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Title: Epizootic due to Yellow Fever Virus in Sao Paulo State: preliminary epidemiological and phylogenetic data and viral dissemination to new areas (2016-2017)

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Abstract
Yellow Fever virus is an RNA virus that belongs to family *Flaviviridae*, genus *Flavivirus.* In humans, yellow fever may vary from inapparent to a fatal disease, with patients presenting fever, prostration, hepatic, renal and myocardial injury, hemorrhage and shock. In Brazil, the virus is maintained by a sylvatic transmission cycle involving non-human primates and forest canopy-dwelling mosquitoe. Urban yellow fever was erradicated in 1942. This is a preliminary descriptive study encompassing epizootic events between July 2016 and March 2017, in São Paulo State, Brazil. Fresh and fixed tissues and tissues fixed in 10% neutral buffered formalin from NHP found dead or serum from live animals were sent to Adolfo Lutz Institute, São Paulo, for YFV molecular detection and immunohistochemistry. A total of 67 NHP were YFV positive during this time. Until December 2016, only cities within the vaccine recommendation area reported epizootic events due to YFV. However, in the beggining of 2017, YFV was detected in areas not deemed to be at risk for yellow fever. A Phylogenetic analysis showed similarity between the current epizootic with the past one, occured in 2008. This work shows the importance of NHP surveillance in order to initiate quickly the vaccination in local susceptible population.

Keywords: Yellow fever virus, non-human primates, epidemiology

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Introduction

Yellow fever virus (YFV) is the prototype of the *Flaviviridae* family, belonging to the *Flavivirus* genus. 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 at el., 1990). The virus is transmitted by the bite of infected mosquitoes to a susceptible host, and the disease is considered endemic in parts of South America and Africa. Due to the variety of clinical symptons and assymptomatic cases, yellow fever is under reported, and the incidence is believed to be 10-50 fold higher (Monath, 2001). Historically, there are two transmission cycles: sylvatic and urban. In Brazil, the sylvatic cycle involves the vectors *Haemagogus* sp. and *Sabethes* sp., and several species of non-human primates (NHP), mainly *Alouatta* sp., *Sapajus* sp., and *Callithrix* sp. (Fialho et al., 2012), that serves as sentinels for virus detection prior to human cases, and therefore they are an important tool to prevent human disease by vaccination of the local population (PAHO, 2005). NHP function as amplifiers of the disease, once when infected, they either die or become cured. In this case, they become immune for the rest of their lives (Vasconcelos, 2003).

In Brazil, during the late XX century, intense YFV circulation extended from the Amazon region to contigous states of Goiás and Mato Grosso do Sul (Central Brazil) throughout the first decade of the XXI century. Phylogenetic analysis demonstrated that YFV has two genotypes in South America (SA): SA genotype I, that includes strains recovered from Brazil, Panama, Colombia, Ecuador, Venezuela, and Trinidad, and SA genotype II, that includes virus mainly from Peru (Wang et al., 1996; Bryant and Barrett, 2003). In Brazil, Vasconcelos et al. (2004) reported that Brazilian strains were divided into two major subclades with four clusters (1A-1D) within clade SA I, showing a complex pattern of relationships, demonstrating geographic and temporal associations. In 2008-2009, a new spread was observed, when the virus reached the south and the southeast of the country (Monath and Vasconcelos, 2015), which were outside the recognized endemic/enzootic area, with no vaccine recommendation. Souza et al. (2010) showed that a new subclade 1E within the clade 1 of the South American genotype was responsible for the epizootic/epidemic in the State of São Paulo, where a total of 28 cases human cases with 11 deaths and 99 epizootic events were reported between March and April, 2009, with 174 animals distributed in 36 different counties (Moreno et al., 2013). This article's purpose is to describe the current epizootic due to YFV in São Paulo State and its expansion to new areas where vaccination was not recommended.

Materials and Methods

The study was conducted in the state of São Paulo, Brazil, which is composed of 645 counties divided in 15 administrative regions, occupies an area of approximately

248,196,960 km2 and has 44.749.699 inhabitants, concentrated mainly in the coastal zone (IBGE, 2016). This is a preliminary descriptive study encompassing epizootic events between July 2016 and March 2017. Tissue, serum or blood samples from NHP were sent to the reference laboratories at Instituto Adolfo Lutz, São Paulo, Brazil. Fresh tissues were sent to Núcleo de Doenças de Transmissão Vetorial for molecular detection, while tissues fixed in 10% neutral buffered formalin were sent to Núcleo de Patologia for routine histopathology with haematoxylin and eosin (H&E) and immunohistochemistry. Viral RNA was extracted from blood or tissues samples from NHPs using QIAamp RNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) and serum samples using Invitrogen PureLink™ Viral RNA/DNA Mini Kit, following manufacturer's instructions. Amplification of YF virus fragment was performed employing the protocol designed by Drosten et al. 2002 and Weidmann et al., 2010, with a positive Ct cut-off value of 38. At least two different organs were tested. For IHC analysis, liver tissue sections were tested with an *in house* primary polyclonal anti-YF antibody (1:40.000), signal amplification was achieved with Super HighDef™ (Enzo Life Sciences, Farmingdale, USA) and visualization was done with diaminobenzidine (D-5637; Sigma, St. Louis, MO, USA). Known NHP and human positive and negative control tissues with omitted first-layer antibody were included. Viral metagenomics was used to analyze 3 cell culture specimens on which YFV was isolated after one passage in C6/36 cell line. The specimens were clarified by centrifugation at 12,000×g for ten minutes and then filtered through a 0.45-µm filter (Millipore). The filtrates were treated with a mixture of nuclease enzymes to digest unprotected nucleic acids. Viral nucleic acids were extracted using a Maxwell 16 automated extractor (Promega). Viral cDNA synthesis from extracted viral RNA/DNA was performed 50 pmol of a octamer of random primer was used in a reverse transcription reaction with SuperScript III (Life

Tech). The 2nd strand cDNA synthesis was performed using DNA Polymerase I Large (Klenow) Fragment (Promega), followed by the use of a Nextera XT Sample Preparation Kit (Illumina) to construct a DNA library with each sample identifiable using dual barcodes. For size selection we used a Pippin Prep (Sage Science, Inc) to select a 400bp insert (range 200-600bp). The library was deep-sequenced using the MiSeq Illumina platform with 300bp paired ends. Datasets were then trimmed according the quality (99,9% coverage) and length (reads <30bp were removed) of each read using Geneious R9 software (Biomatters). Near full genome sequences were then reconstituted by mapping the reads to a reference sequence from GenBank using Geneious (majority base at each position) [Kearse M]. Sequences obtained were examined to ensure that the mapping to a reference sequence did not generate a biased consensus sequence.

Sequences of the prM/E junction retrieved from GenBank were aligned with the YFV sequence generated in this study from a *Cebus* monkey using ClustalW in Bioedit software v.7.2.6 (Hall, 1999). Phylogenetic tree was constructed using a maximum-likehood method with bootstrap resampling of 1,000 replicate using MEGA version 7. The Kimura 2– parameter was used as a nucleotide substitution model (Kimura, 1980).

Results

A total of 428 non-human primates from different counties of São Paulo State were received by Núcleo de Doenças de Transmissão Vetorial, Centro de Virologia, and 540 by Centro de Patologia, Instituto Adolfo Lutz, São Paulo, Brazil, between June 2016 and March 2017. Genera received belonged mainly to genus *Alouatta* spp., *Callithrix* spp., and *Sapajus* spp., but also a smaller proportion of *Callicebus nigrifrons*

and the endangered specie *Leontopithecus rosalia* were tested. A total of 67 NHP were positive for YFV during the time studied, being 31 *Alouatta* spp., 9 *Sapajus* spp. and 6 *Callithrix* spp. Twenty one animals were not identified. We found 91% of correlation when comparing tissue samples that were tested by both methodologies, as 9 animals were positive only by molecular detection, being 3 due to autolysis, five belonging to genus *Callitrix* spp. and one *Alouatta* spp., with Ct values in liver ranging from 17 to 38, and in brain ranging from 26 to 36. During the time studied, positive NHP were detected in 25 cities. Figure 1 shows the map of São Paulo State with positive YFV counties during the time studied and its dissemination by trimester. Black asterisks shows cities with YFV isolation (Ribeirão Preto, Tabapuã and Catanduva).

Phylogenetic analysis by alignment of the prM/E junction with 37 sequences retrieved from GenBank revealed that sample Span SPAn 20170003 (acession number MF987820) clustered within YFV group 1E of South American genotype I, with isolates from Venezuela in 2005, all NHP isolates in São Paulo during the 2008 epizootic and a Brazilian human strain isolated in 2004 from Amazonas, as shown in Figure 2.

Discussion

In 2008/2009, a total of 147 NHP were collected for YFV detection in São Paulo State, with ninety-one epizootic events in 36 different counties (Moreno et al., 2013).
The first YFV epizootic detected in São Paulo State was in july 2016, in a *Callithrix spp.* found dead at the center of Ribeirão Preto (RP), a city located in the southeast region with about 600.000 inhabitants. During the reemergence of YFV in 2008, with two autochnous case confirmed, no positive monkey was detected in this region (Moreno et al., 2011). The second confirmed epizootic occured in August 2016, detected in an

Alouatta spp. in São José do Rio Preto (SJRP), a city located in the northeast of the State, distant 200 Km from RP. In March 2016, an autochnous human fatal case was confirmed in Bady Bassit, a small county adjacent to the city of SJRP (Promed, 2016). No PNH from this region was sent to the reference laboratory during the first half of that year.

By the end of January 2017 until March 2017, YFV expanded towards the Atlantic coast of Brazil in areas not deemed to be at risk for yellow fever, as a new epizootic wave was confirmed in the center of the State, the most populous area and closer to the capital, as shown in Figure 1. The cities with positive NHP included São Roque, Águas da Prata, Amparo, Monte Alegre do Sul and Campinas, which has a population of more than 1 million inhabitants (13. IBGE, 2016). The detection of NHPs positive to YFV at Sousas district, within a 10 Km distance to an important urban center, poses a serious risk for the reintroduction of urban yellow fever, due to high number of local naive population and the presence of the vector *Aedes aegypti*. The current epizootic has also demonstrated some differences between the direct methods used for YFV confirmation, especially in *Callitrix* spp., once most of the negative *Alouatta* spp. samples in IHC were in advanced stage of autolysis. It is of the utmost importance that field professionals are trained for the collection and shipping samples in good conditions to the reference latoratory in order to avoid false negative results. In addition, the present work detected YFV in 9 *Sapajus* spp. monkeys, popular known as “prego”, by both methods. This specie was considered less sensitive to the disease, with low fatality rate (Davis and Shannon, 1929), and recent studies failure to detect YFV in *Sapajus* spp. monkeys (Moreno et al., 2013; Tranquilin et al., 2013; 28. Rocha et al., 2015).

The phylogenetic tree generated herein revealed that Brazilian sample IAL 14 clustered with other YFV isolates from NHP isolated in 2008, a human sample (SPH258595) from 2004 and Venezuelan isolates from 2005, showing therefore an occurence with temporal pattern, with an older clade that appears to have become extinct and another that has become the dominant lineage in recent years, as previously described by Vasconcelos et al. (2004). As Brazil was identified as the major source of YFV introductions into Venezuela (Auguste et al, 2015), propably the Amazon Region is the source for viral dispersal within Brazil.

Conclusions

The identification of the epizootics events prompted vaccination in local susceptible population. Phylogenetic analysis indicated that the current outbreak YFV is similar with those occured in the last 10 years, belonging to E1 genotype. This finding reiterates the endemicity of yellow fever in Brazil and emphasizes the need for consistent routine mass vaccination of the at-risk population to prevent future outbreaks, once several positive animals were found in urban areas or near them. As differences were found in *Callithrix* monkeys, more studies must be done in order to evaluate infection by YFV in this species. This report also highlights the importance of Adolfo Lutz Institute for YFV epizootics identification, an important tool for the prevention of human cases of sylvatic yellow fever.

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Fig 1. Yellow Fever Virus distribution in non-human primates by trimester (2016-2017) in São Paulo State.

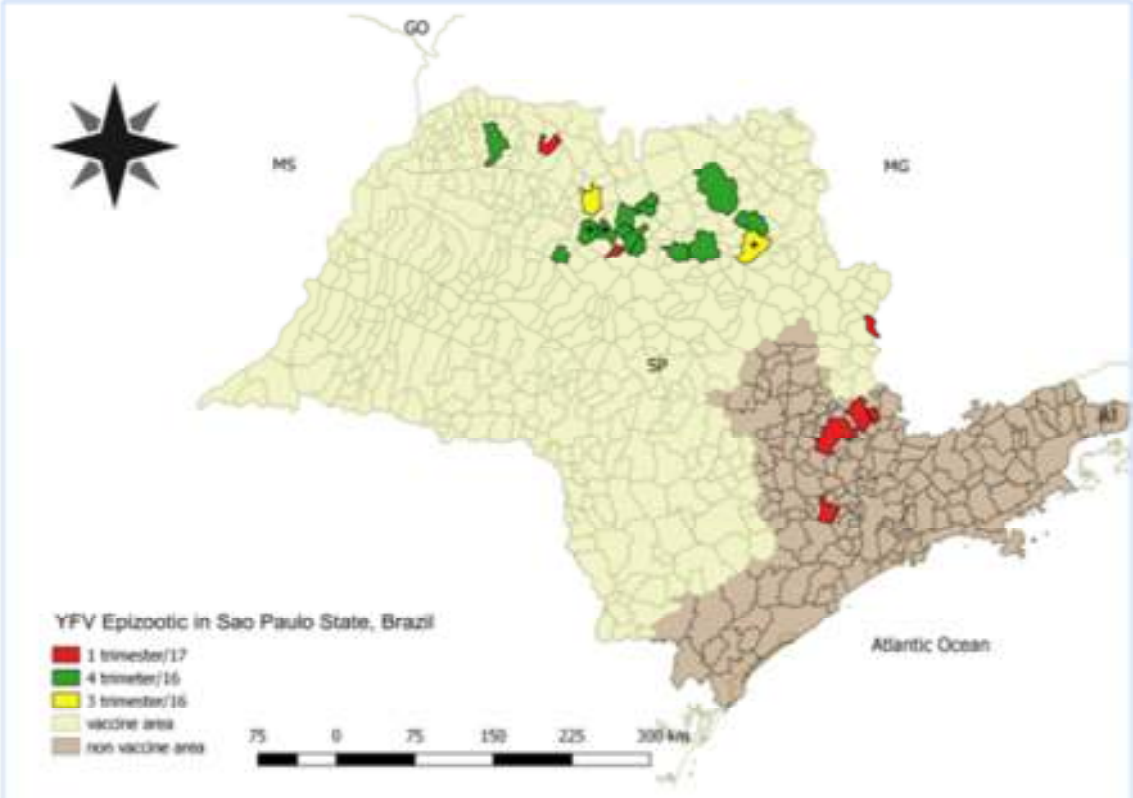

Fig 2. Phylogenetic analysis based on partial sequence of prM/E junction of a Brazilian strain of YFV. The tree was inferred using the maximum likelihood algorithm based on the Kimura two-parameter model with invariant sites as implemented in MEGA 7. The numbers shown to the left of the nodes represent bootstrap support values (1,000 replicates). Branch lengths do not represent genetic distance. Strains were labelled according to GenBank accession /strain/source/country/year of isolation.