Journal Pre-proof

Understanding Sabiá virus infections (*Brazilian mammarenavirus*)

Ana Catharina Nastri, Amaro Nunes Duarte-Neto, Luciana Vilas Boas Casadio, William Marciel de Souza, Ingra M. Claro, Erika R. Manuli, Gloria Selegatto, Matias C. Salomão, Gabriel Fialkovitz, Mariane Taborda, Bianca Leal de Almeida, Marcello C. Magri, Ana Rúbia Guedes, Lauro Vieira Perdigão Neto, Fatima Mitie Sataki, Thais Guimarães, Maria Cassia Mendes-Correa, Tania R. Tozetto-Mendoza, Marcilio Jorge Fumagalli, Yeh-Li Ho, Camila Alves Maia da Silva, Thaís M. Coletti, Jaqueline Goes de Jesus, Camila M. Romano, Sarah C. Hill, Oliver Pybus, João Renato Rebello Pinho, Felipe Lourenço Ledesma, Yuri R. Casal, Cristina T. Kanamura, Leonardo José Tadeu de Araújo, Camila Santos da Silva Ferreira, Juliana Mariotti Guerra, Luiz Tadeu Moraes Figueiredo, Marisa Dolhnikoff, Nuno R. Faria, Ester C. Sabino, Venâncio Avancini Ferreira Alves, Anna S. Levin

PII: S1477-8939(22)00097-7

DOI: https://doi.org/10.1016/j.tmaid.2022.102351

Reference: TMAID 102351

To appear in: *Travel Medicine and Infectious Disease*

Received Date: 17 March 2022

Revised Date: 27 April 2022

Accepted Date: 28 April 2022

Please cite this article as: Nastri AC, Duarte-Neto AN, Boas Casadio LV, Marciel de Souza W, Claro IM, Manuli ER, Selegatto G, Salomão MC, Fialkovitz G, Taborda M, Leal de Almeida B, Magri MC, Guedes AnaRú, Perdigão Neto LV, Sataki FM, Guimarães T, Mendes-Correa MC, Tozetto-Mendoza TR, Fumagalli MJ, Ho Y-L, Maia da Silva CA, Coletti ThaíM, Goes de Jesus J, Romano CM, Hill SC, Pybus O, Rebello Pinho JoãRenato, Ledesma FelipeLourenç, Casal YR, Kanamura CT, Tadeu de Araújo LeonardoJosé, Santos da Silva Ferreira C, Guerra JM, Moraes Figueiredo LT, Dolhnikoff M, Faria NR, Sabino EC, Ferreira Alves VenâAvancini, Levin AS, Understanding Sabiá virus infections (*Brazilian mammarenavirus*), *Travel Medicine and Infectious Disease* (2022), doi: https://doi.org/10.1016/j.tmaid.2022.102351.

AUTHORSHIP STATEMENT

Manuscript title: Understanding Sabiá virus infections (*Brazilian mammarenavirus*) - TMAID-D-22-00134 (TMAID 102351)

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

- Conceptualization: A.C.N., A.N.D-N., L.V.B.C., E.C.S., V.A.F.A. and A.S.L.
- Data curation: A.C.N., A.N.D-N., L.V.B.C., W.M.S. ,I.M.C., E.C.S., V.A.F.A. and A.S.L.;
- Formal analysis: A.C.N., A.N.D-N., L.V.B.C., W.M.S., I.M.C., S.C.H., O.P., J.R.R.P., C.T.K., L.J.T., C.S.F., J.M.G., L.T.F., M.D., N.R.F., E.C.S., V.A.F.A. and A.S.L.
- Funding acquisition: E.C.S.;
- Investigation: A.C.N., A.N.D-N., L.V.B.C., W.M.S., I.M.C., Y-L.H., E.C.S., V.A.F.A. and A.S.L.;
- Methodology: A.C.N., A.N.D-N., L.V.B.C., W.M.S., C.T.K., L.J.T., C.S.F., J.M.G., L.T.F., M.D., N.R.F., E.C.S., V.A.F.A. and A.S.L.
- Project administration: A.C.N., L.V.B.C. and A.S.L.
- Resources: A.C.N., A.N.D-N., L.V.B.C., W.M.S., E.R.M., G.S., M.C.S., G.F., M.T., B.L.A., M.C.M., A.R.G., L.V.P., F.M.S., T.G., M.C.M.C.., T.R.T.M., M.J.F., Y.H., C.A.M., T.M.C., J.G.J., C.M.R.; S.C.H., O.P., J.R.R.P., F.L.L., Y.R.C. and C.T.K.
- Software: W.M.S., I.M.C., N.R.F. and E.C.S.
- Supervision: E.C.S., V.A.F.A. and A.S.L.
- Visualization: A.N.D-N., L.V.B.C., W.M.S. and I.M.C.;
- Writing – original draft: A.C.N., A.N.D-N., L.V.B.C., W.M.S., I.M.C., C.T.K., L.J.T., C.S.F., J.M.G., L.T.F., M.D., N.R.F., E.C.S., V.A.F.A. and A.S.L.
- Writing - review & editing: A.C.N., A.N.D-N., L.V.B.C., W.M.S, I.M.C., E.C.S. and A.S.L.

These authors contributed equally to this study: A.C.N., A.N.D-N., L.V.B.C. and I.M.C.

These authors contributed equally to this study: N.R.F., E.C.S., V.A.F.A. and A.S.L.

1 **Title: Understanding Sabiá virus infections (*Brazilian mammarenavirus*)**

2 **Authors and affiliations:**

	Author	ORCID	Affiliation
1	Ana Catharina Nastri*	0000-0001-5415-6156	Division of Infectious Diseases, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
2	Amaro Nunes Duarte-Neto*	0000-0001-6659-7186	Department of Pathology, Faculdade de Medicina, Universidade de Sao Paulo, Brazil Núcleo de Anatomia Patológica, Instituto Adolfo Lutz, Sao Paulo, Brazil
3	Luciana Vilas Boas Casadio*	0000-0001-8341-8960	Division of Infectious Diseases, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
4	William Marciel de Souza	0000-0002-0025-8293	World Reference Center for Emerging Viruses and Arboviruses and Department of Microbiology and Immunology, University of Texas Medical Branch at Galveston, Galveston, Texas, USA

5	Ingra M. Claro*	0000-0001-8637-2910	Department of Infectious Diseases, Faculdade de Medicina, Universidade de Sao Paulo, Brazil Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
6	Erika R. Manuli*	0000-0003-2376-8488	Department of Infectious Diseases, Faculdade de Medicina, Universidade de Sao Paulo, Brazil Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
7	Gloria Selegatto	0000-0001-5038-4135	Division of Infectious Diseases, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
8	Matias C. Salomão	0000-0001-5507-0063	Infection Control Department, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
9	Gabriel Fialkovitz	0000-0002-3638-454X	Division of Infectious Diseases, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil

10	Mariane Taborda	0000-0002-9900-9894	Division of Infectious Diseases, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
11	Bianca Leal de Almeida	0000-0002-2742-5880	Division of Infectious Diseases, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
12	Marcello C. Magri	0000-0001-7996-8265	Division of Infectious Diseases, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
13	Ana Rúbia Guedes	0000-0002-6358-2912	Infection Control Department, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
14	Lauro Vieira Perdigão Neto	0000-0002-7681-8090	Infection Control Department, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
15	Fatima Mitie Sataki	0000-0002-7444-6983	Division of Infectious Diseases, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
16	Thais Guimarães	0000-0002-7282-5453	Infection Control Department, Hospital das Clínicas, Faculdade de Medicina,

			Universidade de Sao Paulo, Brazil
17	Maria Cassia Mendes-Correa	0000-0001-5655-8108	Department of Infectious Diseases, Faculdade de Medicina, Universidade de Sao Paulo, Brazil Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
18	Tania R. Tozetto-Mendoza	0000-0002-5659-1052	Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
19	Marcilio Jorge Fumagalli	0000-0002-0207-5423	Centro de Pesquisa em Virologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil
20	Yeh-Li Ho	0000-0001-8719-2508	Division of Infectious Diseases, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
21	Camila Alves Maia da Silva	0000-0002-4298-6782	Department of Infectious Diseases, Faculdade de Medicina, Universidade de Sao Paulo, Brazil Instituto de Medicina Tropical, Faculdade de Medicina, Universidade

			de Sao Paulo, Brazil
22	Thaís M. Coletti	0000-0003-1693-7984	Department of Infectious Diseases, Faculdade de Medicina, Universidade de Sao Paulo, Brazil Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
23	Jaqueline Goes de Jesus	0000-0002-5404-272X	Department of Infectious Diseases, Faculdade de Medicina, Universidade de Sao Paulo, Brazil Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
24	Camila M. Romano	0000-0003-4550-1987	Department of Infectious Diseases, Faculdade de Medicina, Universidade de Sao Paulo, Brazil Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
25	Sarah C. Hill	0000-0002-2995-2596	Department of Zoology, University of Oxford, United Kingdom Department of Pathobiology and Population Sciences, The Royal Veterinary

			College, UK.
			Department of Pathobiology and Population Sciences, Royal Veterinary College, Hatfield, United Kingdom
26	Oliver Pybus	0000-0002-8797-2667	Department of Zoology, University of Oxford, United Kingdom
27	João Renato Rebello Pinho	0000-0003-3999-0489	Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
			Hospital Israelita Albert Einstein, São Paulo, SP, Brazil
28	Felipe Lourenço Ledesma	0000-0001-7857-7318	Department of Pathology, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
29	Yuri R. Casal	0000-0002-0488-5307	Department of Pathology, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
30	Cristina T. Kanamura	0000-0003-2537-2356	Núcleo de Anatomia Patológica, Instituto Adolfo Lutz, Sao Paulo, Brazil
31	Leonardo José Tadeu de Araújo	0000-0002-5427-4053	Núcleo de Anatomia Patológica, Instituto Adolfo Lutz, Sao Paulo, Brazil

32	Camila Santos da Silva Ferreira	0000-0002-0386-5486	Núcleo de Anatomia Patológica, Instituto Adolfo Lutz, Sao Paulo, Brazil
33	Juliana Mariotti Guerra	0000-0002-0326-7187	Núcleo de Anatomia Patológica, Instituto Adolfo Lutz, Sao Paulo, Brazil
34	Luiz Tadeu Moraes Figueiredo	0000-0003-4544-6594	Centro de Pesquisa em Virologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil
35	Marisa Dolhnikoff	0000-0002-9073-9989	Department of Pathology, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
36	Nuno R. Faria†	0000-0001-8839-2798	Department of Zoology, University of Oxford, United Kingdom MRC Centre for Global Infectious Disease Analysis, J-IDEA, Imperial College London, London, United Kingdom Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
37	Ester C. Sabino†	0000-0003-2623-5126	Department of Infectious Diseases,

			Faculdade de Medicina, Universidade de Sao Paulo, Brazil Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
38	Venâncio Avancini Ferreira Alves†	0000-0001-5285-4460	Department of Pathology, Faculdade de Medicina, Universidade de Sao Paulo, Brazil
39	Anna S. Levin†	0000-0003-2427-8368	Division of Infectious Diseases, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil Department of Infectious Diseases, Faculdade de Medicina, Universidade de Sao Paulo, Brazil Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de Sao Paulo, Brazil Infection Control Department, Hospital das Clínicas, Faculdade de Medicina, Universidade de Sao Paulo, Brazil

* These authors contributed equally to this study.

† These authors contributed equally to this study.

Corresponding author

Luciana Vilas Boas Casadio

Hospital das Clínicas - Faculdade de Medicina da Universidade de São Paulo

Rua Dr. Ovídio Pires de Campos, 225 – 6th floor (sala 629) - A/C Sueli Ferreira

Cerqueira César - ZIP Code 05403-010

São Paulo – SP – Brazil

E-mail: lvbcasadio@usp.br

Phone/fax: +55 11 2661 7066

Abstract

Background: Only two naturally occurring human Sabiá virus (SABV) infections have been reported, and those occurred over 20 years ago.

Methods: We diagnosed two new cases of SABV infection using metagenomics in patients thought to have severe yellow fever and described new features of histopathological findings.

Results: We characterized clinical manifestations, histopathology and analyzed possible nosocomial transmission. Patients presented with hepatitis, bleeding, neurological alterations and died. We traced twenty-nine hospital contacts and evaluated them clinically and by RT-PCR and neutralizing antibodies. Autopsies uncovered unique features on electron microscopy, such as hepatocyte "pinewood knot" lesions. Although previous reports with similar New-World arenavirus had nosocomial transmission, our data did not find any case in contact tracing.

Conclusions: Although an apparent by rare, Brazilian mammarenavirus infection is an etiology for acute hemorrhagic fever syndrome. The two fatal cases had peculiar histopathological findings not previously described. The virological diagnosis was possible only by contemporary techniques such as metagenomic assays. We found no subsequent infections when we used serological and molecular tests to evaluate close contacts.

Word count: 171 words

Keywords: Arenaviruses, Viral hemorrhagic fever, Disease Transmission.

Introduction

Approximately 20 years after the last [1] reported *Brazilian mammarenavirus* (BM) infection (an arenavirus known as Sabiá virus, SABV), we detected two new cases of *Mammarenavirus* in 2019. Arenaviruses are divided into two serocomplexes: New-World (NW) and Old-World (OW) arenaviruses, divided into four genera in the *Arenaviridae* family [2]. Genus *Mammarenavirus*, whose hosts are mammals, presents third-nine species, including the *Brazilian mammarenavirus* [3].

Arenaviruses have been known to infect rodents for thousands of years, and wild rodents are the primary host of most NW arenaviruses. Regardless, since 1950 human hemorrhagic fevers caused by these agents have been described in South America [2]. The animal reservoir of *Brazilian mammarenavirus* (BM) remains unknown besides a dedicated search [4].

Many aspects of the pathophysiology of BM infection remain unknown. The pathogenicity of the infection differs among the different arenaviruses as Machupo, Chaparé, and Junín, but mortality rates for all of them range around 30% [5–7]. The initial symptoms of arenavirus infections are nonspecific and include flu-like symptoms, mainly fever, and gastrointestinal symptoms such as nausea, vomiting, and diarrhea [2]. Approximately 25% to 30% of patients develop hemorrhagic phenomena and neurological disorders, configuring a differential diagnosis for other tropical hemorrhagic fever such as dengue and yellow fever (YF) [8–10].

The infection route of mammarenaviruses is suspected to be by aerosol inhalation or direct mucosal contact with rodent excrements as in other South American hemorrhagic fevers caused by *Arenaviridae* family [11]. Possible person-to-person transmission is still unclear but there are reports of healthcare workers handling infected patients and becoming ill. Considering this, the Centers for Disease Control and Prevention in their Biosafety in microbiological and biomedical laboratories guidelines, recommend biosafety level 4 for some hemorrhagic fevers, including SABV [12].

Before this study, only four BM infections had been previously described. The two reported

natural infections occurred in the city of Cotia in 1990 and the city of Espirito Santo do Pinhal in 1999. Both cities are in the rural area of the state of Sao Paulo and are approximately 155 km apart. These two were infected rural workers. Two others were laboratory workers thought to be infected while handling viruses. The two infected rural workers died, and two individuals infected by manipulating virus samples in the laboratory survived [1,13–15]. The Figure 1 shows the probable localities of the autochthone SABV infections, and the year in which they were reported.

In 2016-17, YF cases through Brazilian territory started extending their historical area, reaching the State of Sao Paulo, mostly previously considered YF-free [16]. In 2019, YF cases also arrived at an impoverished area of the state of São Paulo called "Vale do Ribeira" - Ribeira de Iguape River Valley and where the city of Eldorado is located [17]. This region has one of Brazil's most well-preserved Atlantic forest areas, and its population lives in very close contact with the forest itself [18,19].

The two cases of BM reported here were initially admitted to Hospital das Clínicas, city of São Paulo, Brazil, with the diagnostic hypothesis of severe YF cases. However, they tested negative for YF by molecular assays in blood and urine samples, but positive for BM. This investigation aims to describe these two cases of BM infection, their clinical presentation, autopsies, and the investigation of the contacts to evaluate for potential nosocomial transmission.

Material and methods

In 2018 and 2019, the southeast Brazilian region witnessed a large YF outbreak in an area in which without a YF vaccination was not previously recommended. At that period, 2,585 cases of YF were reported in Brazil and 571 cases in the state of São Paulo. Hospital das Clínicas (HC), São Paulo, Brazil, treated severe YF during the epidemic, receiving 133 cases [20] from January 2018 to April 2019. Sixty-seven patients were included in a randomized controlled clinical trial with sofosbuvir for YF treatment ("SOFFA" clinical trial) [21]. The inclusion criteria were patients with a history of fever (axillary temperature > 37.8°C), exposed to geographic areas with YF transmission, and aminotransferases above 500 U/L. YF infection was confirmed by YF viral detection in serum and/or urine samples. In patients inclusion criteria, but YF negative tests for YF, we investigated other common causes of acute hemorrhagic diseases, including dengue fever, malaria, leptospirosis; viral hepatitis A, B, C, Delta, and E; acute infections such as HIV, toxoplasmosis, cytomegalovirus, Epstein-Barr virus, herpes simplex virus, and bacterial septic shock. The differential hypotheses were ruled out by serology and molecular methods and culture, when applicable. The metagenomic investigation was done when all tests were negative. All YF-negative cases were subjected to this investigation.

AUTOPSIES AND HISTOLOGY

Autopsies were done for both patients following Letulle's technique [22]. Tissue samples were fixed in buffered 10% formalin, embedded in paraffin, and stained. Autopsy examiners wore personal protective equipment, but the autopsy room was not a biosafety level 3 facility.

Immunohistochemistry for arenavirus antigens in liver tissue was performed at Instituto Adolfo Lutz. After deparaffinization and heat-induced epitope retrieval (citrate buffer ten mM / pH6), slides were incubated with a primary antibody, a rabbit hyperimmune serum for Amapari virus (AMAV) strain BeAn 70563 (Serra do Navio mammarenavirus), optimized at a dilution of 1: 2,000 with incubation for 18 h at 4°C, previously validated with negative and positive controls. The amplification signal was achieved by alkaline phosphatase-conjugated polymer (Polink-2 AP Broad

Detection System; GBI Labs, WA, USA, cat. D24-110), revealed by Fast Red chromogen (GBI Permanent Red Kit; GBI Labs, WA, USA, cat. C13-120). Finally, the slides were counterstained with Mayer's hematoxylin (Polysciences, Inc.; PA, USA). We further tested this primary antibody in liver samples from cases with hepatitis due to other etiologies, with negative results.

Transmission electron microscopy (TEM) of liver tissue samples was performed as previously described [23], using Philips Tecnai 10, Hillsboro, OR, USA, 80 kV microscope.

SABV RNA SEQUENCING METAGENOMIC TECHNIQUE

Briefly, patients' samples were collected in EDTA tubes. RNA extraction, treatment, and sequencing were conducted in a BSL3 Laboratory at IMT-FMUSP using the MinION platform from Oxford Nanopore Technologies (ONT) as previously described [24] with small modifications, specifically the metagenomic amplicon fragments were prepared for sequencing using the genomic sequencing kit LSK109 without barcoding. Metagenomic amplicon fragments were prepared for sequencing using the genomic sequencing kit LSK109 without barcoding. Each sample was subjected to two technical replicates (RNA extraction, cDNA synthesis, PCR, library generation) before running on separate, fresh ONT flowcells (one per replicate) to help rule out the possibility of between-sample or barcode contamination. This way, we could generate a large data volume to analyze and characterize data for each SABV case. Finally, mapping parameters were used to increase sensitivity during genome analysis, and a threshold of 5x was used for variant calling before building the SABV genome consensus.

MOLECULAR STUDY IN PARAFFIN-EMBEDDED TISSUES

RNA extraction

Each FFPE liver sample was sectioned in two tapes of 9 μm thickness, conditioned in sterile microtubes of 1.5 mL. To perform RNA extraction, both FFPE and FF samples underwent QIAmp Viral RNA Mini kit (Qiagen, Hilden, Germany) protocol according to the manufacturer's instructions. Dewaxing and lysis were conducted as previously described [25].

Nested RT-cPCR assays for Mammarenavirus

For the detection of Arenavirus, nested RT-cPCR was performed in the Veriti Thermal Cycler instrument (ThermoFischer Scientific, Massachusetts, EUA) using the GoTaq® Probe 1-Step System (Promega, Wisconsin, USA). Primary reactions used 5 µL of extracted RNA samples as a template in a total volume of 25 µL. The assay includes a set of oligonucleotide primers described previously [26]. RNAse P (IDG, Iowa, USA) was run as a positive control gene in another assay as described [25]. All reactions included two types of no template negative controls (NTC), one added during RNA extraction (NTCe) and the other added during PCR reactions (NTC). Thermal cycling conditions were one cycle at 45°C for 30 min, RT inactivation at 95°C for 5 min and 95°C 30 s, 55°C 30 s (Pos_29F_outer and Pos_381R_outer) and 69°C 30 s (S_outer_1_f and S_outer_1_r), 72°C 1 min for 35 cycles and final extension occurred at 72°C for 7 min. Nested amplification used 5 µL of the primary PCR product as the template in a total volume of 25 µL. Cycling conditions were one cycle at 95°C for 5 min and 95°C 30 s, 52°C 30 s (Pos_171F inner and Pos_381R_inner) and 68°C 30 s (S_inner_1_f and S_inner_1_r), 72°C 1 min for 35 cycles and final extension occurred at 72°C for 7 min. PCR products were loaded in a 2% agarose gel using 1X TBE buffer (LGC, Sao Paulo, Brazil), following the protocol described.

CONTACT INVESTIGATION

Both patients were under standard precautions while hospitalized. Contacts of Case A were traced and followed up. Case B was identified one month after his death; thus, contact tracing was not performed. Contacts were classified according to exposure. *High exposure* was defined as contact with respiratory secretions (orotracheal intubation, airways aspiration), exposure of mucous membranes to blood/bodily fluids, needle-stick, and acute injuries with exposure to blood/bodily fluids; participation in the autopsy; or cleaning of the patient's room. *Moderate exposure* was defined as physical examination, administration of medication, or collection of exams. *Minimal exposure* was defined as entering the patient's room without contact with the patient or his body fluids. Contacts were questioned about symptoms: fever, odynophagia, conjunctivitis, rash, tiredness, malaise, muscle pain, headache, neurological disorders, diarrhea, jaundice, and bleeding.

All high exposure contacts and those who presented any symptom underwent physical examination and testing (RT-PCR for SABV and clinical blood tests). Neutralizing antibodies were tested initially and 4-6 weeks later.

PLAQUE REDUCTION NEUTRALIZATION TEST (PRNT50)

The PRNT is the gold-standard for the differential diagnosis of several emerging viruses (e.g., arenaviruses, flaviviruses, orthohantaviruses) and allows for determining and quantifying the neutralizing antibodies of a previous viral infection. Thus, based on the small number to be tested, we chose to perform the PRNT instead of the ELISA [27,28].

Human serum samples were tested by 50% plaque reduction and neutralization tests (PRNT50) for Amapari virus (AMAV) strain BeAn 70563 was undertaken for contacts' serum samples as previously described [29]. Briefly, 5×10^4 Vero E6 cells were seeded in each well of a 48-well plate one day before infection. All serum samples were heat-inactivated at 56°C for 45 min and serially diluted from 1:5 to 1:10,240 in DMEM (Dulbecco's Modified Eagle's Medium). Serum dilutions were mixed with 102 PFU of AMAV and incubated for one hour at 37°C. After incubation, 50 µl of the serum-virus mixture was inoculated into the Vero cell monolayers and incubated for one hour at 37°C under gentle rocking for viral adsorption. Subsequently, 500 µl of prewarmed 1.5% Carboxymethylcellulose (CMC)–DMEM containing 2% fetal bovine serum was gently added to each well, and the plates were incubated at 37°C in a 5% CO_2 atmosphere for two days. Finally, cells were fixed with 2 ml of 10% formaldehyde solution for two hours and stained with 1% naphthalene black dye for 30 min. Plaque reduction was calculated by comparing the number of plaques in wells inoculated with serum-virus mixtures with those inoculated with only 102 PFU of AMAV (positive control) assayed simultaneously.

Results

The two clinical cases of BM were very similar to severe yellow fever cases and were only investigated for a different cause because of negative RT-PCR for YF. Both diseases cause acute hepatitis and depressed consciousness. On the other hand, histopathology for *mammarenavirus* infection was distinctive and remarkable.

Eight of sixty-seven were YF-negative, and two were diagnosed with BM infection (25%). The remaining six cases had no clear infectious cause. The timeline of the investigation of these cases is shown in Figure 2. The individual case reports are described in detail.

CASE REPORTS

Case A

On December 30, 2019, a 52-year-old man from the city of Sorocaba, in the State of São Paulo (100 km west of the city of São Paulo), who had hiked in the forest in city of Eldorado (170 km south of the city of São Paulo), presented with odynophagia, myalgia, abdominal pain, and dizziness. The following day, symptoms worsened, and he developed conjunctivitis. At a local hospital, he was medicated and released. Four days later, he was admitted to a regional hospital with a high fever and drowsiness. YF was suspected, and he was transferred to HC with fever, bilateral conjunctivitis, bilateral cervical lymph node enlargement, dry cough, drowsiness, lethargy, and mental confusion. Laboratory tests showed lymphopenia, thrombocytopenia, and increased creatine phosphokinase and transaminases (Figure 2). He tested negative for dengue by non-structural protein 1 (NS1) point-of-care test. CT scan showed cervical lymph node enlargement, small bilateral pleural effusion, low perihepatic and pelvic fluid, normal liver, and a duodenal ulcer. He then deteriorated neurologically. Bleeding occurred at sites of puncture for a myelogram and cerebrospinal fluid. He was transferred to the intensive care unit (ICU) ten days after the onset of symptoms, with significant bleeding, renal failure, decreased consciousness, and hypotension subsequently developed oliguria, and died two days later. SABV was diagnosed by metagenomic testing three days after his death. This case was the object of a preliminary report [26]. Since the

cause of death remained unrecognized at that time, and all YF tests were negative, we requested a metagenomic evaluation that identified SABV infection. Samples from the other seven YF-negative patients were subjected to metagenomic sequencing. The genomic analyses, including developing a diagnostic RT-PCR, are described elsewhere [*Claro et al*., submitted data].

Case B

The second case of BM was then diagnosed, which had occurred a month before Case A. On December 1st, 2019, a 63-year-old male rural worker from Assis, a small town of the State of São Paulo (440 km west of the city of São Paulo), presented with fever, generalized myalgia, nausea, and prostration. Symptoms worsened, and eight days later, he was admitted to HC with depressed consciousness and respiratory failure requiring intubation. Laboratory tests showed high transaminases and thrombocytopenia (Figure 2). Severe left ventricular dysfunction, measured by echocardiography, led to refractory shock and death on the 11th day after the onset of symptoms. At that moment, the cause of death was mainly organ dysfunction as BM had not yet been described in the region where the patient proceeded. Cases A and B were infected in regions located 315 Km apart.

AUTOPSY FINDINGS

In HC we encourage autopsies in patients who died without an apparent explanation or in an unexpected way should be referred to autopsies studies for elucidating the cause of death, especially in teaching hospitals such as HC. During the YF outbreak, we performed autopsies attempting to understand defined and investigate the disease. Both cases were deceased without a precise cause, and the physician team ordered the autopsies before the BM diagnosis was made.

Main autopsy findings were common to both patients (Figure 3): enlarged liver with a yellowish orange cut surface; pulmonary edema, hemorrhages, and areas of consolidation; visceral congestion. Microscopy showed pan-lobular hepatitis with the rare confluence of damaged mid-zonal or centrilobular hepatocytes; enlarged or normal hepatocytes, exhibiting

macro/microvesicular steatosis ("moruliform aspect"); and apoptotic bodies scattered in the plates or within sinusoids. Nuclei were frequently enlarged, and numerous cells exhibited PAS-positive eosinophilic bodies. Multinucleation was frequent. Sinusoids were congested with neutrophils and mononuclear cells. Kupffer cells were hypertrophic with engorged, eosinophilic cytoplasm. Inflammatory infiltrates scant, mainly in the portal area. Lobular fibrosis was absent, and the reticulin framework was preserved. Other vital organs were relatively preserved from direct damage caused by BM. We did not find myocarditis, encephalitis, nephritis, or pancreatitis.

The immunohistochemistry for detecting BM-antigens was positive, in both patients, in the liver and labeled steatotic or apoptotic hepatocytes, Kupffer cells, and inflammatory portal cells. TEM showed viral particles in hepatocytes and Kupffer cells cytoplasmic vesicles. A nuclear eosinophilic inclusion corresponded to an electron-dense body with peripheric radial layers, resembling a "pinewood-knot." Kupffer cells showed fragments of phospholipids in their cytoplasm, indicating cytophagocytosis.

Both patients had diffused acute alveolar damage. One case had Gram-positive cocci bronchopneumonia, the other angioinvasive aspergillosis. These results were only seen on autopsy, and no clinical data had indicated those points while the patient was alive. It is impossible to rule out these secondary infections' participation in the outcome, but they probably contributed to the lethal result.

Other findings included acute hemophagocytosis in bone marrow, lymph nodes, spleen, and liver; reactive splenic lymphoid cells; reactive microglia; foci of perivascular cerebral hemorrhage; acute tubular injury; foci of lymphomononuclear adrenalitis, and arterial hypertension-associated vascular alterations. Case A's FFPE liver sample was positive for SABV (all four Pos-primers), Case B was negative.

CONTACT INVESTIGATION WITH SEROLOGICAL AND MOLECULAR TESTS

One-hundred-seventeen people were exposed to Case A: one patient, 21 doctors, 57 nursing professionals, four physiotherapists, three dentists, 21 cleaning workers, and ten laboratory

253 professionals; 20 had high exposure (seven of them presented mild symptoms during follow-up).
254 Nine professionals with moderate/minimal exposure had symptoms. Symptoms were headache (6);
255 diarrhea (5); sore throat (4); arthralgia (3); myalgia, upper respiratory symptoms, fever, malaise,
256 vomiting (2 each); abdominal pain (1). All resolved spontaneously within a few days. All 29 (20
257 high exposure, nine symptomatic) tested RT-PCR-negative. Twenty-eight (97%) tested initially,
258 and 23 (79%) tested 4-6 weeks later had undetectable neutralizing antibodies.

Discussion

We describe two naturally occurring fatal infections caused by BM, similar to severe yellow fever, presenting with hepatitis, severe bleeding, and neurological alterations. Two (3%) of 67 cases thought to be severe YF were caused by BM, suggesting that BM disease may not be as rare as believed. No secondary cases were detected among contacts. On electron microscopy, autopsies uncovered unique features, mainly “pinewood-knot" lesions in hepatocytes.

BM infection is a differential diagnosis in areas where other hemorrhagic fevers occur. Although our two cases were diagnosed by chance during a YF outbreak, our findings suggest that clinicians should be aware of this unusual diagnosis. Both patients lived in different areas of the state of Sao Paulo, but both were exposed to the countryside, as one was a rural worker and the other had been hiking in the Atlantic Forest. The natural reservoir for BM is still unknown. Thus, it is difficult to determine the exposure to rodents or other small mammals due to the lack of surveillance for *mammarenavirus* animal infections in this region.

YF and BM may have similar clinical presentations, with an acute onset of liver damage and hemorrhagic events leading to hospitalization in the intensive care unit. *Mammarenavirus* has the potential to cause very aggressive and lethal infections. However, there probably is an unknown spectrum of undiagnosed presentations, with mild symptoms such as fever, myalgia, and fatigue that may be interpreted as unspecified viral infections. Zoonotic infections occur in regions with poor healthcare services and underdevelopment areas, thus are probably neglected. Even severe cases may not be diagnosed due to the lack of molecular assays in most countryside regions.

Molecular diagnostic tools for low-income scenarios should be a research and development priority. An offer of robust diagnostic assays will presumably raise the observation and detection of other cases. MinION is a technique that has grown significantly in recent years, both in terms of the availability of sequencing kits and the volume of data collected. As previously described in other articles [30–35], it has also been frequently used for whole-genome sequencing and genomic-epidemiological surveillance of viruses. In light of this, we recognized the potential for developing

a quick metagenomics technique based on MinION technology, which has already been assessed and compared to targeted methods [24]. When we compared the genomes obtained by metagenomics sequencing to prior references and accessible primers, we discovered that the currently employed PCR methods were unable to detect circulating SABV strains due to the virus's genetic variability in primer binding sites. Using the genomic data obtained, we built a PCR scheme to test the Case A close contacts for SABV, as well as a more sensitive nested PCR methodology that effectively found SABV in a low viral titer sample from Case B. In each of these conditions, the available PCR primers were unsuccessful and as a response, a new PCR was created. [*Claro et al*., submitted data]. Another explanation for the small number of cases described to date is that the reservoir, currently unknown, may not have significant interactions with humans. Finally, the infectiousness of SABV may be very low, accounting for its rarity in humans.

Current knowledge suggests that the transmission route of mammarenaviruses is the inhalation of aerosolized viral particles, mainly present in dry rodent excretions, with viral replication after reaching the lung [8,36]. This fact was the hypothesized route of accidental human infections during the handling of SABV cultures [37]. Since our both cases had close contact with rural areas and no other suspicion for BM or YF cases was reported at the same time to the Public Health authorities, we suspected that the infection source might be a wild exposure and not an infected human source.

Our two cases were initially thought to be infected with YF and were not isolated, and they only standard precautions were used. While it is impossible to rule out person-to-person transmission, none of Case A's contacts had evidence of infection, suggesting that person-to-person transmission in health care settings may be infrequent and preventable using standard precautions. We have used infection control measures developed for hemorrhagic infections as those used for Ebola virus management had BM been suspected. Other possible determinants for viral transmissions may be viral load in body fluids and disease evolution stage [38,39].

BM-associated lesions were mainly centered in the liver and the reticuloendothelial system at the pathology studies. In the liver, liver damage was characterized by finding macro and

microvesicular steatosis and apoptosis of hepatocytes, with minimal inflammatory reaction. TEM detected only scarce virions. Taken together, these aspects suggest:

1. rapid viral clearance in the liver, with few virions producing severe hepatocyte damage;
2. the host immune system may be involved in the induction of hepatocyte apoptosis, and
3. oxidative lipid metabolism may be involved in liver pathogenesis.

BM induced intense macrophage reactivity in the reticuloendothelial system, corroborated by the cellular phagocytosis in the liver by Kupffer cells, sinusoidal cells in the spleen, and macrophages in the bone marrow and lymph nodes. This finding may explain the profound lymphocyte depletion observed, possibly resulting in opportunistic and nosocomial secondary infections (aspergillosis and nosocomial bacterial infection). Moreover, hemophagocytosis might result in inflammatory status, leading to multiple organ failure. Both patients presented secondary infections at autopsy. These probably contributed to the fatal outcome.

The similarity between the SABV virus hepatitis and YFV hepatitis presents a diagnostic challenge in areas where hemorrhagic fevers are relatively common and occurs seasonally. In addition to dengue, leptospirosis, and reemergent yellow fever, we must consider mammarenavirus infections in Brazil. Macroscopically, the lesions caused by the SABV are similar to YF and dengue. On a rapid examination, histology can lead to a misdiagnosis of yellow fever, with essential repercussions on control and preventive health actions. The cases described herein allowed us to standardize ancillary techniques in pathology, such as immunohistochemistry and RT-PCR in paraffinized samples. Not less importantly, we detailed the histological and EM studies to aid and instruct pathologists to diagnose. The enlarged hepatocytes, the cytopathic change in the hepatocyte nucleus, and a non-frequent mid-zonal lesion are suggestions of SABV hepatitis and not YFV hepatitis.

The IHC for detection of YFV antigens in the BM cases showed intense unspecific staining, but this signal is restricted to the cytoplasm of Kupffer cells, attributed to rests of phagocytized cells, which are PAS-positive. Such a pattern is different from our previous report on YFV

hepatitis, where significant positive signals were found in the cytoplasm of hepatocytes, including those undergoing steatosis or apoptosis [40,41].

BM may be more widespread than previously described. Cases A and B's probable place of infection is approximately 315 Km apart. Serological testing of people living in the rural areas from where these cases originated, and molecular testing of YF-negative cases should be performed to understand BM mode of transmission, pathogenicity, and transmissibility. Additional research should focus on investigating rodent reservoir species of BM in or around the probable location of the infections. Furthermore, it is necessary to address how ecological changes may be associated with a possible increase in the number of cases in humans [9].

Our study has limitations as we observed only two cases. However, considering how little is known about BM infection, we feel that knowledge about this disease has substantially increased.

Conclusion

In conclusion, the finding of 2 severe cases of SABV infection allowed us to characterize its clinical manifestations, histopathology, and evaluate the possibility of nosocomial transmission. Detection of these two BM cases in YF suspected cases must alert clinicians for the possible diagnosis in patients with acute illness with hemorrhagic phenomena.

Word count: 4107 words

ACKNOWLEDGMENTS

Janaína Loureiro da Costa, Fernanda Oliveira Santos, Fernanda Spadão for their help in evaluating contacts. Deyvid Amgarten, Fernanda Malta for their insights in molecular methods.

ETHICAL APPROVALS

HC-FMUSP Ethics in Research Committee (Processes #2.669.963; #3.258.615; and #3.371.745).

FUNDING

São Paulo Research Foundation (FAPESP)-UK-Brazil Joint Center for Arbovirus Discovery, Diagnosis, Genomics, and Epidemiology (CADDE) (grant 2018/14389-0).

MJF is supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grant 2018/09383-3).

WMS is supported by the Global Virus Network fellowship and the NIH (AI12094).

SCH is supported by a Wellcome Trust Sir Henry Wellcome Postdoctoral Fellowship 220414/Z/20/Z.

MD is supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico, 304987/2017-4.

NRF is supported by a Wellcome Trust and Royal Society Sir Henry Dale Fellowship (204311/Z/16/Z).

374 **Figures and captions**

375 **Figure 1.** State of São Paulo, Brazil, South America. In the figure's legend, the distance between the
376 cities is in kilometers. The black star is for the city of São Paulo, the state capital, and where
377 Hospital das Clínicas is located. The green line delimiters the State of São Paulo borders. The red
378 pins show the probable localities of the autochthone SABV infections, and the year reported. #1
379 Cotia, 1990 (Coimbra et. al 1994); #2 Espírito Santo do Pinhal, 1999 (Coimbra et al. 2001); #3
380 Assis, 2019; #4 Eldorado, 2020. Distance between #1 to #2: 155 km; #2 to #3: 380 km; #3. #3 to
381 #4: 315 km. Figure made by WMS with "Scientific colour maps" software [42].

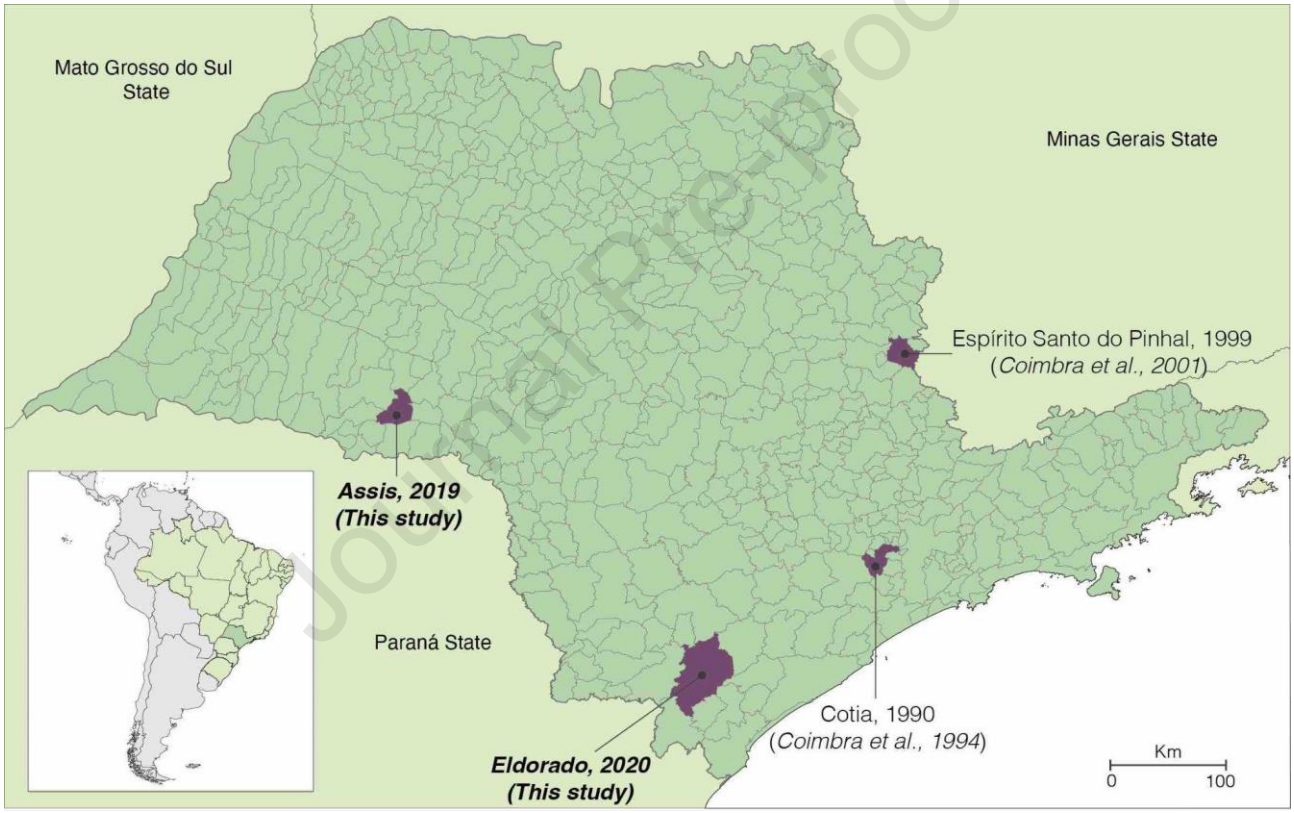

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Figure 2: Timeline of investigation and results of clinical laboratory tests of 2 patients with fatal infection caused by Sabiá virus (SABV). (Laboratory normal reference ranges: AST < 41 U/L; ALT < 37 U/L; Platelets 150 to 400 x103; INR 0,96 to 1,20; R 0,8 to 1,17; Factor V 62 to 139%; DHL 135 to 225 U/L; CPK < 190 U/L). (AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; CPK: creatine phosphokinase; PT/INR: prothrombin time/ international normalized ratio; APTT: activated partial thromboplastin time; HC: Hospital das Clinicas; CSF: cerebrospinal fluid; ICU: intensive care unit; H.C.W.: healthcare workers; RT-PCR: polymerase chain reaction; NS1: nonstructural protein 1).

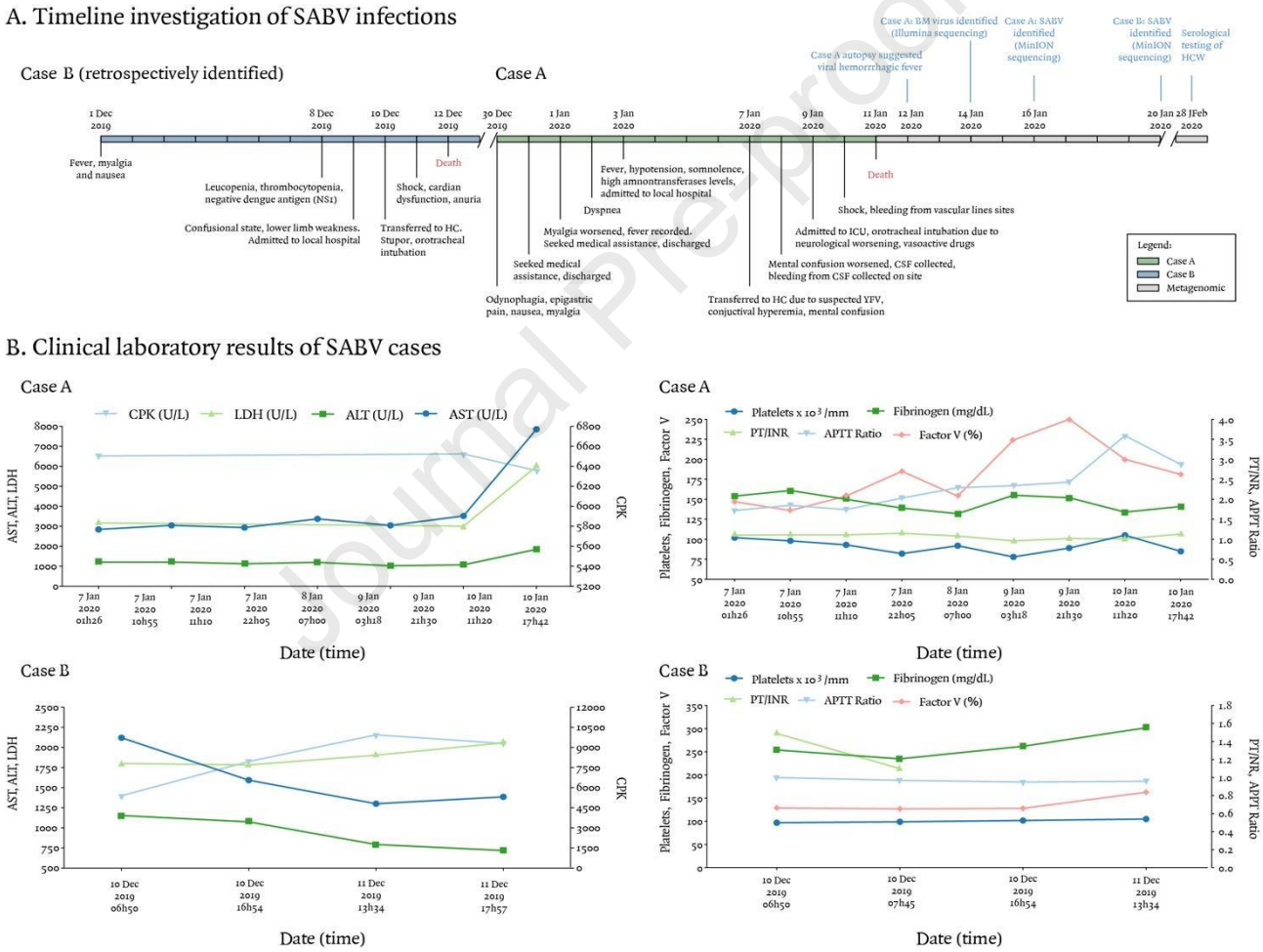
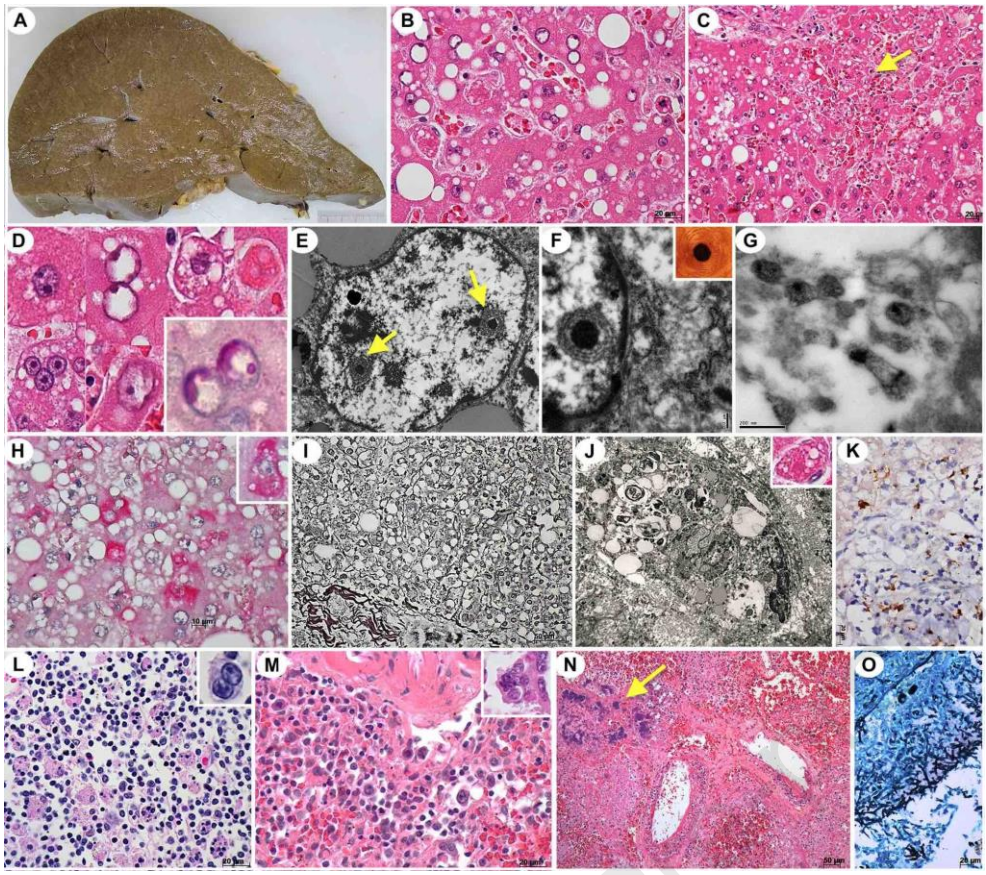


Figure 3. Pathology of severe Sabiá virus (SABV) infection. A. Gross exam showing enlarged liver with steatotic and icteric cut surface. B. Pan lobular hepatitis with hepatocytes exhibiting macrovesicular steatosis, apoptosis, hypertrophic, and eosinophilic Kupffer cells. Inflammation is scant. C. The confluence of apoptotic hepatocytes in midzonal/centrilobular areas is not frequent, contrary to yellow fever hepatitis. D. Cytopathic change characterized by enlarged uni or multinucleated hepatocytes with an eosinophilic nuclear dot, centric, eccentric, single or double, and apoptotic body with some fat drops. Nuclear dots may be PAS positive E-F. Steatotic hepatocyte with two round nuclear bodies (E), with a dense core, surrounded by concentric layers (F), resembling a pinewood knot (F, inset). G. Viral particles, pleomorphic, with a dense core, measuring 30-400nm, within cytoplasmic vesicles. H. The immunohistochemistry-staining the cytoplasm of some large, steatotic, and apoptotic hepatocytes. I. Preserved liver reticulin framework. J. EM shows that engorged Kupffer cells (inset) have fragments of cellular membranes in their cytoplasm. K. Macrophages mainly mediate the immune response in situ. CD68 expression in Kupffer cells and the portal inflammatory infiltrate. L. Lymph node showing intense hemophagocytosis and reactive lymphoid cells resembling Reed-Stenberg cell (inset). M. White pulp of the spleen with lymphoid depletion and reactive cells. The inset shows emperipolesis by megakaryocytes in the bone marrow). N. Suppurative bronchopneumonia due to Gram-positive cocci (arrow) and associated necrotizing vasculitis, with pulmonary hemorrhages and tissue ischemia. O. Invasive pulmonary aspergillosis.



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