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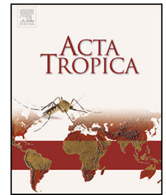

Isolation and characterization of a Brazilian strain of yellow fever virus from an epizootic outbreak in 2009

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ABSTRACT

During a series of epizootics caused by *Yellow fever virus* in Brazil between 2007 and 2009, a monkey was found dead (May 2009) in a sylvatic area in the State of Paraná. Brain samples from this animal were used for immunohistochemical analysis and isolation of a wild-type strain of YFV. This viral strain was characterized, and sequence analyzes demonstrated that it is closely related with YFV strains of the recently identified subclade 1E of the South American genotype I. Further characterization included indirect-immunofluorescence of different infected cell lines and analysis of the kinetics of virus replication and infectivity inhibition by type I IFN. The generated data contributes to the knowledge of YFV evolution and phylogeny. Additionally, the reagents generated and characterized during this study, such as a panel of monoclonal antibodies, are useful tools for further studies on YFV. Lastly, this case stresses the importance of yellow fever surveillance through sentinel monkeys.

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1. Introduction

Yellow fever (YF) is an arboviral disease endemic in many tropical countries of the African and American continents (Vasconcelos, 2003). The etiological agent, the *Yellow fever virus* (YFV), is the prototype of the *Flaviviridae* family and belongs to the *Flavivirus* genus. As with other flaviviruses, it has a positive single-stranded RNA genome enclosed in particles with a diameter of approximately 40 nm. The genome has a single open reading frame, which is translated into a polyprotein that is 3411 amino acids long and is cleaved by viral and host proteases into 3 structural proteins (C, prM/M, E) and 7 non-structural proteins (NS1, NS2a, NS2a, NS3, NS4a, NS4b, NS5) (Chambers et al., 1990).

YF in South America is transmitted to humans and monkeys by mosquito bites. Historically, there are two transmission cycles: sylvatic and urban. The sylvatic cycle involves vectors, such as *Haemagogus* sp. and *Sabethes* sp., and different species of non-human primates such as *Alouatta* sp., *Sapajus* sp., and *Callithrix* sp. (Fialho et al., 2012). On the other hand, the urban cycle is characterized by an alternation between vectors, mainly *Aedes aegypti* and humans. Although the latter has not been reported in South America in the last 45 years (Jentes et al., 2011) the wide distribution of competent vectors and the availability of susceptible hosts, in addition to favorable climate conditions, form a suitable environment for the re-emergence of the disease (Vasconcelos, 2010). Despite the existence of an effective vaccine, a recent study estimates only in Africa between 51,000–380,000 severe cases of yellow fever and 19,000–180,000 deaths due to this disease annually (Garske et al., 2014).

The present work discusses the isolation and characterization of a wild-type strain of YFV from a monkey found dead in May 2009 in the state of Paraná. This case was one of 1971 epizootic cases reported between 2007 and 2009 (Araújo et al., 2011) and stresses the importance of yellow fever surveillance through sentinel monkeys.

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2. Materials and methods

2.1. Cells and viruses

BHK-21 (ATCC® CCL-10™), Huh7.5 (ATCC® PTA-8561™), THP-1 (ATCC® TIB-202™), and VERO-E6 (Sigma, 85020206) cells were cultivated in Dulbecco's Modified Eagle Medium/Nutrient Ham F12 (DMEM F12–Gibco, USA) with 7% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (P/S). C6/36 *Aedes albopictus* cells (ATCC CRL-1660) were cultured in Leibovitz's L15 medium (Gibco, USA) with 5% FBS, 0.26% tryptose, and 25 μg/ml of gentamicin. YFV vaccine strain 17DD stock was obtained from two passages of commercial vaccine (Biomanguinhos, Fiocruz, Brazil) in Huh7.5 cells. The wild-type YFV strain isolate used in the *in vitro* assay corresponds to the first passage in C6/36 cells. Multiplicity of infection (MOI) was determined according to titers measured in the same cell line used for the infection assays, unless otherwise specified. The virus titration was performed by plaque assays and focus immunodetection in mammalian and insect cells, respectively.

2.2. Virus isolation

A small fraction of cerebral tissue from an infected *Alouatta* sp. member was mixed with 500 μl of extraction buffer (PBS, 100 U/ml P/S) and ground with a Precellys (Bertin Technologies, France) using default program 1. The homogenate was centrifuged at 800 x *g* for 2 min and then filtered through 0.45 μm and 0.22 μm filters. The filtrate, 100 μl in 400 μl of culture medium without FBS, was added to culture flasks containing 10^6 cells (Huh7.5, VERO-E6, or C6/36). The infection inocula were discarded after incubation for 1 h at the appropriate temperature (37 °C or 28 °C), and cell culture medium was added up to 10 ml/25 cm^2 flask. Ten days after infection (dpi) the culture supernatants were tested for the presence of YFV using RT-PCR.

2.3. Monoclonal antibody production and purification

Animal procedures were approved by the Ethical Committee on Animal Research of the Fiocruz Foundation under the protocol n°. LW-51/11. Three young adult male (30–45 days) BALB/c mice were immunized every 2 weeks with the equivalent of a 10-fold human dose of YFV 17DD vaccine: four times through the intraperitoneal route and the last dose through intravenous route. Fusion of spleen cells with myeloma cells was performed following a previously described protocol (Mazzarotto et al., 2009). The resultant hybrid cells were screened for the production of monoclonal antibodies (MAbs) against YFV using an immunofluorescence assay (IFA) after incubation of cell culture supernatants with YFV 17DD-infected Huh7.5 cells. After clonal selection, the selected monoclonal antibody was isotyped with a SBA Clonotyping kit (SouthernBiotech, USA), precipitated with saturated ammonium sulfate solution, and purified by affinity chromatography with a HiTrap Protein G HP column (GE Healthcare, UK).

2.4. PCR for viral detection, sequencing, and phylogenetic analysis

Viral RNA was extracted from cell culture supernatants using a commercial kit according to the manufacturer's instructions (QIAamp Viral RNA Mini Kit, Qiagen, Germany). cDNA was synthetized with ImProm-II (Promega, USA) using random primers (Invitrogen, USA). For viral detection, a PCR fragment of 987 bp was generated with a QIAGEN LongRange PCR Kit using the following primer pair: YF1+ (5′-ATCGAGTTGCTAGGCAATAAACAC-3′ annealing in position 41–64 of U17066 Genbank sequence as reference), and YF1- (5′-TCCTCCATGCACCCCCTC-3′, annealing in position 1010–1027). PCR for genome sequencing was performed using the same enzyme but a different set of primers, YF1+ and YF2- (5′- GAGCTCTCTCTTGCCAAAGTT-3′, annealing in position 2471–2491), for a larger amplicon (2451 bp). Strands were sequenced by the commercial Macrogen Inc. services (Seoul, Republic of Korea) and manually assembled. The sequences used in the multiple sequence alignment were selected based on the results of a BLAST search. Sequences, which presented a ≥99% query cover, were selected. Sequences of recombinant viruses, infectious clones, or plaque variants were then excluded. The complete data set encompasses 69 YFV sequences including the seven genotypes that have already been described (Supplementary Table 1). The multiple sequence alignment was carried out using muscle as implemented in MEGA 6.06 software. Identical sequences were excluded and the tree was inferred using the maximum likelihood algorithm as implemented in MEGA 6.06 software (Tamura et al., 2013) as described by Hall, (2013) with 1000 bootstrap replicates. A neighbor joining tree was used as the initial tree for the heuristic search which was carried out through nearest neighbor interchange. Tree edition was carried out on FigTree v.1.1.2. The sequence of Sepik virus (NC_008719) was included as an outgroup as it has been shown to be closely related to YFV (Kuno and Chang, 2006).

2.5. Immunofluorescence assay

For immunofluorescence assay (IFA), Huh7.5 cells were infected with M17/09 at a multiplicity of infection of 0.5. Forty-eight hours after infection, cells were fixed and permeabilized with methanol:acetone (1:1) for 20 min at −20 °C. Immunostaining was carried out with monoclonal antibodies, DAPI (Invitrogen) and secondary goat anti-mouse antibodies labeled with Alexa 488 (Invitrogen) diluted in PBS/1% bovine serum albumin for incubation periods of 30 min at 37 °C followed by washing with PBS. Photomicrographs were captured with Operetta System (Perkin Elmer) using 20 x magnification lens.

2.6. Flow cytometry quantification

For quantification of infected cells, immunolabeling was performed with 4G2 Flavivirus-specific monoclonal antibody and goat anti-mouse IgG (H + L) secondary antibody conjugated with Alexa Fluor 633 (Invitrogen). Flow cytometry analysis was performed with FACSCanto (BD). The percentage of infected cells was determined by comparison with mock cells.

2.7. Immunohistochemistry

A formaline-fixed paraffin-embedded (FF-PE) frozen brain sample was stained using a conventional hematoxylin-eosin technique. Representative areas were marked on the H&E-stained section. Sections from this block were cut using a microtome, mounted on a microscope slide, and analyzed by immunohistochemistry following a previously described protocol (Noronha et al., 2016). For staining the sections specific anti-YFV NS1 monoclonal antibody (3A8-C12) was used; a non-related monoclonal antibody (anti-Chikungunya virus envelope protein, 1G1) was used as a negative control. The immunostained slides were observed using an optical microscope, an Olympus BX50 (Tokyo, Japan). For each sample, photomicrographs were taken in HPF (high power field = 400x) using a Zeiss Axio Scan.Z1.

2.8. Kinetics of YFV replication

Twenty-four-well cell plates containing 2×10^5 Huh7.5 or C6/36 cells per well were infected with YFV M17/09 wild-type strain. The

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