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Developing and validating SARS-CoV-2 assays for nonhuman primate surveillance

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Abstract

Introduction: In early 2020, the California National Primate Research Center implemented surveillance to address the threat of SARS-CoV-2 infection in its nonhuman primate colony.

Materials/Methods: To detect antiviral antibodies, multi-antigen assays were developed and validated on enzyme immunoassay and multiplex microbead immunofluorescent assay (MMIA) platforms. To detect viral RNA, RT-PCR was also performed.

Results/Conclusion: Using a 4plex, antibody was identified in 16/16 experimentally infected animals; and specificity for spike, nucleocapsid, receptor binding domain, and whole virus antigens was 95.2%, 93.8%, 94.3%, and 97.1%, respectively on surveillance samples. Six laboratories compared this MMIA favorably with nine additional laboratory-developed or commercially available assays. Using a screen and confirm algorithm, 141 of the last 2441 surveillance samples were screen-reactive requiring confirmatory testing. Although 35 samples were reactive to either nucleocapsid or spike; none were reactive to both. Over 20000 animals have been tested and no spontaneous infections have so far been confirmed across the NIH sponsored National Primate Research Centers.

KEYWORDS
antibody, diagnostic testing algorithm, RT-PCR

1 | INTRODUCTION/BACKGROUND

Realizing the potential threat to nonhuman primates (NHP), the California National Primate Research Center (CNPRC) initiated enhanced management practices and sentinel surveillance testing to survey the Macaca mulatta colony for SARS-CoV-2 infection within months of the start of the COVID 19 pandemic. The Primate Assay Laboratory (PAL) supported colony-wide efforts by conducting both serological testing for antibody and RT-PCR testing for virus. In experimental infections, antibody responses have been observed as early as 14 days post-infection.[1–5] A positive antibody test indicates that an infection has occurred but unless testing includes both IgG and IgM and/or longitudinal samples, an acute infection cannot be distinguished from the convalescent stage of a past infection. In contrast, the RT-PCR assay to detect viral RNA provides a more direct indication of possible viral replication and shedding at the time the sample is collected. Although the RT-PCR assay provides a real-time result, its window of positivity may be short and variable in SARS-CoV-2 infections.[2,5–7] These findings suggests a possible need for multiple repeat tests before ruling out infection. A positive RT-PCR result confirms an infection, but a negative RT-PCR result only suggests that a swab collected from the animal at a specific location

and specific time did not have detectable viral RNA above the limit of detection. Ideally, both serology and PCR would be incorporated into a surveillance program, as has been successful in the development of specific pathogen free nonhuman primate colonies for other agents.[8]

Our initial testing relied on commercially available Xpress Bio antibody reagents[1,9] and a modification of the United States Centers for Disease Control and Prevention RT-PCR protocol for human testing.[1,10,11] Over the last 2 years PAL has developed and validated multiplex antibody assays and established a sensitive, specific, efficient, and economical multi-step testing algorithm, which this report describes.

So far, no evidence of infection has been found in our colony.[1] Similarly, the Breeding Colony Management and Pathogen Detection Working Groups (PDWG) at all the NIH sponsored National Primate Research Centers (NPRCs) have reported no spontaneous infections in their colonies; and they continue to work collaboratively on surveillance.[12] However, the continued presence of virus in the surrounding human population, as well as increasing prevalence and case reports of transmission of SARS-CoV-2 in great apes, companion animals, captive, wild (including a reservoir in North American white-tailed deer), and farm animals with varying habitats and degrees of exposure to humans[13,14] underscore the need for a continued "one health" approach incorporating sentinel surveillance and appropriate management practices to monitor the status of NHP colony animals and provide an early alert to any potential breakthrough.

2 | MATERIALS AND METHODS

The study was reviewed and approved by the University of California, Davis Institutional Animal Care and Use Committee.

2.1 | Animals

All animals were maintained in fully AAALAC-accredited outdoor enclosures or indoor HVAC temperature controlled, negative pressure rooms in accordance with the Animal Welfare Act, Regulations, and the Guide for the Care and Use of Laboratory Animals.[15,16] CNPRC Personal Protective Equipment (PPE) standard protocols for accessing animal areas require wearing disposable gloves, disposable protective sleeves, protective eyewear, protective face shield, face mask, uniform/coverall/scrubs, and designated shoes/boots/shoe covers.

2.2 | Samples

Biological samples for antibody and virus testing were opportunistically collected from animals either being accessed for routine blood draws; immobilized for an examination; or admitted to one of the conventional or specific pathogen free hospitals for various reasons. Additional samples from animals with suspect respiratory disease based on clinical signs or pathological findings at necropsy were also collected. Similarly, samples were collected from experimentally SARS-CoV-2 infected or vaccinated animals for use as controls. One millilitre whole blood was collected by venipuncture and serum was separated by centrifugation at 1000 *g* for 15 min and frozen at −20°C for antibody testing. Nasal and oropharyngeal swabs were collected using a sterile polyester tipped 6″ applicator stick (Puritan) inserted sequentially in both nares approximately 1 cm until there was resistance and then rotated one to two times. The swab head was then submerged in 1 ml of TRIzol Reagent (Thermo Fisher Scientific), and stored frozen at ≤−80°C for RNA extraction. TRIzol is a monophasic solution of phenol and guanidine isothiocyanate commonly used to fix and preserve blood and body fluid samples for nucleic acid isolation. RNA was extracted using Maxwell RSC viral TNA reagents and corresponding semi-automated instrument (Promega) following the manufacturer's instructions. Briefly, 200 µl of swab eluate was lysed and proteinase K treated for 10 min at 56°C, before being added to a reagent cartridge and loaded into the instrument for automated extraction of total nucleic acid using paramagnetic particles as a mobile solid phase for sample capture, purification, washing, and elution in 50 µl final volume.

2.3 | Antibody assays

Commercially available SARS-CoV-2 strain Wuhan-Hu-1 recombinant spike, nucleocapsid, and RBD + M antigen-coated microtiter plates (Xpress Bio)[9] were used for the initial testing and later confirmatory testing. The spike antigen is a mixture of recombinant spike S1 and spike S2 glycoproteins produced in HEK293 cells and purified with Protein G chromatography. The S1 protein contains the amino acids 1–674 with a C-terminal sheep Fc-tag, and the S2 protein has amino acids 685–1211 with a C-terminal sheep Fc-tag. The spike S1 protein contains the receptor-binding domain that has affinity to the angiotensin-converting enzyme 2 region, and the spike S2 protein contains the fusion machinery and is anchored to the virus membrane. The recombinant nucleocapsid protein antigen is produced in *E. coli* and derived from the Wuhan-Hu-1 strain. This recombinant encompasses amino acids 1–419. The RBD + M fusion antigen is a recombinant protein consisting of 234 amino acids from the RBD region of the SARS CoV-2 spike protein as well as the Virion Surface Domain of Membrane Protein. The plates also included wells coated with an uninfected cell control antigen. A protocol based on other ELISAs already in use by PAL was established: Briefly, serum samples were diluted 1:50 in a commercially available phosphate-buffered saline with goat and bovine serum for blocking (Xpress Bio). After 45 min of incubation at 37°C, the wells were washed five times with a Tris buffered saline containing surfactant. Peroxidase-conjugated anti-simian IgG (Xpress Bio) was then added and incubated for an additional 45 min at 37°C. Wells were washed again and finally incubated with 2′2′azino-di[3-ethyl-benzothiazoline sulfonate] (ABTS)

for 30 min at room temperature. The reaction was stopped by adding 2 N sulfuric acid to change the pH and then read at 405 nm with a 600 nm reference wavelength in a Tecan Sunrise Reader.

After preliminary optimization and validation using enzyme immunoassays,[1] commercially available SARS-CoV-2 antigens were used to format a multiplex microbead fluorescent immunoassay on the Luminex Xmap 200 (Diasorin). The target antigens were (i) Spike protein trimers from SARS-CoV-2 (BetaCoV/Wuhan/IVDC-HB-05/2019) (aa 1–1208; GIAID# EPI_ISL_402121) (Immune Technology Corp), (ii) SARS-CoV-2 (2019-nCoV) nucleocapsid His tag recombinant proteins expressed in insect cells (Sino Biological), (iii) Receptor Binding Domain (RBD) Arg319-Ser591 with Avi and Poly-His Tag expressed in human 293 cells (GenScript), and (iv) heat inactivated, clarified, and diluted cell lysate and supernatant from Vero E6 cells infected with SARS-CoV-2, isolate USA-WA1/2020 (BEI). Briefly, the antigens and cell, IgG, and anti-IgG control antigens were covalently bound to polystyrene microspheres using carbodiimide chemistry which is a two-step process during which microsphere carboxyl groups are first activated with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride reagent in the presence of Sulfo-NHS (N-hydroxysulfosuccinimide) to form a Sulfo-NHS-ester intermediate. The reactive intermediate was then replaced by reacting with the primary amine of the coupling molecule (antibody, protein, linker, or peptide) to form a covalent amide bond.[17] For antibody detection, 50 µl of the antigen panel coupled beads were combined with 50 µl serum or plasma diluted 1:50 in PBS with 0.05% Tween and 2% Prionex[18] blocking agent and incubated on a shaker at 500 rpm in the dark at room temperature for 2 h. Following two washes with PBS with 0.05% Tween the bead mix was incubated with 1:500 diluted R-PE (Jackson Immuno Research) conjugated goat anti-human IgG diluted in PBS with 0.05% Tween on a shaker at 500 rpm in the dark at room temperature for 1 h. After a final wash, the beads were resuspended in PBS with 0.05% Tween and the median fluorescent index (MFI) was read using a Luminex Xmap fluorescence reader which employs two lasers that identify each antigen conjugated bead and determines if specific antibody was bound.

In addition, the in-laboratory conjugated SARS-CoV-2 beads were further multiplexed with Simian Tracking and Assessment Profile reagents from Charles River Laboratories. Following the manufacturer's instructions,[18] serum or plasma samples were reacted with a panel of Simian immunodeficiency virus (SIV), Simian betaretrovirus (SRV), Simian T cell lymphotropic virus (STLV), measles virus (MV), Herpes B virus (BV), Rhesus rhadinovirus (RRV), Simian Foamy Virus (SFV), and Rhesus cytomegalovirus (rhCMV) test antigen beads; and cell, IgG and anti-IgG control antigen beads. Antigen–antibody complexes were subsequently detected using a biotinylated goat anti-human IgG followed by streptavidin phycoerythrin. The suspension microarray was read using the Luminex Xmap fluorescence reader.

The antibody reagents and protocol were validated using sera from experimentally infected or vaccinated macaques as positive controls and PAL archived normal sera collected from 2017 to 2018 before the emergence of SARS-CoV-2 as negative controls.

2.4 | RT-PCR

Multiplex PCR reactions used the CDC-designed oligonucleotide primers and FAM-labeled probes for the N1 and N2 virus nucleocapsid gene segments[10] (IDT) along with the diploid oncostatin M (OSM) primers and VIC-labeled probes[19] (Thermo Fisher) as an internal control for the presence of amplifiable DNA. Five microlitre of viral RNA was transcribed to cDNA and subsequently amplified using the TaqPath RT-qPCR or TaqMan Fast Virus 1-step Master Mix (Thermo Fisher) in a 20 µl total reaction volume. Cycling conditions were 2 min at 25°C, 15 min at 50°C, 2 min at 95°C, 45 cycles of amplification for 3 s at 95°C, and 30 s at 55°C. The reactions were carried out in a QuantStudio 12 K Flex Thermocycler (Thermo Fisher). A plasmid for the SARS-CoV-2 (Wuhan-Hu-1) nucleocapsid gene (IDT) and TRIzolinactivated SARS-CoV-2 (USA-WA-1) tissue culture RNA were used as positive controls. Amplification was measured as cycle threshold (Ct) reflecting the linear phase of the amplification curve as the fluorescence intensity increases due to the reporter dye molecules being cleaved from the probes.

3 | RESULTS

Antibody was initially assessed on samples from 1096 different animals using a commercially available SARS-CoV-2 Spike enzyme immunoassay (Xpress Bio). Samples from 21 animals were reactive. Positive antibody reactivity in 18 of the 21 initially reactive samples was not confirmed when the samples were tested on ELISA and the multiplex assays using additional SARS-CoV-2 antigens under development in our laboratory. However, additional reactivity was detected in the remaining three samples which were subsequently subjected to supplemental testing courtesy of Xpress Bio. These three samples were found to be reactive to either the HEK cell line or the sheep Fc purification tag used in antibody production.[1]

After initially optimizing and validating SARS-CoV-2 spike, nucleocapsid, RBD, and viral lysate antigens in an enzyme immunoassay, the antigens were used to format a multiplex microbead immunofluorescent assay (MMIA) on the Luminex platform. Antibody to one or more antigens was correctly identified in 16/16 samples from experimental infections (>10 days post inoculation); and not identified in 35 historical (pre-2018) archived samples. Specificity for spike (S), nucleocapsid (NC), RBD, and whole virus antigens was 95.2, 93.8, 94.3, and 97.1, respectively. This data is summarized in Figure 1. Initial use of this multiplex for routine surveillance resulted in reactivity greater than two standard deviations above the mean in 102 of 711 prospective samples. These 102 samples were subjected to confirmatory testing using the Xpress Bio Spike and Nucleocapsid ELISAs: 19 samples reacted to spike only and 18 to nucleocapsid only; no samples were confirmed reactive for both S and NC. Reactivity was not confirmed in the remaining 65 samples. This multiplex antibody assay was shared with six other PDWG laboratories for comparison testing on nine additional laboratory-developed or commercially available assays using shared panels of known positive

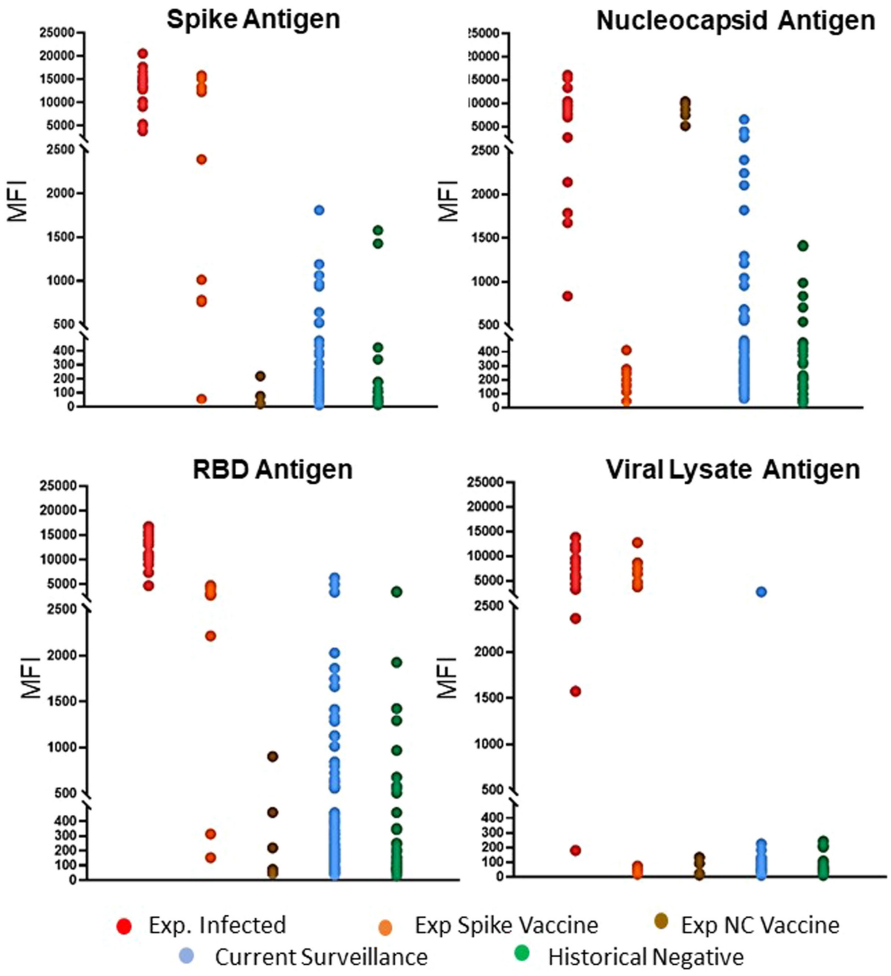

FIGURE 1 SARS-CoV-2 antibody reactivity of experimentally infected or immunized positive controls, archived historical (pre-2018) negative controls, and surveillance study samples. The reactivity for each sample is shown as the MFI against a panel of SARS-CoV-2 Spike, Nucleocapsid, RBD and whole viral lysate antigens in a MMIA on the Luminex platform

and negative samples. Although there were some minor differences all the assays performed satisfactorily and positive/negative interpretations agreed.[12] The PDWG has now tested surveillance samples from over 20000 animals across the seven NIH sponsored NPRCs and no spontaneous infections have been confirmed. The survey population included samples from Macaca mulatta, Macaca nemistrina, Macaca fasicularis, Chlorocebus aethiops, Cercocebus atys, Pan troglodytes, Papio species. and Saimiri species.

After analyzing and evaluating the collated 4-plex data, we found that the RBD and viral lysate antigens did not provide any additional sensitivity but decreased specificity when compared to interpretation based on spike and nucleocapsid only. Thus, for the next time period we reduced the MMIA to a 2-plex including only the spike and nucleocapsid antigens for use as the screening assay with the Xpress Bio Spike and Nucleocapsid enzyme immunoassay as the confirmatory assays in a two-step algorithm. No known positive samples were missed. Of the 1241 surveillance samples, 100 were MMIA screen reactive requiring enzyme immunoassay confirmatory testing. Twenty-four of the 100 confirmatory tests were reactive to NC only and 2 to S only; none were reactive to both. All other confirmatory test results were non-reactive.

In an effort to streamline laboratory workflow and improve efficiency and economy, we added the in-laboratory conjugated SARS-CoV-2 spike and nucleocapsid conjugated beads into the simian tracking profile assay routinely used for SIV, SRV, STLV, Herpes B Virus, Measles Virus, RhCMV, RRV, and SFV antibody screening in our laboratory. No known SARS-CoV-2 positive control samples were missed in the combined assay for simultaneous detection. Of 1200 surveillance samples, 41 were MMIA screen reactive requiring EIA confirmatory testing. Nine confirmatory tests were reactive to NC only and all others were non-reactive.

Initial studies comparing the Xpress Spike and RBD + M enzyme immunoassay as the confirmatory test were inconclusive. Sensitivity was equivalent with 26 positive controls (animals experimentally infected or vaccinated for at least 10 days) reactive against both antigens. Forty surveillance samples were also tested: Five were Spike reactive but RBM + M nonreactive; and six were Spike nonreactive but RBD + M reactive. The remaining 29 surveillance samples gave concordant (five reactive and 24 nonreactive) results against both Spike and RBD + M antigen coated plates.

Nasopharyngeal swab samples were collected and tested by RT-PCR from 940 animals for either routine surveillance or to investigate reported respiratory signs, necropsy pathology, or suspect (did not confirm as positive) antibody results. Nine hundred and twenty-two were nonreactive to both N1 and N2; and 18 were reactive to either N1 or N2, but the signal was not reproducible on repeat testing.

Surveillance data generated using PAL's evolving assays and testing algorithm are shown in Figure 2.

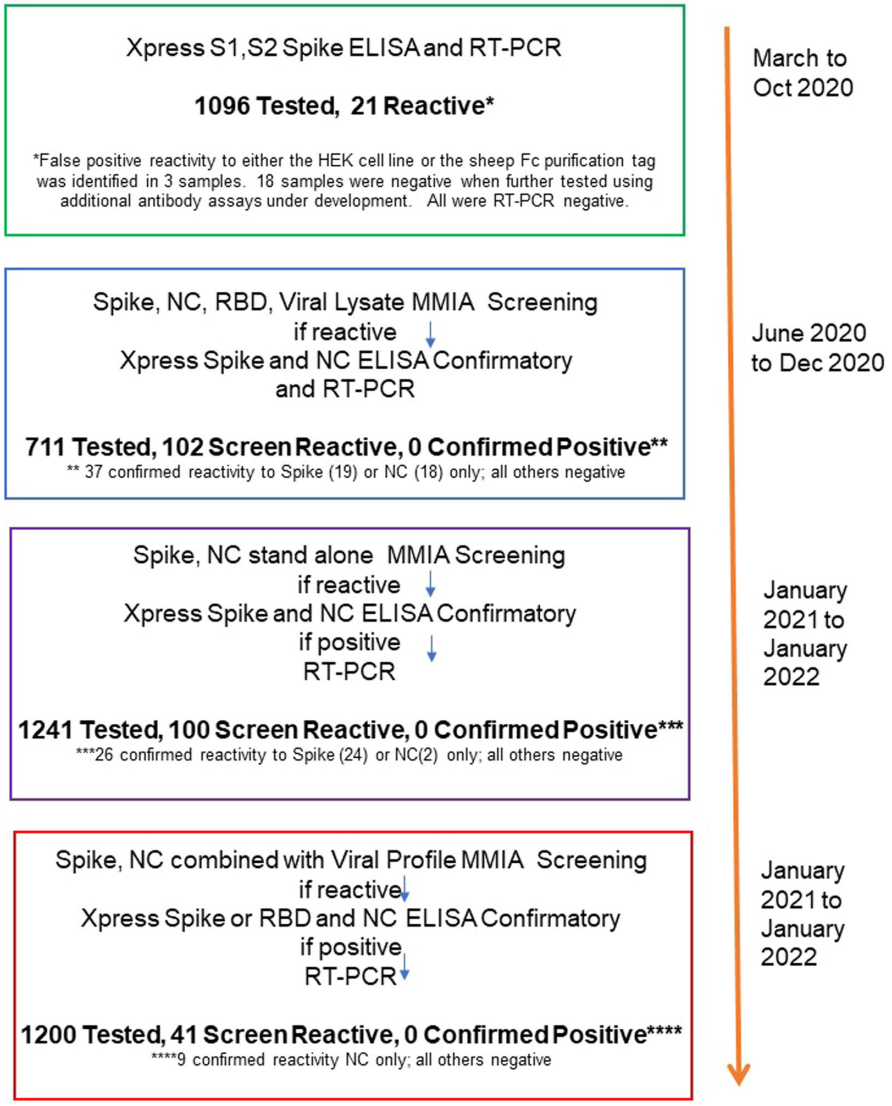

FIGURE 2 Timeline showing SARS-C0V-2 surveillance testing algorithms and results from 2018 to 2021. The results were generated using algorithms incorporating combinations of screening and confirmatory antibody immunoassays and RT-PCR that were developed, refined, and validated by PAL during this time period

4 | DISCUSSION AND CONCLUSIONS

The data indicate that current colony management practices, including restricted access, PPE, personnel vaccination and testing requirements has been adequate to prevent SARS-CoV-2 infection of the CNPRC NHP colony. Although viral antibody or RNA has not been detected, our knowledge and understanding of this virus and its epidemiology and transmission continues to evolve. The literature shows that NHPs are susceptible; and it is reasonable to assume that NHP populations continue to be at some risk of infection.[13,14] Thus, continuing a scaled back surveillance program is warranted. Given the logistical and resource limitations and lack of active infection, we will continue opportunistic antibody testing of 10% of the colony for general ongoing surveillance, with RNA detection reserved for animals showing respiratory signs, relocation to sensitive locations, and pre-research study screening.

The assays and algorithm presented here are validated tools for routine colony management of captive NHP populations at various research and other institutions. They have also been used to monitor basic biology and applied prevention and therapeutic research studies. We are now collaborating with colleagues to apply them to epidemiologic studies in wild caught NHP colonies. PAL will continue to analyze the surveillance data and use the collected samples to refine the assays and algorithm to ensure sensitivity and improve specificity even as new strains for SARS-CoV-2 emerge. The goal is to continue to improve specificity with no loss of sensitivity. The initial data comparing the Xpress Bio Spike S1, S2 to the RBD-M ELISA plate is an example. Although the initial sensitivity is adequate for both; testing larger numbers of false positive samples as they are identified, will be necessary to determine which antigen has better specificity. These efforts to evaluate newer reagents and assays as they become available will be aided by participation in collaborative studies with the PDWG. The PDWG has successfully developed testing algorithms and shared protocols, reagents, controls, and proficiency testing for a panel of specific pathogen free agents and is now applying similar efforts to SARS-CoV-2 screening and confirmatory assays for both the virus and the host immune response and effectively support the management captive nonhuman primate colonies. Thus far this group has compared our assay favorably along with nine other laboratory- developed or commercially available

assays. Over 20000 animals across the seven NPRC's have been tested, with no detection of spontaneous infections.[12]

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data that supports this study are available from the corresponding author upon request.

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