RESEARCH ARTICLE

In-Vitro Screening of Repurposed Drug Library against Severe Acute Respiratory Syndrome Coronavirus-2

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ABSTRACT
The current pandemic caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) demands rapid identification of new antiviral molecules from the existing drugs. Drug repurposing is a significant alternative for pandemics and emerging diseases because of the availability of preclinical data, documented safety in clinic and possibility of immediate production and scalable capacity and supply. Several drugs such as ivermectin and hydroxy chloroquine have been repurposed as anti-SARS-CoV-2 agents, but the effect of these compounds in treating the COVID-19 patients remains sub-optimal. In the present study repurposed drug libraries consisting of 560 compounds from two different sources have been screened against SARS-CoV-2 isolate USA-WA1/2020 in Vero E6 cell line and 24 compounds were found active. The SARS-CoV-2 virus propagated in Vero E6 cell line and used in screening the drug libraries was sequenced by Next Generation Sequencing to identify any mutations that may have accumulated in the virus genome. The whole genome sequencing data of SARS-CoV-2 showed 9 and 6 single nucleotide polymorphisms in spike protein with reference to Wuhan-Hu-1(NC045512.2) and USA/WA-CDC-WA1/2020 (MN985325.1) isolates respectively. The present study identified 24 compounds active against SARS-CoV-2 isolate USA-WA1/2020 out of 560 repurposed drugs from two libraries. The IC-50 values of the identified hits range from 0.4 µM to 16 µM. Further studies on the repurposed drugs identified in the present screen may be helpful in the rapid development of antiviral drugs against SARS-CoV-2.

Keywords: COVID-19; dose response curve; IC-50; NGS; repurposed drugs; SARS-CoV-2; SNPs; Vero E6
Introduction
Coronaviruses (CoVs) belonging to the order Nidovirales, and family Coronaviridae, are enveloped and genetically diverse viruses, consisting of four genera such as alpha, beta, gamma, and delta. Multiple animal species are susceptible to coronavirus infections with several cross-species transmission incidences. Furthermore, zoonotic transmission is thought to be the source of all human CoV infections. However, only alpha and beta CoVs are known to infect humans with varying pathology. In the past two decades, two CoVs outbreaks of zoonotic origin have been witnessed, such as severe acute respiratory syndrome (SARS) caused by SARS-CoV in 2002, and Middle East respiratory syndrome (MERS) in 2012, caused by MERS-CoV. The SARS-CoV outbreak reported 8,098 confirmed cases with a fatality rate of around 10% (774 deaths), while 2,502 confirmed cases with a case fatality rate higher than 30% has been reported with MERS-CoV outbreak. The ongoing pandemic known as coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) belonging to beta CoV has reportedly originated in Wuhan, China, in the early December 2019. The rapid spread of COVID-19 throughout the world in a very short time prompted the World Health Organization (WHO) to declare COVID-19 as a pandemic on 11 March 2020. Mutations in the spike protein of SARS-CoV-2 has led to the emergence multiple variants such as B.1.1.7 (alpha), B.1.351 (beta), B.1.1.28.1/P.1 (gamma), B.1.617.2 (delta), and B.1.617.1 (kappa). The currently circulating variant, B.1.1.529 (omicron) with five distinct sub-lineages such as BA.1, BA.2, BA.3, BA.4, and BA.5.1 along with subvariant such as BA.2.12.1 (a subvariant of BA.2) are still causing significant health concern globally.

The severity and clinical symptoms of SARS-CoV-2 infection varies from asymptomatic or mild disease to severe respiratory distress with significant mortality and populations with underlying medical conditions are highly vulnerable. The COVID-19 disease caused due to SARS-CoV-2 infection has claimed millions of human lives since its emergence and pushed the economy of the world to a significantly low level. While the development of vaccines and their emergency usage approval followed by swift vaccination programs across the world helped in managing the COVID-19 severity. Furthermore, the specific antivirals or therapies against any human CoVs, which minimizes disease severity/death are not yet available. Several drugs, such as interferon, ribavirin, lopinavir-ritonavir have been used as therapeutic options in the treatment of SARS and MERS patients. Remdesivir, a nucleotide analogue with a broad antiviral activity against human and animal CoVs and originally developed against Ebola virus exhibited antiviral activity against SARS-CoV-2 in the in-vitro studies. Further, based on the preclinical studies, and clinical trials on COVID-19 patients, remdesivir received FDA approval as a therapy for COVID-19 patients, however, the therapy remained suboptimal.

The severity of COVID-19 disease and continuous emergence of SARS-CoV-2 variants with varying pathology, along with unstoppable spread necessitated the development of potent antivirals against SARS-CoV-2. It is certainly impossible to develop new antivirals to meet the pace of SARS-CoV-2 pandemic spread, hence repurposing drugs have gained much attention and is expected to deliver new antiviral treatments for COVID-19 at a rapid pace. The present study is designed to screen the chemically diverse library of repurposed drugs to identify actives against SARS-CoV-2 in a Vero E6 cell-based system. The SARS-CoV-2 isolate used in the present study was sequenced by Next Generation Sequencing (NGS) to identify any mutations accumulated in the virus genome and its impact on the antiviral screening.

Materials and Methods
2.1. Cell line
Vero E6 (C1008), the African green monkey kidney epithelial cell was obtained from Elabscience Biotechnology Inc. (Cat no. EP-CL-0491). The Vero E6 cells were cultured in Dulbecco’s modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotic antimycotic solution containing penicillin (100 units/mL), streptomycin (100 µg/mL) and Amphotericin B (0.25 µg/mL) at 37°C in a humidified CO₂ (5%) incubator. All cell culture reagents were obtained from Sigma-Aldrich.

2.2. Propagation of virus
The SARS-CoV-2 isolate USA-WA1/2020 used in the present study was obtained from BEI Resources, USA (Cat No; NR-52281). The virus stock was prepared by propagating SARS-CoV-2 in Vero E6 cells by following the standard protocol. In brief, the virus inoculum at multiplicity of infection (MOI) 0.01 was prepared in a minimum volume of DMEM (5 mL) containing 2% FBS and added on to preformed Vero E6 monolayer cells (T-75 flask) and incubated at 37°C for 1 h in a humidified CO₂ (5%) incubator with shaking at every 15 minutes. After 1 h incubation the flask was supplemented...
with 5 mL of DMEM (2% FBS) and incubated at 37°C for 1 h in a humidified CO₂ (5 %) incubator. The flask was observed for virus induced cytopathic effect (CPE) at every 24 h in comparison to mock flask. With the clear CPE at 72-hour post infection (hpi), the supernatant was collected and centrifuged to clarify the supernatant to harvest the virus. The virus stock was stored at −80°C in small aliquots until use.

2.3. Quantification of SARS-CoV-2 by standard plaque assay

Plaque assay is considered as the gold standard to quantify the infectious virus particles and has been well established for SARS-CoV-2 quantification 19. For plaque assay, Vero E6 cells (~2.5 × 10⁵ cells/well) were plated into 12 well plate in DMEM supplemented with FBS (10%) and incubated for 24 h at 37°C in a humidified CO₂ (5 %) incubator. Tenfold serial dilutions of virus stock were prepared in DMEM supplemented with FBS (2%). The existing medium from the plate was removed and inoculated with virus dilutions in duplicate. The plate was then transferred to CO₂ incubator and incubated for 1 h for the adsorption of virus. After the incubation period the infection medium was removed and overlayed with DMEM-Carboxy Methyl Cellulose (CMC) medium. The DMEM-CMC overlay medium was prepared by mixing equal volume of DMEM (2X) supplemented with FBS (4%) and sterile CMC (2%). The plate was then incubated at 37°C for 3 days in a humidified CO₂ (5 %) incubator 19. On day 3, the cells were fixed with 4% formaldehyde solution and stained using crystal violet to observe the plaques. The SARS-CoV-2 infection study was carried out in a high containment (BSL-3) facility. Schematic representation (Fig. 1) depicts the SARS-CoV-2 propagation, titration, and antiviral screening strategy.

A. SARS-CoV-2 propagation

B. Cytotoxicity assay

C. Antiviral screening by cytopathic effect (CPE) assay

Fig. 1: Schematic representation of SARS-CoV-2 propagation, titration, and antiviral screening in Vero E6 cells. The SARS-CoV-2 was propagated in Vero E6 cells and titrated by plaque assay followed by determination of cytotoxicity and antiviral screening of repurposed drug library against SARS-CoV-2 by cytopathic (CPE) assay.

2.4. Next Generation Sequencing and bioinformatics analysis of SARS CoV-2 genome

The SARS-CoV-2 isolate propagated in Vero E6 cells at our laboratory was sequenced by NGS followed by bioinformatics analysis to identify any mutations accumulated in the virus genome. For NGS, the viral RNA was extracted from the infected cell culture supernatant using QIAamp Viral RNA Mini Kit (Qiagen, USA) by following the manufacturer’s instructions. The whole genome sequencing of the isolated RNA was done using NextSeq500 platform. In brief, the quantity and quality of the RNA samples was checked by Nanodrop and Qubit fluorometer followed by preparation of paired end (PE) viral genome sequence libraries using illumine Truseq total RNA library preparation kit. The PCR enriched libraries were quantity and quality checked on Agilent 4200
Tape Station (Agilent Technologies), followed by loading on to NextSeq500 for cluster generation and sequenced in both forward and reverse directions.

The sequenced raw data was processed to obtain high quality clean reads using Trimmomatic v0.38 to remove adapter sequences, ambiguous reads (reads with unknown nucleotides “N” larger than 5%), and low-quality sequences (reads with more than 10% quality threshold (QV) < 20 phred score). The high-quality reads of the samples were aligned to the reference sequences (Wuhan-Hu-1 (NC045512.2) and USA/WA-CDC-WA1/2020 (MN985325.1) using BWA MEM (version 0.7.17). Consensus sequence was extracted using Samtools mpileup. Further, the single nucleotide polymorphisms (SNPs) were identified using mpileup utility of Samtools (v 0.1.18) from the sorted BAM file. The SNPs were filtered based on a minimum read depth of 5, quality threshold of 25. The mostly likely lineage to the sample consensus sequence is assigned using the Pangolin tool. The schematic representation of bioinformatics workflow in shown in Fig.2.

2.5. Repurposed drugs collection

The repurposed drugs for SARS-CoV-2 antiviral screening were obtained from two different sources. Medicines for Malaria Venture (MMV) kindly supplied 160 different drugs with known or predicted activity against SARS-COV-2 called MMVs COVID box. The compounds in the MMV’s COVID box were made available as solubilized 10 mM solutions in dimethyl sulfoxide (DMSO) within a 96-well plate and as a free Open Access box and screened primarily at 10 µM concentration. Further, 400 compounds were purchased from Micro Source Discovery system, all the compounds were supplied at 10 mM concentration in DMSO and screened initially at 25 µM concentration. The compounds received from both the sources were stored at -80°C until use.

2.6. Antiviral screening and determination of IC-50 values by plaque assay

The repurposed drug libraries were screened against SARS-CoV-2 by standard plaque assay in Vero E6 cells. For primary antiviral screening, Vero E6 cells were seeded in 96 well plate at a density of ~30000 cells per well in 200 µL DMEM supplemented with 10% FBS in a 96 well flat bottom tissue culture plate. The plate was incubated at 37°C for 18-24 h, in a humidified CO2 (5%) incubator. Next day, medium was removed from the wells and the compounds were added to respective wells in duplicate at a final indicated concentrations.
followed by infection with SARS-CoV-2 at approximately 30 PFU/well. The antiviral compounds and virus were diluted in infection medium containing DMEM supplemented with 2% FBS. The plate was incubated at 37°C for 1 h, in a humidified CO₂ (5%) incubator for adsorption of virus. The final volume of infection medium containing compounds and virus was maintained at 40 μL per well to maximize the virus adsorption. The virus only wells (with infection and without test compound) and cell only wells (without infection/test compound) were maintained as positive and negative controls, respectively. After 1 h incubation, the infection medium was removed from the wells and overlayed with DMEM-CMC medium and incubated for 72 h. After the completion of incubation, the plates were processed, and number of plaques were counted in each well. The percentage reduction of virus in test compound treated wells were calculated in comparison to positive control (virus only well). The hit molecules identified in the primary screen were subjected to dose response curve (DRC) generation and IC-50 determination at indicated concentrations in Vero E6 cells. The IC-50 of the drug was calculated by GraphPad Prism 9.2.0.

2.7. Determination of cellular cytotoxicity of the compounds.

The cellular toxicity of the test compounds at 10 μM and 25 μM respectively with MMVs COVID box and Micro Source Discovery system was determined by measuring the cytosolic lactate dehydrogenase (LDH) enzyme. LDH present in many different cell types, releases into the surrounding cell culture media upon plasma membrane damage and is a reliable indicator of cytotoxicity. The Vero E6 were seeded in 96 well flat bottom tissue culture plate in 200 μL of DMEM supplemented with 10% FBS and incubated for 18-24 h for the monolayer formation. Next day, the medium was removed, and the compounds were prepared at indicated concentrations in DMEM (supplemented with 10% FBS) and added to the respective wells and incubated for 72 h. After the completion of incubation, the released LDH was measured using CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen) by following the manufacturer’s instructions.

Results

3.1. Genome sequencing and analysis identified mutations in spike protein.

The SARS-CoV-2 obtained from the BEI resources was propagated in Vero E6 (passage 2). The main goal of the whole genome sequencing of SARS-CoV-2 used in the present study was to identify the genomic similarity of the passaged virus stock (P2) with that of the original SARS-CoV-2 isolate (USA-WA1/2020). To identify any mutations accumulated in the propagated SARS-CoV-2 genome, the RNA was subjected to NGS followed by bioinformatics analysis. Pangolin tool was used in the identification of mostly likely lineage in comparison to reference sequences. The genome sequencing and analysis showed that the present SARS-CoV-2 isolate belongs to lineage A. The mapping and consensus statistics showed 99.9% genome coverage in comparison to reference sequence NC045512.2 and MN_985325., with a total length of 29,903 and 29,882 bp respectively (Table I).

<table>
<thead>
<tr>
<th>Reference Sequence</th>
<th>Mapping (%)</th>
<th>Genome coverage (%)</th>
<th>Total length of consensus (bp)</th>
<th>No. of SNPs</th>
<th>Lineage</th>
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<td>99.9933</td>
<td>29,882</td>
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Table I: Mapping and consensus statistics of NGS data: The SARS-CoV-2 isolate of the present study was mapped with reference sequences to identify the Single Nucleotide Polymorphisms (SNPs) and lineage. The analysis identified that the isolate belongs to lineage A.
The NGS data of SARS-CoV-2 genome revealed 9 and 6 SNPs (in spike protein, ORF-1b and ORF-2) in comparison to the reference sequences (Wuhan-Hu-1(NC045512.2) and USA/WA-CDC-WA1/2020 (MN985325.1)) respectively. Spike protein showed 5 SNPs, ORF 1ab and ORF 8 had shown 3 and 1 SNPs respectively. The summary of SNPs identified in the genome is given in table II.

<table>
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<tr>
<th>Position in NC_045512.2</th>
<th>Position in N985325.1</th>
<th>Reported NC_045512.2 sequence</th>
<th>Reported MN985325.1 sequence</th>
<th>Identified Alternate Base</th>
<th>Quality</th>
<th>Gene Name</th>
<th>Protein change</th>
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</table>

Table II: Summary of Single Nucleotide Polymorphisms (SNPs) identification: The nucleotide changes with respect to reference sequences showed 5 SNPs in spike protein, 3 SNPs in ORF 1ab and 1 SNP in ORF 8. The corresponding amino acid changes in the protein molecule is given.

3.2. Preliminary screening and IC-50 determination of potential hit molecules. Primary screening of repurposed drug libraries in Vero E6 cell-based system identified 24 molecules active against SARS-CoV-2. The summary of primary hits from antiviral screening is given in Fig. 3. The number of primary hit molecules identified from ‘MMV COVID box’ at 10 µM concentration and Micro source discovery system at 25 µM concentration (Table-III) against SARS-CoV-2 were found to be 12 each.
Table III: The IC-50 and non-cytotoxic concentrations of hit molecules: The IC-50 of the hit molecules identified from MMV COVID Box and Microsource repurposed drug library were determined by 6-point dose response curve (DRC) using plaque assay. The cytotoxicity was determined by LDH assay.

The six-point DRCs were generated for the active molecules starting with highest concentrations of 10 µM (MMV COVID box) and 25 µM (Micro source discovery system). The IC-50 was calculated by non-regression analysis using GraphPad prism version 9.2.0. The IC-50 of hit molecules from MMV COVID box ranged from 0.4 µM to 7.1 µM (Fig. 4). The IC-50 of Almitrine and Remdesivir were found to be low among the screened compounds with 0.4 µM and 0.7 µM, respectively. While Hydroxychloroquine and Amiodarone showed IC-50 value > 6.0 µM. The IC-50 of hit molecules from Micro source discovery system ranged from 0.7 µM – 16 µM (Fig. 5). Ethacridine and Hydroquinidine showed lowest IC-50 value of 0.76 µM and 1.1 µM, respectively. While the IC-50 values of Pimethixene, Tolperosone and Gliquidone were found to be >10 µM.
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Fig. 4: The IC-50 of hit molecules identified from “MMV COVID Box”: Antiviral screening of “MMV COVID Box” repurposed drug library against SARS-CoV-2 and DRC for the hit molecules identified from the preliminary screening was established by cytopathic assay. The IC-50 values of hit molecules were determined by 6-point DRC starting with highest concentration at 10 µM.

Fig. 5: Generation of DRC and determination of IC-50: The DRC for the hit molecules identified from the “Micro source discovery system” repurposed drug collection was generated against SARS-CoV-2 by cytopathic assay and IC-50 was determined. The IC-50 values of hit molecules was determined by 6-point DRC starting with highest concentration at 25 µM.

3.3. Non-cytotoxic concentration of hit molecules.
The cytotoxicity assay was carried out for the hit molecules at the top concentrations used in the DRC generation. The cytotoxicity was tested at 10 µM for MMV COVID Box and 25 µM for Micro source discovery system. All the 24 primary hit molecules were found to be non-cytotoxic to Vero E6 cells at 10 µM (MMV COVID Box) and 25 µM (Micro source discovery system) concentrations (Table III).

4.0. Discussion
The emergence and severity of COVID-19 disease has intensified the effort to repurpose existing drugs to rapidly identify antivirals against SARS-CoV-2. Drug repurposing approach reduces the time required for the development of novel antivirals apart from reducing failure risk associated with safety of the drug due to the availability of preclinical and clinical safety data of majority of repurposing drugs. Repurposed drugs have been found to reduce the hospitalization of COVID-19 patients, reduced severity and time of hospital stay, however their benefit in hospitalized patients is arguable. Undoubtedly, the use of repurposed drugs and fast track repurposing is a great option to counter emerging infections in near future. In the present study, we have screened 560 repurposed drugs obtained from two different sources by standard plaque assay. The whole genome sequencing of the SARS-CoV-2 isolate which was propagated and used in the present study showed accumulation of 5 SNPs in spike protein, 3 SNPs in ORF 1ab and 1 SNP in ORF8. The NGS data showed that the passaged virus (P2) used in the present screen belonged to the same lineage as that of the original SARS-CoV-2 isolate, USA-WA1/2020. Further, the NGS data helps to understand that repurposed drugs active against SARS-CoV-2 isolate USA-WA1/2020 in the present screen are specific to the SARS-CoV-2
isolate USA-WA1/2020. Several earlier studies report that, the propagation and passage of SARS-CoV-2 in Vero and Vero E6 cells is known to accumulate mutations/SNPs in the viral genome. Further, the passaging of SARS-CoV-2 in Vero E6 cells showed accumulation of adaptive mutations in the spike protein under selective pressure from different passages.

The current study identified 12 active molecules against SARS-CoV-2 in the primary screen from MMV COVID box. Remdesivir, targeting the RNA-dependent RNA polymerase of SARS-CoV-2 showed IC-50 value of 0.7 μM in Vero E6 cells and is on par with the earlier studies which reported the IC-50 value between 0.77 μM to 1.6 μM. The present study results are comparable with the reported IC-50 value of celecoxib (3.3 μM), ivermectin (2.8 to 5.5 μM) and hydroxychloroquine (5.0 μM). The comparative agreement of the hit molecules from the present study with earlier studies provides validation of the assay. Ferroquine had shown broad spectrum anti-coronavirus activity including 229E, OC-43, SARS-CoV-1, and SARS-CoV-2 with varying IC-50 values, and the IC-50 value against SARS-CoV-2 was reported as 2.6 ± 0.6 and results of the current study falls within the reported values. An enzymatic inhibition assay had shown 3CLpro inhibitory activity of clinical drug Ebselen and plaque reduction assay showed strong inhibitory activity with an IC-50 value of 4.67 ± 0.80 μM/L. The IC-50 value of Ebselen obtained in the present study is 4.6 μM and agrees with the previous studies.

Anti-hepatitis C virus drug sofosbuvir demonstrated the inhibition of SARS-CoV-2 viral replication with an IC-50 >10 μM in Vero E6 cells and 7.3 ± 0.5 μM in Calu-3 cells; however, the present study showed IC-50 value of 5 μM in Vero E6. Celecoxib, a non-steroidal anti-inflammatory drug had shown anti-SARS-CoV-2 activity with an IC-50 value of 0.04 μM and 13.02 μM in Vero cells and in Vero E6 cells respectively, compared to 2.5 μM in Vero E6 cells in the present study. Some of the molecules identified in the present screen such as Ivermectin and hydroxychloroquine have been moved to clinical trial, however the clinical data showed the limited application of these drugs in different forms of COVID-19 treatment. Hence, the rapid screening, identification and clinical use of repurposed drugs is the need of the hour to fight emerging infectious diseases. The present study involving screening of repurposed drugs is done against ancestral strain of SARS-CoV-2 isolate USA-WA1/2020. Hence, the screening of the hit molecules against delta and omicron variants would help in the identification of potential candidate molecules against the currently circulating variants and help in the rapid development of clinical antivirals.

The development of effective and safe COVID-19 vaccines has significant prophylactic potential in limiting the spread of SARS-CoV-2 infections. However, several concerns such as duration of immunity, and effectiveness of vaccines against different variants of SARS-CoV-2 needs attention. Moreover, therapeutic agents and preferably broad-spectrum antivirals are in immediate necessity to contain emerging viral infections. Hence, the identification of existing drugs targeting new mechanisms of SARS-CoV-2 virus/repurposing presents a quick option to develop effective anti-SARS-CoV-2 therapeutics. Moreover, the availability of human safety and pharmacokinetic data for these repurposed compounds helps immediate use in clinical management of COVID-19.

5.0. Conclusion
The SARS-CoV-2 isolate passedag in Vero E6 is highly identical to the reference sequences NC045512.2 and MN985325.1. However, NGS data showed 9 and 6 SNPs respectively with NC045512.2 and MN985325.1. The Vero E6 cell line based in vitro screening offers a reliable method to identify new or repurposed anti-SARS-CoV-2 drugs. The data obtained in the current screen identified 24/560 repurposed drugs active against SARS-CoV-2. Hence, larger screens based on repurposed drug libraries is the need of the hour for the faster development of new drugs to treat COVID-19 patients. Further, the testing of the hit molecules against other variants of SARS-CoV-2 such as, delta and omicron and determination of mode of action will help in developing these drugs as clinical candidates to treat COVID-19.

Declarations
Availability of data and materials
The datasets generated from the “MMVs COVID box” during the current study is shared with Medicines for Malaria Venture (MMV). The datasets generated in the current study is available from the corresponding author on reasonable request and with the permission of MMV for “MMVs COVID box datasets.

Competing interests

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The authors declare that they have no competing interests.

**Funding**
Not applicable

**Authors’ contributions**
All authors conceptualized and designed the study. Gudepalya Renukaiah Rudramurthy, performed investigation, methodology, and original draft writing (manuscript). The review and editing of manuscript were done by Radha Krishan Shandil and Shridhar Narayanan.

**Acknowledgement**
"The SARS-CoV-2 isolate USA-WA1/2020, was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH: SARS-Related Coronavirus 2, Isolate USA-WA1/2020, NR-52281.”
The “MMVs COVID box” was a kind gift from Medicines for Malaria Venture (MMV).
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