, conjunctivitis and arthralgia (Zanluca et al. 2015). In addition to other symptoms, microcephaly in newborns, apparently as resulting of their mothers being infected during pregnancy, has been observed (Higgs 2016). ZIKV currently circulate in 21 states, and is estimated to have infected between 440,000 to 1,300,000 people in Brazil in 2015. As of 30 January 2016, 4,783 cases of microcephaly have been reported from 22 Brazilian states. Of these, 1,113 cases have been investigated: 404 cases were confirmed to have microcephaly or other congenital disorders of the nervous system, including 17 cases confirmed to be associated with ZIKV infection, and 709 cases have been discarded. 3,670 cases are still under investigation (<http://combateaedes.saude.gov.br/images/pdf/informe-epidemiologico-11-2016.pdf>, <http://zikainfection.tghn.org>). In studies performed by Slovenian researchers, ZIKV was found in the microcephalic fetal brain tissue on reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay, with consistent findings on electron microscopy. The complete genome of ZIKV was recovered from the fetal brain. The expectant mother has had a febrile illness with rash at the end of the first

trimester of pregnancy while she was living in Brazil (Mlakar et al 2016). Since Brazil reported Zika virus in May 2015, infections have occurred in at least 20 countries in the Americas. The Pan American Health Organization (PAHO) issued a series of epidemiological updates and alerts in 2015 urging enhanced surveillance for ZIKV, as well as neurological, auto-immune and congenital malformation associations (PAHO, 2015; Lucey & Gostin, 2016).

Material and Methods

In the present study monolayers of the Vero cell line were inoculated with ZIKV positive patient sample and analyzed by transmission electron microscopy (TEM) for detection of ZIKV particles. The supernatant of these cells were tested by real time reverse transcriptase polymerase chain reaction (RT-PCR) technique for detection of ZIKV genome.

The blood sample was obtained from one patient resident of Vitória, Espírito Santo, Brazil in July of 2015, that presented fever, myalgia, arthralgia, nausea, pruriginous exanthema and hands and feet joint pain. The ZIKV was originally isolated in *Aedes albopictus* cell line (C6/36 cells) from a patient sample blood and propagated in Vero cells by inoculating 200µL of C6/36 fluid that was adsorbed for 1 hour at 37⁰C. After the incubation period Minimum Essential Medium Eagle (MEM) supplemented with 2% fetal bovine serum was added and cells incubated at 37⁰C. After six days of inoculation the cell culture fluid was tested for molecular technique and the cell monolayer was processed for morphological analysis.

For the molecular test, the Vero cell culture fluid was subjected to quantitative ZIKV-specific real-time RT-PCR (Lanciotti et al., 2008). Viral RNA was extracted from 140µL from the culture fluid with the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) in accordance with the manufacturer's suggested protocol.

For morphological analysis the cells were fixed in cacodylate buffer (0.2 M, pH 7.2), post-fixed with 1% buffered osmium tetroxide, dehydrated in acetone, embedded in epoxy resin and polymerized at 60⁰C during three days (Sesso 2007, Barreto-Vieira et al. 2010, Barreto-Vieira et al. 2015). The blocks were cut of 50-70 nm thick ultrathin sections. The sections were picked up onto copper grids and stained with uranyl acetate and lead citrate and observed in a Zeiss EM-900 TEM.

Results

Ultrastructural analysis by transmission electron microscopy showed cell injury of Vero cell monolayers six days post-inoculation with ZIKV. Numerous lysosomes, vacuoles and mitochondria fulfilled the cell cytoplasm. Viral particles of approximately 50 nm diameter occurred as clusters inside vesicles and viral nucleocapsids were found inside cisterns of the endoplasmic reticulum. Viral RNA was detected in the supernatant of these cell monolayers confirming the presence of ZIKV (Figure 1).

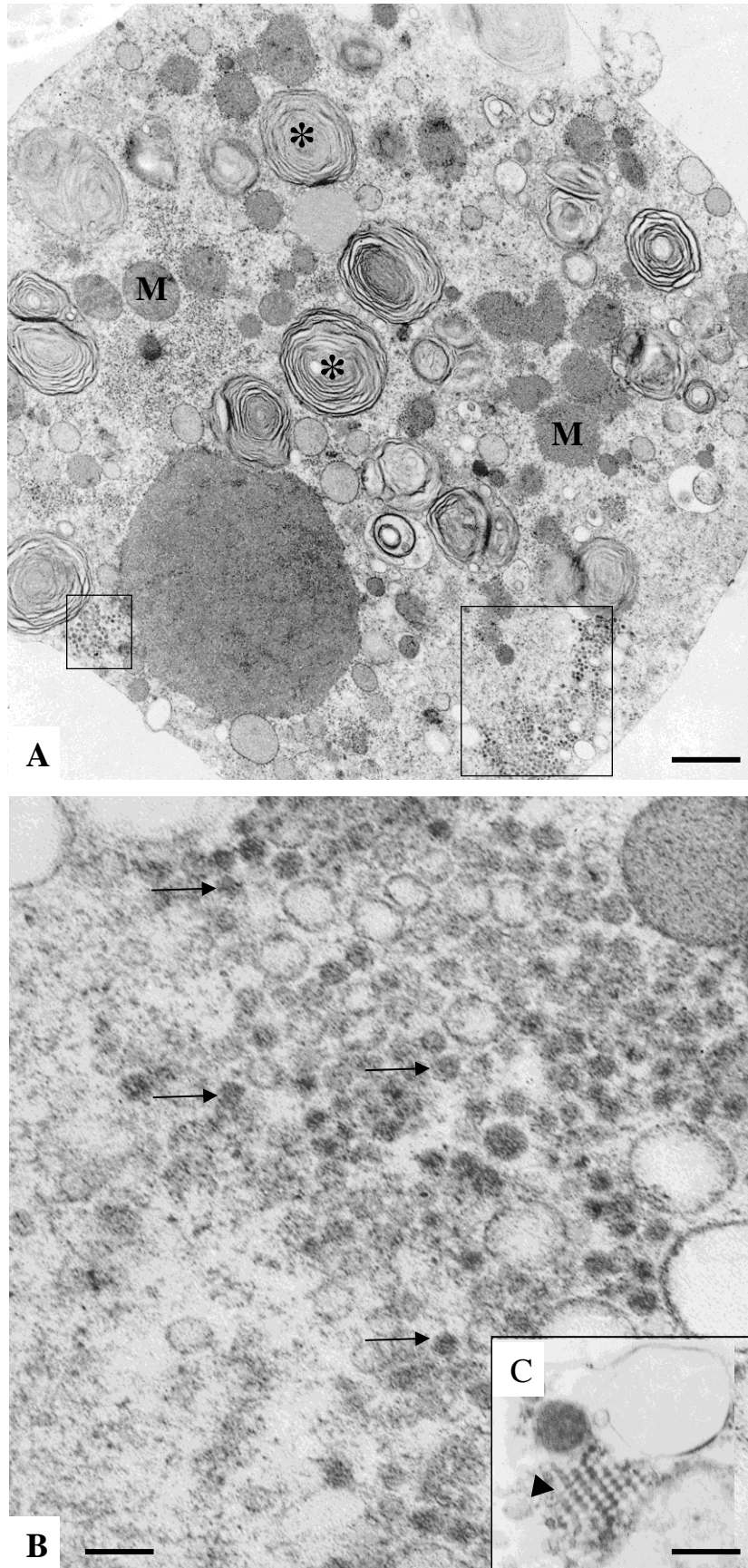


Figure1. Vero cell 6 days post inoculation with a human blood serum sample positive for ZIKV. A – Infected Vero cell presenting numerous lysosomes (*) inclusion bodies

and clusters of ZIKV particles inside the cell cytoplasm (marked areas); bar = 700nm. B – Increased area of figure A showing ZIKV particles (arrows); bar = 120nm. C – Regularly arranged viral nucleocapsids (arrow head); bar = 300nm.

Discussion

ZIKV particles were detected in microcephalic fetal brain tissue cells formerly diagnosed by real time RT-PCR. They showed a typical flavivirus feature and of 50 to 57 nm diameter (Mlakar et al. 2016). In the present study, Vero cell cultures inoculated with a ZIKV positive human blood sample, showed clusters of viral particles inside cytoplasm vesicles. The virus particle diameters averaged 50nm. Nucleocapsids were observed inside cisterns of the endoplasmic reticulum indicating viral replication. These aspects confer with the morphological studies of dengue viruses present in C6/36 cells (Barth 2000). Findings confirm that the ZIKV is able to replicate in the Vero cell line. Vero cells are widely used in studies of Virology for its efficiency in replicating other viruses such as measles, poxviruses, dengue and rubeola (Barth 2000, Schatzmayr et al. 2009, Takata et al. 1994). Real time RT-PCR confirmed the presence of the ZIKV genome inside Vero cell culture (Calvet et al. 2016) corroborating with ultrastructural observations by transmission electron microscopy.

Declaration of interests

We declare no competing interests.

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References

Barreto-Vieira DF, Barth-Schatzmayr OM, Schatzmayr HG 2010. *Modelo animal experimental para o estudo da patogênese dos vírus dengue sorotipos 1 e 2, manual de técnicas*. Interciência, Rio de Janeiro, 82 p.

Barreto-Vieira DF, Takiya CM, Jácome FC, Rasinhas AC, Barth OM 2015. Secondary infection with dengue viruses in a murine model: morphological analysis. *Indian Journal of Applied Research* 5(6): 44-51.

Barth OM 2000. Atlas of dengue virus morphology and morphogenesis. Imprinta Gráfica Ltda., Rio de Janeiro. 209p.

Calvet G, Aguiar RS, Melo ASO, Sampaio SA, Filippis I, Fabri A, Araujo ESM, Sequeira, PC, Mendonça MCL, Oliveira L, Tschoeke DA, Schrago CG, Thompson FL, Brasil P, Santos FB, Nogueira RMR, Tanuri A, Filippis AMB 2016. Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study. *Lancet Infect Dis* 2016. Published Online February 17, 2016, [http://dx.doi.org/10.1016/S1473-3099\(16\)00095-5](http://dx.doi.org/10.1016/S1473-3099(16)00095-5).

Daniel R. Lucey, Lawrence O. Gostin. The Emerging Zika Pandemic Enhancing Preparedness., *JAMA* Published online January 27, 2016. Published Online: January 27, 2016. doi:10.1001/jama.2016.0904.

Dick GW, Kitchen SF, Haddow AJ 1952. Zika virus. I. Isolation and serological specificity. *Trans R Soc Trop Med Hyg* 46: 509-520.

Duffy MR, Chen TH, Hancock WT, Powers AM, et al. 2009. Zika virus outbreak on Yap Island, Federated States of Micronesia. *N Engl J Med* 360: 2536-2543.

Hayes EB 2009. Zika Virus outside Africa. *Emerg Infect Dis* 15(9): 1347-1350.

Higgs S 2016. Zika Virus: Emergence and Emergency. *Vector-Borne and Zoonotic Diseases*. 16 (2): 1-2.

<http://combateaedes.saude.gov.br/images/pdf/informe-epidemiologico-11-2016.pdf>

<http://zikainfection.tghn.org>

Lanciotti RS, Kosoy OL, Laven JJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerg Infect Dis.* 2008; 14: 1232-9.

Loos S, Mallet HP, Leparac Goffart I, Gauthier V, Cardoso T, Herida M 2014. Current Zika virus epidemiology and recent epidemics. *Med Mal Infect* 44(7): 302-307.

Lucey DR, Gostin LO 2016. The Emerging Zika Pandemic: Enhancing Preparedness. *JAMA* 27.

Mlakar J, Korva M, Tul N, Popović M, Poljšak-Prijatelj M, Mraz J, Kolenc M, Rus KR, Vipotnik TV, Vodusek VF, Vizjak A, Pižem J, Petrovec M, Županc TA 2016. Zika Virus Associated with Microcephaly. *The new England Journal of Medicine* 17: 1-8.

Pan American Health Organization (PAHO)/ World Health Organization. Epidemiological Alert: increase of microcephaly in the northeast of Brazil. http://www.paho.org/hq/index.php?option=com_docman&task=doc_view&Itemid=270&gid=32285&lang=en. November 17, 2015. Accessed January 24, 2016.

Schatzmayr HG, Simonetti BR, Abreu DC, Simonetti JP, Simonetti SR, Costa RVC, Gonçalves MCR, Rocha MG, Silva MEV, Farias-Filho JC, Barth OM. 2009 Animal infections by vaccinia-like viruses in the state of Rio de Janeiro: An expanding disease. *Pesquisa Veterinária Brasileira* 29 (7): 509-514.

Sesso A 2007. Fixação de sistemas biológicos. In W de Souza, *Manual Sobre Técnicas Básicas em Microscopia Eletrônica*. Vol. I. Técnicas Básicas. Sociedade Brasileira de Microscopia Eletrônica, Rio de Janeiro, p. 13-21.

Zanluca C, de Melo VC, Mosimann AL, Dos Santos GI, Dos Santos CN, Luz K 2015. First report of autochthonous transmission of Zika in Brazil. *Mem Inst Oswaldo Cruz* 110 (4): 569-572.

Takata CS, Kubrusly FS, Miyaki C, Mendes IF, de Rizzo E 1994. Susceptibility of Vero cell line to vaccine of the measles virus. *Rev Saude Publica* 28(3) 209-12.