SUPPLEMENTAL DIGITAL CONTENT

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1. **METHODS**

**Real-time PCR.**  Samples from tracheal aspirate, urine, CSF and serum, were extracted on the NUCLISENS® easyMag® platform (BioMerieux). All extracts were tested for human RNase P gene by RT-PCR to confirm sample quality. The PCR reaction was carried out with RNA or DNA from each sample with a set of primers and probes with FAM as dye reporter for the probe. Primers/probes for Sars.Cov2 [1], Adenovirus (AdV), Influenza A and B (FluA and B), Respiratory Syncytial Virus A and B (RSV A and B), human Metapneumovirus (hMPV), Rhinovirus (RV), Enterovirus (EV), Parainfluenza Virus 1-4 (PIV 1-4), sazonal Coronavirus OC43, CoV-HKU1, CoV229E and CoVNL63 [2-4], Herpesvirus (HHV1, 2), CMV (HHV-5) [5], Parvovirus B19 (PV-B19) [6], and Measles virus (MV) [7], Zika Virus (ZIKV) [8], Dengue virus (DENV) [9] and Chikungunya virus (CHIKV) [10]. All assays were performed using the AgPath-IDTM One-Step RT-PCR reagents (Applied Biosystems).

**Enzyme-linked immunosorbent assay (ELISA).** Viral diagnostics of the infections was performed for Immunoglobulins (IgA, IgM and IgG) nucleoprotein-based SARS-CoV-2 enzyme-linke immunosorbent assays (ELISAs). Briefly, 96-well plates (Costar) were coated with the nucleoprotein antigen (ncov-PS-Ag7) (0.2 ug/mL in sodium carbonate–sodium bicarbonate buffer) and incubated at 37°C for 1 hour. Unspecific binding of the antibodies was avoided by blocking with BS (Advagen Biotech ltda,Itu, Brazil) at 37°C. After washing three times with PBST, 100 μL of appropriately diluted serum or plasma sample in PBST was added and incubated for 1 h at 37°C. After washing three times with PBST, the bound antibodies were detected by using the following secondary antibodies conjugated with horseradish peroxidase diluted 1:4000 of goat anti-human IgA, IgM and IgG (Sigma-Aldrich Co., Deisenhofen, Germany). After incubation for 1 h at room temperature and three PBST washes, 100 μL of 3, 3′, 5, 5′- tetramethylbenzidine (Sigma) was added to each well and the mixture was incubated for 10 min at room temperature. The reaction was stopped by adding 0.2 N sulfuric acid to the mixture, and the optical density at 450 nm was measured.

**Cytopathic effect-based virus neutralization test (CPE-VNT).** The CPE-VNT was carried out with SARS-CoV-2 (MT126808.1) in 96-well plates containing 5x10 4 cells/mL of Vero cells (ATCC CCL-81). Plasma samples were, initially, inactivated for 30 min at 56°C. We used 8 dilutions (two-fold) of each plasma (1:20 to 1:2560). Subsequently, plasma was mixed vol/vol with the virus (100 TCID 50 per well) and pre-incubated at 37°C for 1 hour to allow virus neutralization. Then the plasma+virus mixture was transferred onto the confluent cell monolayer and incubated for 3 days at 37°C and under 5% CO 2 . After 72 hours, the plates were analyzed directly with transmitted-light bright-field microscopy (Olympus Co., Tokyo, Japan). Gross cytopathic effect can be observed on Vero cells, being able to distinguish the presence/absence of viral cytopathic effect caused by SARS-CoV-2. Virus neutralization titer referred to VNT 100 is described as the highest dilution of serum that neutralized virus growth. Alternatively, for double check of the titers, the plates were fixed and stained for 30 min with 0.2% Naphthol blue black solution (Sigma-Aldrich Co., Deisenhofen, Germany) and then photographed for documentation of culture morphology. In each assay, a strong, assured internal positive control serum (RT-qPCR positive + PRNT 90 &gt;640) (11) was used, as a negative pre-outbreak serum sample. The method here described was adapted from Nurtop et al., 2018 (12) and has been widely used for SARS-CoV serologic studies (13–18). All the procedures related to CPE-VNT were performed in a biosafety level 3 laboratory, following WHO recommendations (9).

1. **ADDITIONAL REFERENCES**
2. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020;25(3):1–8.
3. De Onis M, Habicht JP, Kodani M, Yang G, Conklin LM, Travis TC, et al. Application of TaqMan low-density arrays for simultaneous detection of multiple respiratory pathogens. J Clin Microbiol. 2011;49(6):2175–82.
4. Heim A, Ebnet C, Harste G, Pring-Åkerblom P. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. J Med Virol. 2003;70(2):228–39.
5. Dare RK, Fry AM, Chittaganpitch M, Sawanpanyalert P, Olsen SJ, Erdman DD. Human Coronavirus Infections in Rural Thailand: A Comprehensive Study Using Real‐Time Reverse‐Transcription Polymerase Chain Reaction Assays. J Infect Dis. 2007;196(9):1321–8.
6. van Doornum GJ, Guldemeester J, Osterhaus AD, Niesters HG. Diagnosing herpesvirus infections by real-time amplification and rapid culture. *J Clin Microbiol*. 2003;41(2):576-580. doi:10.1128/jcm.41.2.576-580.2003.
7. Javanmard D, Ziaee M, Ghaffari H, et al. Human parvovirus B19 and parvovirus 4 among Iranian patients with hemophilia. *Blood Res*. 2017;52(4):311-315. doi:10.5045/br.2017.52.4.311
8. Hummel KB, Lowe L, Bellini WJ, Rota PA. Development of quantitative gene-specific real-time RT-PCR assays for the detection of measles virus in clinical specimens. Journal of Virological Methods. 2006 Mar;132(1-2):166-173. DOI: 10.1016/j.jviromet.2005.10.006.
9. 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.
10. Wagner D, de With K, Huzly D, et al. Nosocomial acquisition of dengue. Emerg Infect Dis 2004;10:1872-3.
11. Lu X, Li X, Mo Z, Jin F, et al. Rapid Identification of Chikungunya and Dengue Virus by a Real-Time Reverse Transcription-Loop-Mediated Isothermal Amplification Method. Am J Trop Med Hyg. 2012 Nov7; 87(5): 947-953.
12. Almeida, Flávia Jacqueline MD, PhD; Olmos, Rodrigo Diaz MD, PhD; Oliveira, Danielle Bruna Leal PhD; Monteiro, Cairo Oliveira MsC; Thomazelli, Luciano Matsumiya PhD; Durigon, Edison Luiz PhD; Sáfadi, Marco Aurélio Palazzi MD P. Hematuria Associated With SARS-CoV-2 Infection in a Child. Pediatr Infect Dis J. 2020;3668.
13. Nurtop E, Villarroel PMS, Pastorino B, Ninove L, Drexler JF, Roca Y, et al. Combination of ELISA screening and seroneutralisation tests to expedite Zika virus seroprevalence studies. Virol J. 2018;15(1):1–6.
14. Tan CW, Chia WN, Chen MI-C, Hu Z, Young BE, Tan Y-J, et al. A SARS-CoV-2 surrogate virus neutralization test (sVNT) based on antibody-mediated blockage of ACE2-spike (RBD) protein-protein interaction. Nat Res [Internet]. 2020; Available from: https://www.researchsquare.com/article/rs-24574/v1
15. Shen C, Wang Z, Zhao F, Yang Y, Li J, Yuan J, et al. Treatment of 5 Critically Ill Patients with COVID-19 with Convalescent Plasma. JAMA - J Am Med Assoc. 2020;323(16):1582–9.
16. Zhang Q, Zhang H, Huang K, Yang Y, Hui X, Gao J, et al. SARS-CoV-2 neutralizing serum antibodies in cats: a serological investigation. bioRxiv. 2020;2020.04.01.021196.
17. Tu C, Crameri G, Kong X, Chen J, Sun Y, Yu M, et al. Antibodies to SARS coronavirus in civets. Emerg Infect Dis. 2004;10(12):2244–8.
18. Zhao S, Li W, Schuurman N, Van Kuppeveld F, Bosch BJ, Egberink H. Serological screening for coronavirus infections in cats. Viruses. 2019;11(8).
19. Meyer B, Drosten C, Müller MA. Serological assays for emerging coronaviruses: Challenges and pitfalls. Virus Res. 2020;194(January):175–83.
20. World Health Organization (WHO). Laboratory biosafety guidance related to the novel coronavirus ( 2019-nCoV ). 2020;(February):1–12. Available from: https://www.who.int/docs/default-source/coronaviruse/laboratory-biosafety-novel-coronavirus-version-1-1.pdf?sfvrsn=912a9847\_2