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Detection of arboviruses in salivary glands and midgut of mosquitoes vector V.1

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ISABELLA FERREIRA DA COSTA[1], CAROLINE PIRES SANTANA[1], Isabela Cinquini Junqueira[1], Juscelino Rodrigues[2], Christian Luz[2], Suleimy Marinho Fernandes[3], Izabela Batista Melo[3], Fabíola Fiaccadori[3], Valéria Christina de Rezende Feres[1]

[1]Laboratory of Molecular Biology and Applied Technologies for Laboratory Diagnosis, Faculty of Pharmacy, Federal University of Goiás (UFG), Goiânia, GO, Brazil.;
[2]Laboratory of Invertebrate Pathology, Institute of Tropical Pathology and Public Health, Federal University of Goiás (UFG), Goiânia, GO, Brazil.;
[3]Laboratory of Virology and Cell Culture, Institute of Tropical Pathology and Public Health, Federal University of Goiás (UFG), Goiânia, GO, Brazil.

ISABELLA FERREIRA DA COSTA

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Abstract

Dengue, Zika and chikungunya viruses are widely disseminated in Brazil and cause infections in humans, with similar clinical manifestations of varying intensity, with relevance in the context of public health.These diseases are mainly transmitted by mosquitoes of the genus Aedes. Therefore, monitoring the circulation of these arboviruses in vectors is a crucial step in epidemiological surveillance. In this protocol, adult mosquitoes were dissected to extract the salivary glands and midgut, which are structures directly associated with viral replication and transmission. These structures were inoculated into cells of the C6/36 lineage of Aedes albopictus for amplification and viral isolation. Our main objective was to develop and evaluate an experimental technical protocol in adult mosquitoes with the aim of enhancing the detection and characterization of these arboviruses.

Troubleshooting

Safety warnings

Please use personal protective types of equipment (such as gloves, safety glasses, a lab coat and masks) during all experiment.

Before starting

1 Prepare pools of 2 to 10 live and engorged an non-engorged adult mosquito specimens, sorted by sex (male/female). Place the pools in 1.5 µl microtubes and store in a freezer at -80°C.

Mosquitoes dissection

2 Pinch one mosquito at a time and remove the legs.

3 Place the mosquito on a micro glass slide under the stereoscopic magnifying glass

4 Add a drop of sterile solution of phosphate buffer saline (PBS) 1% to the micro glass slide to create a conductive medium for the separation of the desired structures.

5 With a needle, immobilize the mosquito's thorax by pinning it against the micro glass slide.

6 Using tweezers, carefully remove the entire abdomen of the mosquito, ensuring that the midgut remains attached to the immobilized thorax.

7 Use a needle to separate and section the midgut from the thorax.

8 Transfer the midgut to microtubes already prepared with 200 µL of PBS solution and store them appropriately.

9 While the mosquito is still fixed on the slide, use another needle to separate the mosquito's head from the rest of the body. This is necessary because the salivary glands, located in the anterior portion of the thorax, may partially adhere to the head.

10 Gently press the head and thorax to extract the salivary glands.

11 Using a pipette, extract the salivary glands from the medium and transfer them to the respective microtube where the intestine has already been stored.

12 Each tube will be homogenized by repeated pipetting (60 seconds). Subsequently, vortexed for 60 seconds to disrupt the cells.

Cell culture

13 Maintain C6/36 cells (Aedes albopictus) in culture using Leibovitz medium (L-15 Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) and 1% Penicillin/Streptomycin (Gibco, Waltham, MA, USA) at 28°C in an incubator.

14 Monitor cell growth and wait until the cells reach approximately 80% of confluence.

15 Completely discard the medium from the cell culture bottle and resuspend the cells in approximately 2 ml of L-15 medium.

16 Transfer the cells to a falcon tube (50 ml) and proceed to cout them using a Neubauer chamber.

17 Prepare a solution combining the cell suspension and Trypan blue dye at a 1:4 dilution ratio.

18 Under an optical microscope, count all living cells present in the four lateral quadrants of the Neubauer chamber.

19 Calculate the average of the cells counted in the four quadrants. Then, multiply the result by the dilution factor and the Neubauer chamber factor (10^4).

20 Aliquot 500 µL of the cell suspension into each well of a 24-well cell culture plate. Incubate the cell culture plate at 28ºC for 48 hours.

21 After confirming the formation of a cell monolayer, inoculate 50 uL of the samples processed as described in item 2. Then, add 500 uL of L-15 medium to each well and incubate the plate for 7 days.

22 At the 7th day, freeze the plates at -80ºC for at least overnight. Then, thaw the plates at room temperature to promote cell disruption and virus release.

23 Aspirate the contents of each well individually and transfer them to centrifuge tubes.

24 Centrifuge the tube at 14.000 rpm for 60 seconds at room temperature. After centrifugation, separate the supernatant and refrigerate it at -80ºC for subsequent viral detection and identification through molecular biology techniques.

25 All stages of cell cultivation must be carried out in a sterile biological safety cabinet and in accordance with biosafety standards.

Arbovirus Molecular identification

26 Extract the viral RNA using the silica/gel membrane column methods, utilizing a commercial kit (QIAamp Viral RNA Mini Kit, Qiagen, Hilden, Germany).

27 Perform reverse transcription and amplify the genetic material using real-time polymerase chain reaction (PCR).

28 This protocol was standardized using two methodologies for real-time RT-PCR. The protocol described by Huhtamo et al. 2010 for Dengue virus (DENV) detection, which empoyed specific primers and a probe for the conserved 3'UTR region (Path-IDtm multiplex One-Step RT-qPCR - Applied Biosystems – Carlsbad, CA, USA). The final reaction volume was 10 µL, and the components inincluded 2.5 µL of extracted material, 5.0 µL of Multiplex RT-PCR buffer, 1.0 µL of Multiplex Enzyme Mix, 100 nM of each primer, 200 nM of TaqMan probe and Nuclease-Free water provided in the kit to complete the volume.

29 The protocol described by Lanciotti et al. 2008 for Zika virus (ZIKV) detection was performed using specific primers and a probe targeting the conserved region of the protein E gene (GoTaq® Probe 1-Step RT-qPCR System, Promega – USA). The final reaction volume was 10 µL, and the components included 2.5 µL of extracted material, 5.0 µL of GoTaq® Probe Master Mix with dUTP, 0.4 µL of GoScript™ RT Mix, 100 nM of each primer, 200 nM of TaqMan probe and, Nuclease-Free water provided in the kit to complete the volume.

30 For the detection of nucleic acids (RNA) from ZIKV, DENV (4 serotypes) and Chikungunya virus, the commercial Kit IBMP Biomol ZDC-Zika, Dengue and Chikungunya (IBMP, Curitiba - PR - Brazil) was utilized, following the manufacturer’s instructions.

Protocol references

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