RESEARCH ARTICLE

Fast Evaluation of Viral Emerging Risks (FEVER): A computational tool for biosurveillance, diagnostics, and mutation typing of emerging viral pathogens

Zachary R. Stromberg¹, James Theiler², Brian T. Foley³, Adán Myers y Gutiérrez⁴, Attelia Hollander⁴, Samantha J. Courtney¹, Jason Gans⁴, Alina Deshpande⁴, Ebany J. Martinez-Finley⁵, Jason Mitchell⁵, Harshini Mukundan¹, Karina Yusim^{3†}, Jessica Z. Kubicek-Sutherland^{1*}

1 Physical Chemistry and Applied Spectroscopy, Los Alamos National Laboratory, Los Alamos, New Mexico, United States of America, **2** Space Data Science and Systems, Los Alamos National Laboratory, Los Alamos, New Mexico, United States of America, **3** Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos, New Mexico, United States of America, **4** Biosecurity and Public Health, Los Alamos National Laboratory, Los Alamos, New Mexico, United States of America, **5** Presbyterian Healthcare Services, Albuquerque, New Mexico, United States of America

† Deceased.

* jzk@lanl.gov

OPEN ACCESS

Citation: Stromberg ZR, Theiler J, Foley BT, Myers y Gutiérrez A, Hollander A, Courtney SJ, et al. (2022) Fast Evaluation of Viral Emerging Risks (FEVER): A computational tool for biosurveillance, diagnostics, and mutation typing of emerging viral pathogens. PLOS Glob Public Health 2(2): e0000207. <https://doi.org/10.1371/journal.pgph.0000207>

Editor: Priyanka Baloni, Institute for Systems Biology, UNITED STATES

Received: June 23, 2021

Accepted: January 23, 2022

Published: February 24, 2022

Copyright: © 2022 Stromberg et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All code written in support of this publication is publicly available at <https://github.com/jt-lanl/fever-probes>.

Funding: This research was funded by Los Alamos National Laboratory Exploratory Research and Development grant number 20190392ER (to J.K.S and K.Y.) and Los Alamos National Laboratory Technology Evaluation and Demonstration Funds. The funders had no role in study design, data

Abstract

Viral pathogens can rapidly evolve, adapt to novel hosts, and evade human immunity. The early detection of emerging viral pathogens through biosurveillance coupled with rapid and accurate diagnostics are required to mitigate global pandemics. However, RNA viruses can mutate rapidly, hampering biosurveillance and diagnostic efforts. Here, we present a novel computational approach called FEVER (Fast Evaluation of Viral Emerging Risks) to design assays that simultaneously accomplish: 1) broad-coverage biosurveillance of an entire group of viruses, 2) accurate diagnosis of an outbreak strain, and 3) mutation typing to detect variants of public health importance. We demonstrate the application of FEVER to generate assays to simultaneously 1) detect sarbecoviruses for biosurveillance; 2) diagnose infections specifically caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); and 3) perform rapid mutation typing of the D614G SARS-CoV-2 spike variant associated with increased pathogen transmissibility. These FEVER assays had a high *in silico* recall (predicted positive) up to 99.7% of 525,708 SARS-CoV-2 sequences analyzed and displayed sensitivities and specificities as high as 92.4% and 100% respectively when validated in 100 clinical samples. The D614G SARS-CoV-2 spike mutation PCR test was able to identify the single nucleotide identity at position 23,403 in the viral genome of 96.6% SARS-CoV-2 positive samples without the need for sequencing. This study demonstrates the utility of FEVER to design assays for biosurveillance, diagnostics, and mutation typing to rapidly detect, track, and mitigate future outbreaks and pandemics caused by emerging viruses.

collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors declare no competing interests. Author Dr. Karina Yusim was unable to confirm their authorship contributions. On their behalf, the corresponding author has reported their contributions to the best of their knowledge.

Introduction

In the last decade, several RNA viruses have emerged from animal reservoirs to threaten global public health [1]. These viruses include influenza A in 2009 [2], Ebola in 2013 [3], Chikungunya in 2014 [4], Zika in 2015 [5], and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) most recently in 2019 [6,7]. Outbreaks and pandemics often occur when a zoonotic viral strain evolves and escapes human immunity [8,9]. The early detection of these pathogens is required to minimize outbreaks and prevent pandemics by guiding early interventions [10]. However, RNA viruses mutate rapidly, so biosurveillance tools must be able to detect groups of viral pathogens with diverse genome sequences and emerging variants, often requiring time-consuming and expensive procedures performed by trained personnel that are not easily accessible in all regions of the world [11,12]. Once spillover occurs and a viral isolate enters human circulation, a rapid and accurate diagnostic test is required to circumvent its spread [13–16]. However, development of accurate diagnostics at the onset of an outbreak can be challenging due to limited data availability depending on the location of the outbreak, and the process for validation and implementation of a new test can also be time-consuming [17]. Having a broadly applicable computational approach to assay design that can accommodate pathogen emergence and variant evolution would help alleviate some of these challenges.

Coronaviruses are positive-sense, single-stranded RNA viruses and a leading cause of the common cold [18]. However, several recent coronavirus outbreaks, including SARS-CoV in 2002–2003, and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012, were associated with significant morbidity and mortality [8,19,20]. In December 2019, SARS-CoV-2 emerged in humans and spread rapidly, resulting in the COVID-19 (coronavirus disease 2019) global pandemic [21–23]. SARS-CoV-2, along with SARS-CoV and SARS-like viruses, belong to the subgenus *Sarbecovirus* [24]. Effective diagnostic and biosurveillance assays are critical to control the spread of SARS-CoV-2 and prevent future *Sarbecovirus* pandemics.

Real-time reverse transcription PCR (RT-PCR) is considered the gold standard for COVID-19 diagnostics [25] with assays developed by the United States Centers for Disease Control and Prevention (U.S. CDC) [26], China CDC [27], Hong Kong University [28], and Charité Institute of Virology [29], along with commercial products [30]. Traditionally, RT-PCR probe and primer design involved selecting a target from a specific gene, often a virulence factor [31]. In contrast, modern probe and primer design involves multiple sequence alignments and algorithms to determine conserved signatures for detection [32–34]. However, if assays are not updated periodically to account for variants, genome mutations can emerge at the primer or probe binding site and limit assay utility [35,36]. The U.S. CDC initially designed a pan-*Sarbecovirus* assay targeting the nucleocapsid gene (2019-nCoV_N3) with broad coverage, however it eliminated this assay from its diagnostic panel to simplify the testing process [37] and because of cross-reactivity [38] and false-negative results on clinical samples [39]. Also, mutations at either a primer or probe binding site (not specified because of proprietary components) yielded false-negative results for the Roche cobas envelope gene pan-*Sarbecovirus* assay [36]. Finally, mutations have made SARS-CoV-2 more transmissible [40,41] and potentially more resistant to immunity induced by vaccination [42]. The current practice of independently introducing biosurveillance efforts, targeted diagnostics, and mutation tracking has hampered our ability to adapt and respond to the dynamic challenges presented by the COVID-19 pandemic. The development of a synchronous approach to integrate these data streams can help mitigate the challenges posed by emerging viral pathogens and support rapid decision making to help combat future outbreaks and pandemics.

There is a critical need for tools that can be used in biosurveillance and diagnostic applications to respond rapidly to emerging pathogens and mitigate their global impact [43]. To this

end, we have developed FEVER (Fast Evaluation of Viral Emerging Risks), a computational approach that can generate both high-coverage or strain-specific diagnostic assays so that the same detection platform can be used for both broad-based biosurveillance and targeted diagnostic applications. Here, we demonstrate the applicability of FEVER for simultaneous biosurveillance of pan-*Sarbecoviruses* and targeted diagnosis of SARS-CoV-2 in a cohort of 100 human patients. We also demonstrate mutation typing of the D614G SARS-CoV-2 spike variant using this approach as an example of a rapid method for tailored surveillance of pathogen evolution and emergence.

Materials and methods

Ethics

This study was designed in alignment with DOE, NIH, and universal HIPAA guidelines. The protocol was reviewed and approved by the Institutional Review Board of the Los Alamos National Laboratory (LANL#000473), per DOE guidelines and policies, and by the Presbyterian Institutional Review Board (PHS IRB#1608836). All samples were collected following written informed consent by the patient.

FEVER surveillance and diagnostic probe and primer design

For the surveillance and diagnostics FEVER assays, four sets of primers and probes were generated (Table 1). Two sets of primers and probes were developed for universal detection of all sarbecoviruses, with one targeting the 5' untranslated region (UTR) termed FEVER_5'UTR and another targeting the envelope gene termed FEVER_Env. Another two sets of primers and probes were developed for specific detection of SARS-CoV-2, with one targeting the ORF1ab

Table 1. FEVER assays for COVID-19.

Gene target	Primer or probe name	Sequence 5' to 3'	Final conc. (nM)
Pan-Sarbecovirus detection			
5'UTR	FEVER_5'UTR_F	CTGCTTACGGTTYCGTCCGTGTTGC	500
	FEVER_5'UTR_R	ACTGAGTTGGACGTGTGTTTCTC	500
	FEVER_5'UTR_P	FAM-CTCTCCATC/ZEN/TTACCTTTCGGTCACACCCGG-IABkFQ	250
Envelope	FEVER_Env_F	CCGACGACGACTACTAGCG	500
	FEVER_Env_R	GTTTAGACCAGAAGATCAGGAAC	500
	FEVER_Env_P	FAM-CGCACACAA/ZEN/TCGAAGCGCAGTAAGGATGGCT-IABkFQ	250
Specific SARS-CoV-2 detection			
ORF1ab	FEVER_ORF1ab_F	ATGAAGTATTTTGTGAAAATAGG	500
	FEVER_ORF1ab_R	CAGCTAGACACCTAGTCATG	500
	FEVER_ORF1ab_P	FAM-TCTGAAGCA/ZEN/GTGGAAAAGCATGTGGCACGT-IABkFQ	250
Spike	FEVER_Spike_F	TCCCTCAGGGTTTTTCGGCTTTAG	500
	FEVER_Spike_R	GTTTCTGAGAGAGGGTCAAGTGCAC	500
	FEVER_Spike_P	FAM-TGCAGCACC/ZEN/AGCTGTCCAACCTGAAGAAGAA-IABkFQ	250
Mutation typing			
Spike	23403_F	CCAGGAACAAATACTTCTAACCAGGTT	900
	23403_R	GCATGAATAGCAACAGGGACTTCT	900
	23403_P1(Wuhan)	VIC-TGTTCTTTATCAGGA <u>A</u> TGTTAAC-NFQ	200
	23403_P2(Italy)	FAM-TCTTTATCAGGG <u>G</u> TGTTAAC-NFQ	200

Bold/underlined nucleotide indicates the position at which the SNP is detected.

<https://doi.org/10.1371/journal.pgph.0000207.t001>

region termed FEVER_ORF1ab and one targeting the spike gene called FEVER_Spike. After the FEVER probes were designed, primers flanking the probes were created by the PrimerDesign-M tool [44,45] using an alignment of SARS-CoV-2 sequences.

Sequence data. As of March 10, 2020 the Global Initiative on Sharing All Influenza Data (GISAID) database (<https://www.epicov.org> or <https://www.gisaid.org/>) had acquired 338 complete, or near-complete, high-quality SARS-CoV-2 genome sequences. After aligning the sequences and eliminating all but one of each set of sequences which were 100% identical to each other to reduce redundancy, our data set consisted of 19 unique sequences of SARS-CoV-2. We also collected all coronaviruses from GenBank that were in the “Sarbecovirus clade” of the beta coronaviruses, using BLAST searches rather than GenBank taxonomy because many sarbecoviruses were listed as “unclassified coronavirus” in the taxonomy table. Again, we reduced redundancy by eliminating identical sequences (primarily many dozens of SARS sequences from the 2003–2004 outbreak), and retained a set of 67 genome sequences. It was these 86 *Sarbecovirus* genomes that were used to design our assays.

FEVER algorithm. The FEVER algorithm does not require sequence alignments prior to probe design. Instead, our probe design goal was to produce a short nucleotide sequence (called a “ k -mer”, *e.g.*, a short DNA sequence, k base pairs long) that binds to SARS-CoV-2 without binding to background material that may be present in a sample that is being tested. Specific desirable properties of the probe included maintaining a high GC-content and reducing the hairpin propensity (*i.e.*, the tendency of the probe string to fold over on itself) by ensuring that any self-complementary pairs of sub-strings in the probe are sufficiently short. The mathematical formalization of the problem was expressed in terms of a constrained optimization, with minimum GC-content and the maximum length of self-complementary sub-strings taken as constraints while maximizing coverage. A probe is said to “cover” a SARS-CoV-2 sequence if the probe’s k -mer appears as a sub-sequence of that sequence. Thus, coverage is defined as the number of sequences in the database covered by the probe. “Exact” coverage corresponds to an exact match of probe and sub-sequence. Hamming distance between two strings is defined as the number of characters that would have to be changed in one string to agree with the other string. Given a database of SARS-CoV-2 sequences, the single probe design problem is to find a string of length k , subject to the constraints of GC-content and hairpin aversion, that covers as many SARS-CoV-2 sequences as possible. For the multi-probe design problem, we seek n probes instead of just one, and a n -probe design is said to cover a SARS-CoV-2 sequence if any one of the probes covers it. The selection of $k = 31$ was used because of practical considerations for molecular beacon synthesis allowing for a 12 bp stem region (6 bp on each side of the k -mer). Also, $k = 31$ was long enough to bind to target sequences in the SARS-CoV-2 database without accidentally covering potential background sequences.

Our FEVER algorithm works by extracting all k -mers from each of the sequences in the database and then restricting consideration to those that exhibit a minimal GC-content and hairpin aversion. For every one of these k -mers, we counted how many viral sequences were covered, being careful not to count a sequence twice even if the k -mer appears twice in it. Finally, we selected the k -mer with the largest count. To ensure that our probes were not susceptible to viral mutation, a multi-probe design approach was applied. The first probe was designed using a naïve approach by producing the optimal probe with highest coverage of the queried sequence database. The next probe is designed by eliminating the viral sequences covered by the first probe and using this truncated database to find the single probe that best covers the remaining viral sequences. This process is repeated until 100% of the database is covered. For both pan-*Sarbecovirus* sequences and SARS-CoV-2 sequences it only took 2 probes to obtain 100% coverage of the sequence database at the time of design.

FEVER mutation typing probe and primer design

A variant carrying the amino acid change D614G spike mutation became globally dominant, and the G614 variant has been demonstrated to have a fitness advantage associated with higher viral loads [40]. A custom TaqMan SNP Genotyping Assay was designed (Thermo Fisher, 4332075) to detect this single nucleotide polymorphism (SNP) in SARS-CoV-2 using Thermo Fisher Scientific's Custom Assay Design Tool. Probe and primer sequences are presented in Table 1.

Analysis of primer and probe coverage

The FEVER biosurveillance and diagnostics assays were computationally characterized using two complementary methods. First, the frequency of SARS-CoV-2 variants in the amplified region of the FEVER and U.S. CDC assays were assessed visually using the Variant Visualizer, which is under development and will be available at <https://cov.lanl.gov>. Second, we used an *in silico* validation tool [46] to assess the inclusivity of the FEVER assays compared with the U.S. CDC N1 and N2 assays [26]. Each assay (forward primer, reverse primer and TaqMan probe) was evaluated against each SARS-CoV-2 sequence from GISAID [47] public database that was greater than 29 kb ($n = 525,708$) to ensure only complete genomes were assessed. The GISAID sequence data used in this analysis was accessed on February 15, 2021. Results are presented in Table 2. For this analysis, a false-negative was assigned if one or more assay oligonucleotides satisfied any of the following conditions: (i) three or more mismatches to a target sequence, (ii) a predicted melting temperature less than 40°C between the oligonucleotide and a target sequence, or (iii) when primer/target mismatches occurred in either of the last two 3' bases of a primer associated with an expected increase of 2 or more in the cycle threshold (C_T) value (as defined previously [48]). True-positives were assigned for any assay/target sequence pairing that did *not* result in a false negative. Results are also reported as the number of genome sequences that contained perfect matches, single mismatches, two mismatches, or three or more mismatches for a given assay.

Patient sample collection

From November 2020 to December 2020, 100 nasopharyngeal (NP) swabs were collected from individuals >18 years of age, presenting to Presbyterian Hospital (Albuquerque, New Mexico, USA) with suspected or confirmed COVID-19. Pregnant women, children, non-English speakers, and prisoners were excluded from this study. Eligible patients were approached by the Presbyterian Clinical Research Team about participating in our research study

Table 2. *In silico* inclusivity test against SARS-CoV-2 sequences.

Assay	Recall (%) ^a	No. sequences with perfect match	No. sequences with 1 mismatch	No. sequences with 2 mismatches	No. sequences that failed ^b
FEVER_5'UTR	96.39	413,603	91,763	1,361	18,981
FEVER_Env	98.63	511,976	6,164	343	7,225
FEVER_ORF1ab	99.72	490,388	33,723	143	1,454
FEVER_Spike	90.51	360,033	115,321	481	49,873
U.S. CDC N1	98.95	506,092	11,454	2,633	5,529
U.S. CDC N2	99.17	507,298	13,901	156	4,353

Oligonucleotides from each assay were assessed against 525,708 sequences of SARS-CoV-2 obtained from GISAID on February 15, 2021.

^aRecall = true-positive (sum of the perfect match, single mismatch, and double mismatch) / (true-positive + false negative).

^bFailure represents SARS-CoV-2 sequences that were not detected (false-negative).

<https://doi.org/10.1371/journal.pgph.0000207.t002>

(IRB#1608836). If the patient was interested informed consent was obtained in their individual room following COVID safe practices. This study's sample is representative of the general adult population. Relevant patient demographical details can be found in [S1 Table](#).

NP swabs were collected following consent using existing U.S. CDC guidelines, labeled with a de-identified bar code, and stored under refrigerated conditions until batch shipping (within 24 hours of sample collection). Specimens were packed and shipped according to U.S. Department of Transportation regulations regarding the shipment of biological substances.

RNA extraction

For spiked samples, RNA extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen). For the NP swab samples, RNA was extracted using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher) and a KingFisher. For both kits, a 200 μ L portion of each sample was used for extraction following the manufacturer's instructions with a 50 μ L elution, and 5 μ L of the eluted template was used in RT-PCR.

RT-PCR protocol

Samples were tested using our FEVER and U.S. CDC 2019-novel coronavirus (2019-nCoV) RT-PCR diagnostic panel (termed U.S. CDC assays) [26]. For the U.S. CDC N1 (2019-nCoV_N1) and N2 (2019-nCoV_N2) assays, each 20 μ L reaction consisted of 8.5 μ L of water, 1.5 μ L of combined primer and probe mix (IDT, 500 nM primer and 125 nM probe, final concentrations), 5 μ L of TaqPath 1-step RT-qPCR master mix (Thermo Fisher), and 5 μ L of the template. For the FEVER assays (FEVER_5'UTR, FEVER_Env, FEVER_ORF1ab, and FEVER_Spike), each 20 μ L reaction consisted of 8 μ L of water, 2 μ L of combined primer and probe mix (IDT, 500 nM primer and 250 nM probe, final concentrations), 5 μ L of TaqPath 1-step RT-qPCR master mix, and 5 μ L of the template. The thermocycling conditions were the same for all assays. Thermocycling conditions using an ABI StepOnePlus or 7500 Fast Dx were 25°C for 2 min, 50°C for 15 min, 95°C for 2 min, followed by 45 cycles of 95°C for 3 s and 55°C for 30 s. For RT-PCR assays, samples were considered positive when the C_T value ≤ 40 . Patient clinical samples were considered positive for SARS-CoV-2 when the two U.S. CDC assays N1 and N2 both had a C_T value ≤ 40 , and negative when both targets had a C_T value > 40 according to CDC guidelines [37]. If only one N1 or N2 result was positive, the result was considered inconclusive and the sample was re-run. If the re-test results were also inconclusive, the final result was reported as inconclusive.

Analytical specificity of RT-PCR

The analytical specificities of the FEVER and U.S. CDC assays were evaluated using genomic RNA from various viruses obtained from BEI resources. We tested 5 μ L of undiluted genomic RNA preparation from SARS-CoV Urbani (NR-52346), avian coronavirus Massachusetts (NR-49096), alphacoronavirus Purdue P115 (NR-48571), porcine respiratory coronavirus ISU-1 (NR-48572), two influenza A viruses (NR-20080 and NR-20081), and two influenza B viruses (NR-45848 and NR-45849). RNA from heat-inactivated SARS-CoV-2 isolate USA-WA1/2020 (BEI Resources, NR-52347) was a positive assay control, and water was a negative assay control.

Analytical sensitivity

The analytical sensitivity of each assay was determined by spiking inactivated SARS-CoV-2 USA-WA1/2020 strain (BEI resources, NR-52286) at 10^4 copies/ μ L, and 10-fold serial

dilutions were performed in an NP swab matrix. NP swabs from a single human donor (Lee Biosolutions, #991-31-NC-COVID-N) were suspended in 0.45% saline and confirmed SARS-CoV-2 negative by the supplier. Each concentration was tested using three biological replicates.

Assessment of NP swab samples

The 100 NP swab samples were transported to the laboratory for RNA extraction and testing. After RNA extraction, samples were stored at -80°C . Samples were tested by our FEVER and U.S. CDC assays for SARS-CoV-2. For the U.S. CDC assays, the 2019-Ncov plasmid (IDT) containing the nucleocapsid gene was used as a positive control, and water was used as a negative control. For the FEVER assays, RNA from heat-inactivated SARS-CoV-2, isolate USA-WA1/2020 (BEI Resources, NR-52347) was used as a positive control, and water was used as a negative control. The RNase P assay described by the U.S. CDC assays [26] was used as an extraction control for each sample.

Mutation typing of SARS-CoV-2 by PCR

Extracted RNA (10 μL) from SARS-CoV-2 positive patient samples was reverse transcribed in a 20 μL reaction using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher, 4368814) according to the manufacturer's instructions. Each 20 μL SNP genotyping PCR reaction consisted of 5 μL of cDNA as the template, 4.5 μL of water, 0.5 μL of 40 $\times$ SNP genotyping mix (900 nM primer and 200 nM probe final concentrations), and 10 μL of 2 $\times$ TaqMan Genotyping Master Mix (Thermo Fisher, 4371353). Amplification was performed using an ABI StepOnePlus RT-PCR machine under the following conditions: 10 min at 95°C and 45 cycles of 15 s at 95°C and 60 s at 60°C in a 96-well plate. The PCR C_T values corresponding with the wild type (VIC) and mutant (FAM) forms were compared, and the lower value (≤ 45) of the two was determined to be the SNP sequence. Samples were considered undetermined when both reactions had a C_T value >45 . Control RNA from Heat-Inactivated SARS-CoV-2, isolate USA-WA1/2020 (BEI Resources, NR-52347) was used as a positive control, diluted to 10^4 copies/ μL , and run with each batch of cDNA synthesis and PCR. Isolate USA-WA1/2020 contains the wild-type spike sequence, and all results with this control showed that the wild-type C_T (≤ 36) was lower than the mutant C_T value (undetermined, >45).

A total of 17 NP swab samples had sufficient sample volume to be used in sequencing to confirm the D614G mutation. The spike gene was amplified using the 23403F_seq and 23403R_seq primers (5'- TGAACCTTCTACATGCACCAGC -3' and 5'- AAACAGCCTGCACG TGTTTG -3', respectively). Each 25 μL reaction consisted of 5 μL of cDNA as the template, 11.75 μL of water, 5 μL of 5X Q5 reaction buffer (New England BioLabs), 0.5 μL of 10 mM dNTPs, 1.25 μL of each primer (0.5 μM final concentration), and 0.25 μL of Q5 hot start high-fidelity DNA polymerase (New England BioLabs). PCR was performed on a thermocycler as follows: 30 s at 95°C and 35 cycles of 10 s at 98°C , 30 s at 65°C , and 30 s at 72°C . Amplified products were purified using the PureLink PCR Purification Kit (Thermo Fisher, K310002) and were sent for sequencing to Eurofins Genomics.

Statistical analysis

Data were analyzed with GraphPad Prism version 8. Confidence intervals were calculated using the modified Wald method. The Pearson correlation coefficient was used to assess the concordance between PCR C_T values. P values < 0.05 were deemed significant.

Results

FEVER computational tool

FEVER combines probes and primers generated to 1) broadly identify an entire group of viruses; 2) specifically detect a strain of interest; and 3) detect SNPs of importance to public health (Fig 1). The computational approach starts with a curated multiple sequence alignment. The FEVER algorithm does not however require sequences to be aligned in order to generate assays. The alignment was performed to avoid overrepresentation of any single isolate and ensure broad coverage of the pathogen of interest. In March of 2020, we developed two alignments using the sequences available in GISAID: one with SARS-CoV-2 sequences and one with *Sarbecovirus* sequences. Of note our FEVER assays were developed within one month of the U.S. CDC assays [41] and also used viral sequence information obtained from GenBank. Next, our FEVER algorithm was applied to design probes using the *Sarbecovirus* alignment for biosurveillance and the SARS-CoV-2 alignment for diagnostics while also screening the results using parameters set for GC-content, hairpin propensity, and match recall to achieve high coverage as well as reagent manufacturing and performance compatibility. The FEVER algorithm identifies sets of probes that cover 100% of each multiple sequence alignment. Two pan-*Sarbecovirus* (FEVER_5'UTR and FEVER_Env) and two SARS-CoV-2 (FEVER_ORF1ab and FEVER_Spike) specific probes were needed to detect all input sequences. After the FEVER probes were designed, primers flanking the probe sites were designed using the PrimerDesign-M tool [44,45] for use in RT-PCR assays (Table 1). Few variants were visually detected in the region of amplification of these primers and probes (S1 Fig). Further, we designed TaqMan SNP Genotyping PCR probes and primers to detect the previously identified A23403G (D614G amino acid change) mutation in the spike gene [40] (Table 1). Mutations in the SARS-CoV-2 genome conferring a fitness advantage have been reported [40]. However, rapid

Fig 1. Schematic of FEVER assay approach and comparison of features to the U.S. CDC SARS-CoV-2 assay. (a) Overview of compiling a database of SARS-CoV-2 and *Sarbecovirus* genome sequences to generate FEVER primer and probe sets. (b) Assays were designed to detect the D614G variant. (c) The tabulated features of the FEVER assays compared with the U.S. CDC SARS-CoV-2 assay.

<https://doi.org/10.1371/journal.pgph.0000207.g001>

methods to characterize SARS-CoV-2 mutations are lacking. Together, the primers and probes for all three components (biosurveillance, diagnostics, and mutation typing) are referred to collectively as the FEVER assays.

***In silico* inclusivity test**

We evaluated our FEVER assays using a public web-based validation tool against 525,708 sequences of SARS-CoV-2 [46]. The *in silico* evaluation only included complete SARS-CoV-2 genome sequences that were at least 29 kb long. In general, a low number of predicted failures (false-negatives) was observed in comparison with the number of successes or true-positives (total of perfect matches, single mismatch, and double mismatches) (Table 2). Recall (true positives divided by the sum of true positives and false negatives) was used to assess relative assay performance. Of the FEVER and U.S. CDC assays, the SARS-CoV-2 specific FEVER probe targeting ORF1ab had the best-predicted performance with a recall of 99.7%. The pan-*Sarbecovirus* probe targeting the 5' UTR and envelope gene were also predicted to be highly inclusive for SARS-CoV-2 with recalls of 96.4% and 98.6%, respectively. In contrast, the SARS-CoV-2 specific probe targeting the spike gene had a 90.5% recall, suggesting that the spike gene is a suboptimal target due to its high genetic variability [40,49]. For instance, the U.S. CDC N1 assay had a recall of 99.0% and the U.S. CDC N2 assay had a 99.2% recall. Compared with the U.S. CDC assays, the FEVER_ORF1ab assay had a ~1% higher recall, FEVER_Env had a similar recall, and the FEVER_5'UTR and FEVER_Spike assays had lower recall values.

Analytical specificity

Cross-reactivity of primers and probes with other microorganisms can reduce the efficacy of RT-PCR [50]. For analysis of specificity, SARS-CoV-2 was the only target virus for the SARS-CoV-2 specific assays, and all sarbecoviruses were considered the targets for the pan-*Sarbecovirus* probes. We initially evaluated cross-reactivity *in silico* and then experimentally with non-target viruses. The specificity of each primer and probe set was tested *in silico* using BLAST, which yielded matches to SARS-CoV-2 only for the assay oligonucleotides specific to SARS-CoV-2 (FEVER_ORF1ab and FEVER_Spike) and matches to sarbecoviruses for the pan-*Sarbecovirus* assay oligonucleotides (FEVER_5'UTR and FEVER_Env). No hits to human DNA or other respiratory viruses were found.

For experimental evaluation of specificity, the FEVER assays were tested against three animal coronavirus strains (alphacoronavirus 1 strain Purdue P115, avian coronavirus strain Massachusetts, and porcine respiratory coronavirus strain ISU-1), four influenza virus strains (A/Brisbane/59/2007, A/Brisbane/10/2007, B/Nevada/03/2011, and B/Texas/06/2011), and a single strain of SARS-CoV (Urbani, isolated in 2003). The SARS-CoV-2 strain USA-WA1/2020 was used as a positive control. The three animal coronavirus and four influenza virus strains all tested negative (Table 3). Excluding SARS-CoV-2, the SARS-CoV Urbani strain was the only commercially available genomic RNA from a *Sarbecovirus* available at the time of testing. The SARS-CoV Urbani strain was correctly tested negative by the U.S. CDC assays (N1 and N2), FEVER_ORF1ab, and FEVER_Spike targets. The SARS-CoV strain Urbani tested positive by the pan-*Sarbecovirus* (FEVER_5'UTR and FEVER_Env) FEVER assays, which was expected because these were designed to detect all sarbecoviruses. The SARS-CoV-2 strain USA-WA1/2020 used as a positive control was correctly identified by both FEVER and U.S. CDC assays.

Analytical sensitivity in a NP swab matrix

The analytical sensitivity and reproducibility of each assay was tested using standard curves generated by three biological replicates of 10-fold serial dilutions of SARS-CoV-2 spiked into

Table 3. Analytical specificity of the FEVER and U.S. CDC assays.

Virus	Strain	GenBank Acc. # or Taxon ID ^a	FEVER				U.S. CDC	
			FEVER_5'UTR	FEVER_Env	FEVER_ORF1ab	FEVER_Spike	N1	N2
Alphacoronavirus 1	Purdue P115	DQ811788	–	–	–	–	–	–
Avian coronavirus	Massachusetts	GQ504724	–	–	–	–	–	–
Influenza A H1N1	A/Brisbane/59/2007	504904	–	–	–	–	–	–
Influenza A H3N2	A/Brisbane/10/2007	476294	–	–	–	–	–	–
Influenza B Victoria lineage	B/Nevada/03/2011	1308200	–	–	–	–	–	–
Influenza B Yamagata lineage	B/Texas/06/2011	1308263	–	–	–	–	–	–
Porcine respiratory coronavirus	ISU-1	DQ811787	–	–	–	–	–	–
SARS-CoV	Urbani	AY278741	+	+	–	–	–	–
SARS-CoV-2	USA-WA1/2020	MN985325	+	+	+	+	+	+

Abbreviations: +, detected;–, not detected.

^aNCBI Taxon IDs were listed for influenza strains, which did not have a GenBank accession number.

<https://doi.org/10.1371/journal.pgph.0000207.t003>

human NP swab samples. The standard curves for RT-PCR in NP swab samples had correlation coefficients (efficiency values) of 0.98 (100.9%) for FEVER_5'UTR, 0.99 (93.7%) for FEVER_Env, 0.99 (96.5%) for FEVER_ORF1ab, 0.99 (98.0%) for FEVER_Spike, 0.99 (103.2%) for N1, and 0.99 (99.1%) for N2, respectively (Fig 2). We demonstrated that both FEVER and U.S. CDC assays reliably detected SARS-CoV-2 at 1 copy/μL spiked into an NP swab sample matrix.

Comparison of FEVER and U.S. CDC RT-PCR results for clinical NP swab samples

Nasopharyngeal (NP) swab sampling is the recommended sampling method by the World Health Organization [51] for nucleic acid amplification testing of SARS-CoV-2. Viral titers measured in NP swabs vary depending on several factors, including disease progression [52–54]. However, NP swabs currently remain the reference sample type and support sensitive detection of SARS-CoV-2 [55]. Nasopharyngeal swabs from 100 COVID-19 patients were collected in New Mexico, U.S. All assays were run under the same thermocycling conditions as per the U.S. CDC protocol [26]. All samples tested positive for the human RNase P gene used as a control to ensure success of the RNA extraction and associated sample collection methods (S2 Table). Of the 100 NP swab samples, 66% (66/100) were considered positive according to the U.S. CDC assays and testing criteria [37], 22 were negative and 12 samples yielded inconclusive results (Table 4). Samples that initially produced an inconclusive result were re-tested and the 12 that remained inconclusive following the second test were removed from the analysis of agreement, sensitivity, and specificity. We compared the four FEVER assays to the results obtained with the U.S. CDC assays and found that all four FEVER assays displayed 100% specificity (no false positives were detected) and varying sensitivities ranging from 74.2% to 92.4% (Table 4 and S2 Table). However, the U.S. CDC assays were performed in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory, while the FEVER assay was performed in a separate facility on a different thermocycler for research purposes only.

To directly compare the performance of the FEVER and U.S. CDC assays, 10 samples that contained sufficient remaining RNA were analyzed using both groups of assays on the same ABI StepOnePlus thermocycler in the same laboratory to avoid inter-instrument and inter-laboratory variability. The C_T values of the FEVER assays were plotted against those from the U.S.

Fig 2. FEVER assays can detect 1 copy/ μ L SARS-CoV-2 spiked into a nasopharyngeal swab sample matrix. Inactivated SARS-CoV-2 strain USA-WA1/2020 was spiked into human nasopharyngeal swab samples (confirmed to be SARS-CoV-2 negative) and serial 10-fold diluted from 10^4 to 1 copies/ μ L. Cycle threshold (C_T) values were determined for FEVER assays (blue dots) (a) FEVER_5'UTR, (b) FEVER_Env, (c) FEVER_ORF1ab, and (d) FEVER_Spike and the (a) N1 and (b) N2 assay described by the U.S. CDC (black dots). Each dot within a given concentration is from an independent experiment and a total of three independent experiments were performed for each assay.

<https://doi.org/10.1371/journal.pgph.0000207.g002>

Table 4. FEVER assays compared to the U.S. CDC assays for detection of SARS-CoV-2 in 100 human patient nasopharyngeal swab samples.

Result ^a	FEVER_5'UTR	FEVER_Env	FEVER_ORF1ab	FEVER_Spike
Positive	61	56	56	49
Negative	39	44	44	51
Agreement ^b	94.3% (87.1–97.9%)	87.5% (78.8–93.0%)	88.6% (80.2–93.9%)	80.7% (71.1–87.7%)
Sensitivity ^c	92.4% (83.1–97.1%)	83.3% (72.4–90.6%)	84.8% (74.1–91.8%)	74.2% (62.5–83.3%)
Specificity ^d	100% (84.6–100%)	100% (84.6–100%)	100% (84.6–100%)	100% (84.6–100%)

^a True values are as measured by the U.S. CDC assay criteria [37]: 66 positive, 22 negative and 12 inconclusive. Samples initially recorded as inconclusive were re-tested and the 12 that remained inconclusive were removed from the analysis of agreement, sensitivity, and specificity.

^b Agreement = no. of samples with identical results for an individual FEVER assay and U.S. CDC assay / 88.

^c Sensitivity = true positive / (true positive + false negative) with 95% confidence intervals.

^d Specificity = true negative / (true negative + false positive) with 95% confidence intervals.

95% confidence intervals were calculated using the modified Wald method.

N/A, not applicable.

<https://doi.org/10.1371/journal.pgph.0000207.t004>

CDC assay (Fig 3). In general, a broad range of C_T values from 13 to 39 was observed. Interestingly, one sample that originally tested positive for the U.S. CDC assay (N2) with a C_T value of 34 on the ABI 7500 Fast Dx, tested negative on the ABI StepOnePlus. There was a significant ($P < 0.001$), correlation between the C_T values of all four FEVER assays compared with each of the U.S. CDC N1 and N2 assays indicating that when run on the same instrument the FEVER and U.S. CDC assays yield highly similar C_T values (Fig 3). It is unfortunate that this

Fig 3. The FEVER and U.S. CDC assays show concordance in detecting SARS-CoV-2 in patient nasopharyngeal swab samples. The U.S. CDC N1 cycle threshold (C_T) values were compared to (a) FEVER_5'UTR, (b) FEVER_Env, (c) FEVER_ORF1ab, and (d) FEVER_Spike RT-PCR assays C_T values (blue dots). The U.S. CDC N2 C_T values were compared to (e) FEVER_5'UTR, (f) FEVER_Env, (g) FEVER_ORF1ab, and (h) FEVER_Spike RT-PCR assays C_T values (purple dots). One sample was removed for the N2 vs FEVER_ORF1ab and N2 vs FEVER_Spike comparisons because it was not detected by these assays. Each dot represents an individual sample. Dotted lines represent the limit of detection. ND, not detected.

<https://doi.org/10.1371/journal.pgph.0000207.g003>

finding occurred after most of the patient samples were exhausted and so not all could be re-tested using the same instrument. These results highlight the importance of using the same instrument when comparing C_T values for assay validation.

D614G mutation typing

High-throughput tracking of SARS-CoV-2 mutations in patient samples may be essential for monitoring the spread of vaccine escape mutants [42]. Sequencing can identify novel variants but is too expensive and time-consuming for high-throughput screening of patient samples to track the spread and movement of these variants on a global scale [40]. To facilitate a rapid and high-throughput method for monitoring the spread of SNPs of interest, we have incorporated a SNP Genotyping PCR assay. For this mutation typing test, we are detecting the A23403G (D614G amino acid change) mutation in the spike gene, which is associated with greater infectivity [40] (Table 1). Control RNA from isolate USA-WA1/2020 (BEI Resources, NR-52347) was used as a positive control, and results showed that USA-WA1/2020 tested positive for D614 (C_T of ≤ 36 for D614 vs C_T not determined [>45] for G614). Of the 59 SARS-CoV-2 positive patient samples (according to at least one SARS-CoV-2 specific FEVER assay), we determined the SNP at position 23,403 for 56 (95%) samples. The G23403 variant was found in 56 samples and 3 samples were undetermined with a C_T cutoff of 45 (S3 Table). A fragment of the spike gene was amplified, and the A23403G SNP was confirmed in a subset of samples ($n = 17$) by sequencing (S4 Table). These results indicate that most (56/59) patient isolates contained the mutated G614 form of SARS-CoV-2 that became prevalent globally in April 2020 [40].

Discussion

Early detection of emerging pathogens is required to mitigate outbreaks and prevent pandemics [10]. Assays used for diagnosing infections are often used for biosurveillance; however, due to their high specificity, they are not usually suited for broad-spectrum surveillance applications or capable of identifying pathogen mutations [43,56]. Therefore, the user must often decide between either obtaining diagnostics or biosurveillance information but not both. FEVER (Fast Evaluation of Viral Emerging Risks) is a computational approach designed to simultaneously facilitate broad-spectrum biosurveillance, pathogen-specific diagnostics, and SNP variant tracking for pandemic response to any viral pathogen of interest. In this manuscript, we demonstrate the application of FEVER to the ongoing COVID-19 pandemic as an initial proof-of-concept. As a proof of concept, we present FEVER assays to detect all sarbecoviruses for future biosurveillance, SARS-CoV-2 for strain-specific diagnostics, and the D614G spike variant SNP mutation associated with increased transmissibility.

Here, we used an RT-PCR format to evaluate the performance of the FEVER assays as compared to the U.S. CDC assays. The success of RT-PCR as a diagnostic approach relies heavily on the design quality of the primers and probes [57–59] as well as factors such as probe format, the ability to incorporate degenerate nucleotides, the number of target genomes that can be included in the design process to account for genetic diversity, and the ability to avoid cross-reactivity with near-neighbor genomes [60]. In the case of SARS-CoV-2 diagnostics, it is also important that assays are able to differentiate infections caused by other common coronaviruses, in which case a false positive could result in unnecessary and costly mitigation procedures [61]. For this reason, the U.S. CDC designed its N3 pan-Sarbecovirus assay; however, it was unfortunately removed from their assay in March of 2020 in order to expedite reagent manufacturing, consequently removing the biosurveillance component of the U.S. CDC COVID-19 assay [35,37]. This situation highlights the urgent need for a computational

approach that can develop both biosurveillance and diagnostics assays that are screened for potential design issues that may potentially affect their performance and manufacturing.

Current probe design methods are also limited by over-representing single isolates in a sequence database as indicated by high coverage *in silico* but assay failure when novel variants emerge [31]. In the case of SARS-CoV-2, the high number of false-negative results reported highlights the need for screening and updating assay designs as sequences become available [62–64], which has been a primary goal of <https://cov.lanl.gov>. Mutations in the probe or primer binding sites are particularly problematic since these can lead to false-negative results and infected individuals may continue to spread the disease [65]. Pandemic preparedness requires a balanced approach combining targeted diagnostics assays designed to detect a specific outbreak strain with high-coverage biosurveillance assays that can account for pathogen mutation, and ideally this approach can be utilized to detect a variety of viral pathogens including those that are highly mutagenic. FEVER was designed to fill this void. In the case of SARS-CoV-2, our assays were developed within a month of the U.S. CDC assays also using publicly available sequence data, but with a different computational approach in mind. The U.S. CDC designed highly specific diagnostics assays by pre-selecting a single genomic target, the viral nucleocapsid gene, aligned a reference SARS-CoV-2 sequence (GenBank accession no. MN908947), and selected probe/primer sets with low similarity to other coronavirus sequences in GenBank [37]. This method has yielded two SARS-CoV-2 specific assays that have contributed to combatting the current pandemic, however it is unclear how useful these assays will be in detecting the next coronavirus outbreak.

FEVER takes a more comprehensive approach to design robust multi-purpose assays to detect circulating viral strains and track their mutations but also to detect entire groups of viral pathogens in order to monitor emerging threats. The FEVER algorithm scans the entire viral genome to identify conserved regions and identifies the lowest possible number of probes necessary to cover all sequences scanned. The diagnostics probe FEVER_ORF1ab displayed the highest coverage of SARS-CoV-2 sequences in this study with a predicted recall of 99.7%. It was predicted to detect all but 1,454 SARS-CoV-2 sequences out of 525,708 deposited in GISAID as of February 15, 2021. In comparison, the U.S. CDC N2 probe displayed 99.17% coverage and was predicted to fail in 4,353 sequences, which is nearly a 3-fold increase in false-negative results.

In addition to optimized sequence analysis, FEVER incorporates physical constraints set by the user to include GC-content, hairpin propensity, sequence length, and even mismatch tolerance. This ensures that probes selected for testing are thermodynamically compatible for increased assay performance as well as future multiplexing. It is critical that assay sensitivity is optimized as a 10-fold increase in the limit of detection for SARS-CoV-2 has shown to result in an increase in the false-negative rate by 13% [66]. Here, the limit of detection for both the FEVER and U.S. CDC assays in NP swab samples was 1 copy/ μ L which matches previously published findings [26]. Additionally, FEVER assay design features can be used to ensure that probes and primers are suitable for large-scale manufacturing and can be tailored to a variety of biosensing platforms, including but not limited to RT-PCR. There is a clear need for the development of rapid and accurate point-of-care diagnostic tools to detect viral infections [67], and a variety of nucleic acid-based viral biosensors are in development for this purpose [43].

FEVER also includes a method for rapid mutation typing to track viral variants of public health importance. SARS-CoV-2 has proofreading mechanisms [68] and therefore relatively lower genetic diversity than other respiratory viruses such as influenza [69]. For SARS-CoV-2, the predicted evolutionary rate is approximately 8×10^{-4} nucleotide substitution per site per year [69]. The SARS-CoV-2 genome is evolving primarily by genetic drift and negative genomic selection. Despite proof reading mechanisms of SARS-CoV-2, over 70% of positions along

the genome have been mutated at least once [70]. In a previous study, it was observed that the non-structural proteins (ORF1ab) had a lower mutation rate than segments coding for structural and accessory proteins [70]. The evolution of the virus can influence diagnostic efficacy as we observed that FEVER_ORF1ab displayed the highest coverage of SARS-CoV-2 sequences, while the FEVER_Spike assay displayed the lowest coverage of SARS-CoV-2 sequences. The spike gene is under immune selective pressure as neutralizing antibodies against the protein product can inhibit viral entry into host cells [40,71]. SARS-CoV-2 acquired the D614G mutation in the spike protein early in the pandemic, and this mutation confers greater infectivity [72–74]. Previously, the detection of this mutation has been based solely on viral sequencing, which is time-consuming and cost-prohibitive in many resource limited regions of the world [40,61,75,76]. FEVER incorporates a rapid PCR-based method for identifying the D614G mutation directly in patient samples. The rise of multiple SARS-CoV-2 variants has led to the need to track multiple variants across time and a typing of a single variant may not be sufficient for this virus. Similarly, mutation typing probes and primers can be designed in the future to track multiple variants of interest including the Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Delta (B.1.617.2) variants [41,77,78] to provide insight and real-time biosurveillance data on the circulation of potential vaccine escape variants.

There are limitations in our evaluation of the FEVER assays. We sampled 100 patients suspected of COVID-19 and this limitation in sample number has been frequently reported in validation studies for COVID-19 diagnostic kits [79]. In addition, the U.S. CDC assay was performed in a separate facility and on a separate thermocycler than the FEVER assays. Differences in laboratories, instruments, and personnel are known to impact diagnostic test results and may have led to slight discrepancies between assay results in the current study [80]. To evaluate this effect, we tested a subset of samples on the same instrument and found strong concordance (Pearson $r \geq 0.95$) between the FEVER and U.S. CDC assays. Samples were tested only once in the concordance experiment due to insufficient sample volumes for repeat testing.

In summary, the FEVER computational approach provides a novel tool to combat emerging viral pathogens. FEVER can be used for simultaneous biosurveillance of viruses, diagnostics of outbreak or pandemic isolates, and mutation typing to rapidly track the spread of variants impacting public health. In addition to PCR, FEVER can generate probes that are compatible with a wide variety of nucleic acid sensing techniques which may be more suitable to point-of-care testing than RT-PCR [43]. Here we show that the FEVER assays are comparable to those designed by the U.S. CDC for the detection of SARS-CoV-2 while simultaneously developing pan-*Sarbecovirus* assays for future biosurveillance applications. Additionally, FEVER mutation typing assays provide a rapid means for monitoring the spread of potential vaccine escape variants that may significantly impact a population. Future work will focus on applying FEVER towards the development of assays for the detection of other upper respiratory pathogens including influenza as well as testing FEVER probes in ultra-sensitive amplification-free nucleic acid biosensing platforms. FEVER provides a holistic computational approach to combine biosurveillance and diagnostics in order to better combat emerging viral pathogens.

Supporting information

S1 Fig. Visualization of SARS-CoV-2 variants in the amplified regions of FEVER and U. S. CDC RT-PCR assays. Near-complete SARS-CoV-2 sequences ($n = 299,404$) were assessed from GISAID.org. The amplified regions of the FEVER assays (a) FEVER_5'UTR, (b) FEVER_Env, (c) FEVER_ORF1ab, and (d) FEVER_Spike and the U.S. CDC assays (e) N1 and (f) N2 were analyzed for SARS-CoV-2 variants. In each plot, no variants (N/A) were indicated by white and genomic positions containing variants were indicated by colored dots (green,

blue, yellow or red). Plots were made using the Variant Visualizer that is under development and will be available at: <https://cov.lanl.gov>.

(DOCX)

S1 Table. Patient demographics.

(DOCX)

S2 Table. Raw C_T values of FEVER and U.S. CDC assays run on 100 human patient nasopharyngeal swab samples.

(XLSX)

S3 Table. Characterization of the D614G mutation among 59 SARS-CoV-2 positive patient nasopharyngeal swab samples.

(DOCX)

S4 Table. Sequence confirmation of the D614G mutation among 17 selected patient nasopharyngeal swab samples.

(DOCX)

Acknowledgments

The authors would like to thank the Presbyterian Clinical Research team (Brigitte Holder, Susan Salazar, and Maria Vahtel) for their contributions to this study. We are also grateful to the patients who volunteered to take part in this study. Dr. Karina Yusim passed away before the submission of the final version of this manuscript. Dr. Jessica Kubicek-Sutherland accepts responsibility for the integrity and validity of the data collected and analyzed.

Author Contributions

Conceptualization: Ebany J. Martinez-Finley, Jason Mitchell, Karina Yusim, Jessica Z. Kubicek-Sutherland.

Data curation: Brian T. Foley, Karina Yusim.

Formal analysis: Zachary R. Stromberg, James Theiler, Brian T. Foley, Adán Myers y Gutiérrez, Attelia Hollander, Samantha J. Courtney, Karina Yusim.

Funding acquisition: Karina Yusim, Jessica Z. Kubicek-Sutherland.

Investigation: Zachary R. Stromberg, James Theiler, Brian T. Foley, Adán Myers y Gutiérrez, Attelia Hollander, Samantha J. Courtney, Jason Gans, Karina Yusim, Jessica Z. Kubicek-Sutherland.

Methodology: Zachary R. Stromberg, James Theiler, Brian T. Foley, Adán Myers y Gutiérrez, Jason Gans, Karina Yusim, Jessica Z. Kubicek-Sutherland.

Project administration: Karina Yusim, Jessica Z. Kubicek-Sutherland.

Resources: Jason Gans, Alina Deshpande, Ebany J. Martinez-Finley, Jason Mitchell, Harshini Mukundan, Karina Yusim, Jessica Z. Kubicek-Sutherland.

Software: James Theiler, Brian T. Foley, Adán Myers y Gutiérrez, Jason Gans, Karina Yusim.

Supervision: Jason Gans, Alina Deshpande, Ebany J. Martinez-Finley, Jason Mitchell, Harshini Mukundan, Karina Yusim, Jessica Z. Kubicek-Sutherland.

Validation: Zachary R. Stromberg.

Visualization: Zachary R. Stromberg, Brian T. Foley.

Writing – original draft: Zachary R. Stromberg, Jessica Z. Kubicek-Sutherland.

Writing – review & editing: Zachary R. Stromberg, James Theiler, Brian T. Foley, Adán Myers y Gutiérrez, Attelia Hollander, Samantha J. Courtney, Jason Gans, Alina Deshpande, Ebany J. Martinez-Finley, Jason Mitchell, Harshini Mukundan, Jessica Z. Kubicek-Sutherland.

References

1. Reperant LA, Osterhaus A. AIDS, Avian flu, SARS, MERS, Ebola, Zika... what next? *Vaccine*. 2017; 35(35 Pt A):4470–4. Epub 2017/06/22. <https://doi.org/10.1016/j.vaccine.2017.04.082> PMID: 28633891
2. Thompson WW, Shay DK, Weintraub E, Brammer L, Bridges CB, Cox NJ, et al. Influenza-associated hospitalizations in the United States. *JAMA*. 2004; 292(11):1333–40. Epub 2004/09/16. <https://doi.org/10.1001/jama.292.11.1333> PMID: 15367555.
3. Feldmann H. Ebola—a growing threat? *N Engl J Med*. 2014; 371(15):1375–8. Epub 2014/05/09. <https://doi.org/10.1056/NEJMp1405314> PMID: 24805988.
4. Delisle E, Rousseau C, Broche B, Leparac-Goffart I, L'Ambert G, Cochet A, et al. Chikungunya outbreak in Montpellier, France, September to October 2014. *Euro Surveill*. 2015; 20(17). Epub 2015/05/09. <https://doi.org/10.2807/1560-7917.es2015.20.17.21108> PMID: 25955774.
5. Ai J-W, Zhang Y, Zhang W. Zika virus outbreak: 'a perfect storm'. *Emerg Microbes Infect*. 2016; 5(3): e21–e. <https://doi.org/10.1038/emi.2016.42> PMID: 26956448.
6. LeDuc JW, Barry MA. SARS, the First Pandemic of the 21st Century. *Emerging Infectious Diseases*. 2004; 10(11):e26–e. https://doi.org/10.3201/eid1011.040797_02
7. Carrasco-Hernandez R, Jácome R, López Vidal Y, Ponce de León S. Are RNA Viruses Candidate Agents for the Next Global Pandemic? A Review. *ILAR J*. 2017; 58(3):343–58. <https://doi.org/10.1093/ilar/ilx026> PMID: 28985316.
8. Morens DM, Fauci AS. Emerging Pandemic Diseases: How We Got to COVID-19. *Cell*. 2020; 182(5):1077–92. Epub 2020/08/28. <https://doi.org/10.1016/j.cell.2020.08.021> PMID: 32846157.
9. Stromberg ZR, Fischer W, Bradfute SB, Kubicek-Sutherland JZ, Hraber P. Vaccine Advances against Venezuelan, Eastern, and Western Equine Encephalitis Viruses. *Vaccines*. 2020; 8(2). <https://doi.org/10.3390/vaccines8020273> PMID: 32503232
10. Madhav N, Oppenheim B, Gallivan M, Mulembakani P, Rubin E, Wolfe N. Pandemics: Risks, Impacts, and Mitigation. In: Jamison DT, Gelband H, Horton S, Jha P, Laxminarayan R, Mock CN, et al., editors. *Disease Control Priorities: Improving Health and Reducing Poverty*. Washington (DC) 2017.
11. Carroll D, Morzaria S, Briand S, Johnson CK, Morens D, Sumption K, et al. Preventing the next pandemic: the power of a global viral surveillance network. *BMJ*. 2021; 372:n485. <https://doi.org/10.1136/bmj.n485> PMID: 33712471
12. Ibrahim NK. Epidemiologic surveillance for controlling Covid-19 pandemic: types, challenges and implications. *Journal of Infection and Public Health*. 2020; 13(11):1630–8. <https://doi.org/10.1016/j.jiph.2020.07.019> PMID: 32855090
13. Kobres P-Y, Chretien J-P, Johansson MA, Morgan JJ, Whung P-Y, Mukundan H, et al. A systematic review and evaluation of Zika virus forecasting and prediction research during a public health emergency of international concern. *PLOS Neglected Tropical Diseases*. 2019; 13(10):e0007451. <https://doi.org/10.1371/journal.pntd.0007451> PMID: 31584946
14. Perkins MD, Dye C, Balasegaram M, Bréchet C, Mombouli JV, Røttingen JA, et al. Diagnostic preparedness for infectious disease outbreaks. *Lancet*. 2017; 390(10108):2211–4. Epub 2017/06/05. [https://doi.org/10.1016/S0140-6736\(17\)31224-2](https://doi.org/10.1016/S0140-6736(17)31224-2) PMID: 28577861.
15. Manore C, Graham T, Carr A, Feryn A, Jakhar S, Mukundan H, et al. Modeling and Cost Benefit Analysis to Guide Deployment of POC Diagnostics for Non-typhoidal Salmonella Infections with Antimicrobial Resistance. *Scientific Reports*. 2019; 9(1):11245. <https://doi.org/10.1038/s41598-019-47359-2> PMID: 31375759
16. Del Valle SY, McMahon BH, Asher J, Hatchett R, Lega JC, Brown HE, et al. Summary results of the 2014–2015 DARPA Chikungunya challenge. *BMC Infect Dis*. 2018; 18(1):245. <https://doi.org/10.1186/s12879-018-3124-7> PMID: 29843621
17. Kelly-Cirino CD, Nkengasong J, Kettler H, Tongio I, Gay-Andrieu F, Escadafal C, et al. Importance of diagnostics in epidemic and pandemic preparedness. *BMJ Glob Health*. 2019; 4(Suppl 2):e001179. <https://doi.org/10.1136/bmjgh-2018-001179> PMID: 30815287.

18. de Wilde AH, Snijder EJ, Kikkert M, van Hemert MJ. Host Factors in Coronavirus Replication. *Curr Top Microbiol Immunol*. 2018; 419:1–42. Epub 2017/06/24. https://doi.org/10.1007/82_2017_25 PMID: 28643204
19. Paules CI, Marston HD, Fauci AS. Coronavirus Infections—More Than Just the Common Cold. *JAMA*. 2020; 323(8):707–8. Epub 2020/01/24. <https://doi.org/10.1001/jama.2020.0757> PMID: 31971553
20. Cui J, Li F, Shi Z-L. Origin and evolution of pathogenic coronaviruses. *Nature Reviews Microbiology*. 2019; 17(3):181–92. <https://doi.org/10.1038/s41579-018-0118-9> PMID: 30531947
21. Wu F, Zhao S, Yu B, Chen Y-M, Wang W, Song Z-G, et al. A new coronavirus associated with human respiratory disease in China. *Nature*. 2020; 579(7798):265–9. <https://doi.org/10.1038/s41586-020-2008-3> PMID: 32015508
22. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N Engl J Med*. 2020; 382(8):727–33. Epub 2020/01/25. <https://doi.org/10.1056/NEJMoa2001017> PMID: 31978945
23. Sharma A, Tiwari S, Deb MK, Marty JL. Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2): a global pandemic and treatment strategies. *International Journal of Antimicrobial Agents*. 2020; 56(2):106054-. Epub 2020/06/10. <https://doi.org/10.1016/j.ijantimicag.2020.106054> PMID: 32534188
24. Fahmi M, Kubota Y, Ito M. Nonstructural proteins NS7b and NS8 are likely to be phylogenetically associated with evolution of 2019-nCoV. *Infection, Genetics and Evolution*. 2020; 81:104272. <https://doi.org/10.1016/j.meegid.2020.104272> PMID: 32142938
25. Green DA, Zucker J, Westblade LF, Whittier S, Rennett H, Velu P, et al. Clinical Performance of SARS-CoV-2 Molecular Tests. *J Clin Microbiol*. 2020; 58(8). Epub 2020/06/10. <https://doi.org/10.1128/JCM.00995-20> PMID: 32513858
26. Centers for Disease Control and Prevention (CDC). CDC 2019-novel coronavirus (2019-nCoV) real-time RT-PCR diagnostic panel 2020 [April 6, 2021]. <https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html>.
27. Centers for Disease Control and Prevention (CDC). National institute for viral disease control and prevention. Specific primers and probes for detection of 2019 novel coronavirus 2020 [April 6, 2021]. http://ivdc.chinacdc.cn/kyjz/202001/t20200121_211337.html.
28. Chu DKW, Pan Y, Cheng SMS, Hui KPY, Krishnan P, Liu Y, et al. Molecular Diagnosis of a Novel Coronavirus (2019-nCoV) Causing an Outbreak of Pneumonia. *Clin Chem*. 2020; 66(4):549–55. Epub 2020/02/08. <https://doi.org/10.1093/clinchem/hvaa029> PMID: 32031583
29. 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 surveillance*. 2020; 25(3):2000045. <https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045> PMID: 31992387.
30. van Kasteren PB, van der Veer B, van den Brink S, Wijsman L, de Jonge J, van den Brandt A, et al. Comparison of seven commercial RT-PCR diagnostic kits for COVID-19. *J Clin Virol*. 2020; 128:104412. Epub 2020/05/18. <https://doi.org/10.1016/j.jcv.2020.104412> PMID: 32416600
31. SantaLucia J Jr., Sozhamannan S, Gans JD, Koehler JW, Soong R, Lin NJ, et al. Appendix Q: Recommendations for Developing Molecular Assays for Microbial Pathogen Detection Using Modern In Silico Approaches. *Journal of AOAC INTERNATIONAL*. 2020; 103(4):882–99. <https://doi.org/10.1093/jaoacint/qsaa045> PMID: 33241357
32. Medina RA, Rojas M, Tuin A, Huff S, Ferres M, Martinez-Valdebenito C, et al. Development and characterization of a highly specific and sensitive SYBR green reverse transcriptase PCR assay for detection of the 2009 pandemic H1N1 influenza virus on the basis of sequence signatures. *J Clin Microbiol*. 2011; 49(1):335–44. Epub 2010/11/19. <https://doi.org/10.1128/JCM.01142-10> PMID: 21084522
33. Sozhamannan S, Holland MY, Hall AT, Negrón DA, Ivancich M, Koehler JW, et al. Evaluation of Signature Erosion in Ebola Virus Due to Genomic Drift and Its Impact on the Performance of Diagnostic Assays. *Viruses*. 2015; 7(6):3130–54. <https://doi.org/10.3390/v7062763> PMID: 26090727
34. Fitch JP, Gardner SN, Kuczmarski TA, Kurtz S, Myers R, Ott LL, et al. Rapid development of nucleic acid diagnostics. *Proceedings of the IEEE*. 2002; 90(11):1708–21. <https://doi.org/10.1109/JPROC.2002.804680>
35. Vogels CBF, Brito AF, Wyllie AL, Fauver JR, Ott IM, Kalinich CC, et al. Analytical sensitivity and efficiency comparisons of SARS-CoV-2 RT-qPCR primer–probe sets. *Nature Microbiology*. 2020; 5(10):1299–305. <https://doi.org/10.1038/s41564-020-0761-6> PMID: 32651556
36. Artisi M, Bontems S, Göbbels P, Franckh M, Maes P, Boreux R, et al. A Recurrent Mutation at Position 26340 of SARS-CoV-2 Is Associated with Failure of the E Gene Quantitative Reverse Transcription-PCR Utilized in a Commercial Dual-Target Diagnostic Assay. *Journal of Clinical Microbiology*. 2020; 58(10):e01598–20. <https://doi.org/10.1128/JCM.01598-20> PMID: 32690547

37. Lu X, Wang L, Sakthivel SK, Whitaker B, Murray J, Kamili S, et al. US CDC Real-Time Reverse Transcription PCR Panel for Detection of Severe Acute Respiratory Syndrome Coronavirus 2. *Emerging Infectious Diseases*. 2020; 26(8):1654–65. <https://doi.org/10.3201/eid2608.201246> PMID: 32396505
38. Babiker A, Myers CW, Hill CE, Guarner J. SARS-CoV-2 Testing: Trials and Tribulations. *American Journal of Clinical Pathology*. 2020; 153(6):706–8. <https://doi.org/10.1093/ajcp/aqaa052> PMID: 32227199
39. Nalla AK, Casto AM, Huang MW, Perchetti GA, Sampoleo R, Shrestha L, et al. Comparative Performance of SARS-CoV-2 Detection Assays Using Seven Different Primer-Probe Sets and One Assay Kit. *J Clin Microbiol*. 2020; 58(6). Epub 2020/04/10. <https://doi.org/10.1128/jcm.00557-20> PMID: 32269100
40. Korber B, Fischer WM, Gnanakaran S, Yoon H, Theiler J, Abfalterer W, et al. Tracking Changes in SARS-CoV-2 Spike: Evidence that D614G Increases Infectivity of the COVID-19 Virus. *Cell*. 2020; 182:812–27.e19. Epub 2020/07/23. <https://doi.org/10.1016/j.cell.2020.06.043> PMID: 32697968
41. Tang JW, Tambyah PA, Hui DS. Emergence of a new SARS-CoV-2 variant in the UK. *J Infect*. 2020; 82(4):e27–e8. Epub 2021/01/01. <https://doi.org/10.1016/j.jinf.2020.12.024> PMID: 33383088
42. Williams TC, Burgers WA. SARS-CoV-2 evolution and vaccines: cause for concern? *Lancet Respir Med*. 2021; 9(4):333–5. Epub 2021/02/02. [https://doi.org/10.1016/S2213-2600\(21\)00075-8](https://doi.org/10.1016/S2213-2600(21)00075-8) PMID: 33524316
43. Courtney SJ, Stromberg ZR, Kubicek-Sutherland JZ. Nucleic Acid-Based Sensing Techniques for Diagnostics and Surveillance of Influenza. *Biosensors (Basel)*. 2021; 11(2):47. <https://doi.org/10.3390/bios11020047> PMID: 33673035.
44. Yoon H, Leitner T. PrimerDesign-M: a multiple-alignment based multiple-primer design tool for walking across variable genomes. *Bioinformatics*. 2015; 31(9):1472–4. Epub 2014/12/20. <https://doi.org/10.1093/bioinformatics/btu832> PMID: 25524896
45. Brodin J, Krishnamoorthy M, Athreya G, Fischer W, Hraber P, Gleasner C, et al. A multiple-alignment based primer design algorithm for genetically highly variable DNA targets. *BMC Bioinformatics*. 2013; 14(1):255. <https://doi.org/10.1186/1471-2105-14-255> PMID: 23965160
46. Li P-E, Myers y Gutiérrez A, Davenport K, Flynn M, Hu B, Lo C-C, et al. A public website for the automated assessment and validation of SARS-CoV-2 diagnostic PCR assays. *Bioinformatics*. 2020. <https://doi.org/10.1093/bioinformatics/btaa710> PMID: 32777813
47. Shu Y, McCauley J. GISAID: Global initiative on sharing all influenza data—from vision to reality. *Euro surveillance*. 2017; 22(13):30494. <https://doi.org/10.2807/1560-7917.ES.2017.22.13.30494> PMID: 28382917
48. Li B, Kadura I, Fu DJ, Watson DE. Genotyping with TaqMAMA. *Genomics*. 2004; 83(2):311–20. Epub 2004/01/07. <https://doi.org/10.1016/j.ygeno.2003.08.005> PMID: 14706460
49. Peñarrubia L, Ruiz M, Porco R, Rao SN, Juanola-Falgarona M, Manissero D, et al. Multiple assays in a real-time RT-PCR SARS-CoV-2 panel can mitigate the risk of loss of sensitivity by new genomic variants during the COVID-19 outbreak. *Int J Infect Dis*. 2020; 97:225–9. Epub 2020/06/15. <https://doi.org/10.1016/j.ijid.2020.06.027> PMID: 32535302
50. Qian J, Boswell SA, Chidley C, Lu Z-x, Pettit ME, Gaudio BL, et al. An enhanced isothermal amplification assay for viral detection. *Nature Communications*. 2020; 11(1):5920. <https://doi.org/10.1038/s41467-020-19258-y> PMID: 33219228
51. World Health Organization. Laboratory testing for 2019 novel coronavirus (2019-nCoV) in suspected human cases, Interim guidance, 2 March 2020 2020 [cited 2021 05/11/2021]. <https://www.who.int/publications/i/item/10665-331501>.
52. Jamal AJ, Mozafarihashjin M, Coomes E, Powis J, Li AX, Paterson A, et al. Sensitivity of Nasopharyngeal Swabs and Saliva for the Detection of Severe Acute Respiratory Syndrome Coronavirus 2. *Clin Infect Dis*. 2021; 72(6):1064–6. Epub 2020/06/26. <https://doi.org/10.1093/cid/ciaa848> PMID: 32584972
53. Wyllie AL, Fournier J, Casanovas-Massana A, Campbell M, Tokuyama M, Vijayakumar P, et al. Saliva or Nasopharyngeal Swab Specimens for Detection of SARS-CoV-2. *N Engl J Med*. 2020; 383(13):1283–6. Epub 2020/08/29. <https://doi.org/10.1056/NEJMc2016359> PMID: 32857487
54. Klausner JD, Kojima N, Butler-Wu SM. Is the Patient Infected with SARS-CoV-2? *Journal of Clinical Microbiology*. 2021; 59(4):e00147–21. <https://doi.org/10.1128/JCM.00147-21> PMID: 33500362
55. Bastos ML, Perlman-Arrow S, Menzies D, Campbell JR. The Sensitivity and Costs of Testing for SARS-CoV-2 Infection With Saliva Versus Nasopharyngeal Swabs: A Systematic Review and Meta-analysis. *Ann Intern Med*. 2021; 174(4):501–10. Epub 2021/01/12. <https://doi.org/10.7326/M20-6569> PMID: 33428446
56. Hammou RA, Benhassou M, Bessi H, Ennaji MM. Chapter 7—Scientific Advances in the Diagnosis of Emerging and Reemerging Viral Human Pathogens. In: Ennaji MM, editor. *Emerging and Reemerging Viral Pathogens*. Academic Press; 2020. p. 93–120.

57. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. 2012; 13(1):134. <https://doi.org/10.1186/1471-2105-13-134> PMID: 22708584
58. Hendling M, Pabinger S, Peters K, Wolff N, Conzemius R, Barišić I. Oli2go: an automated multiplex oligonucleotide design tool. *Nucleic Acids Res*. 2018; 46(W1):W252–W6. Epub 2018/05/03. <https://doi.org/10.1093/nar/gky319> PMID: 29718464
59. Lopez-Rincon A, Tonda A, Mendoza-Maldonado L, Mulders DGJC, Molenkamp R, Perez-Romero CA, et al. Classification and specific primer design for accurate detection of SARS-CoV-2 using deep learning. *Scientific Reports*. 2021; 11(1):947. <https://doi.org/10.1038/s41598-020-80363-5> PMID: 33441822
60. Li D, Zhang J, Li J. Primer design for quantitative real-time PCR for the emerging Coronavirus SARS-CoV-2. *Theranostics*. 2020; 10(16):7150–62. <https://doi.org/10.7150/thno.47649> PMID: 32641984
61. Isabel S, Graña-Miraglia L, Gutierrez JM, Bundalovic-Torma C, Groves HE, Isabel MR, et al. Evolutionary and structural analyses of SARS-CoV-2 D614G spike protein mutation now documented worldwide. *Scientific Reports*. 2020; 10(1):14031. <https://doi.org/10.1038/s41598-020-70827-z> PMID: 32820179
62. Albert E, Ferrer B, Torres I, Serrano A, Alcaraz MJ, Buesa J, et al. Amplification of human β -glucuronidase gene for appraising the accuracy of negative SARS-CoV-2 RT-PCR results in upper respiratory tract specimens. *J Med Virol*. 2021; 93(1):48–50. <https://doi.org/10.1002/jmv.26112> PMID: 32484950
63. Gietema HA, Zelis N, Nobel JM, Lambriks LJG, van Alphen LB, Oude Lashof AML, et al. CT in relation to RT-PCR in diagnosing COVID-19 in The Netherlands: A prospective study. *PLoS One*. 2020; 15(7):e0235844–e. <https://doi.org/10.1371/journal.pone.0235844> PMID: 32645053
64. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, et al. Presenting Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New York City Area. *JAMA*. 2020; 323(20):2052–9. <https://doi.org/10.1001/jama.2020.6775> PMID: 32320003
65. Woloshin S, Patel N, Kesselheim AS. False Negative Tests for SARS-CoV-2 Infection—Challenges and Implications. *N Engl J Med*. 2020; 383(6):e38. Epub 2020/06/06. <https://doi.org/10.1056/NEJMp2015897> PMID: 32502334
66. Arnaout R, Lee RA, Lee GR, Callahan C, Cheng A, Yen CF, et al. The Limit of Detection Matters: The Case for Benchmarking Severe Acute Respiratory Syndrome Coronavirus 2 Testing. *Clin Infect Dis*. 2021. Epub 2021/02/04. <https://doi.org/10.1093/cid/ciaa1382> PMID: 33532847.
67. Ravi N, Cortade DL, Ng E, Wang SX. Diagnostics for SARS-CoV-2 detection: A comprehensive review of the FDA-EUA COVID-19 testing landscape. *Biosens Bioelectron*. 2020; 165:112454–. Epub 2020/07/18. <https://doi.org/10.1016/j.bios.2020.112454> PMID: 32729549.
68. Robson F, Khan KS, Le TK, Paris C, Demirbag S, Barfuss P, et al. Coronavirus RNA Proofreading: Molecular Basis and Therapeutic Targeting. *Mol Cell*. 2020; 79(5):710–27. Epub 2020/08/28. <https://doi.org/10.1016/j.molcel.2020.07.027> PMID: 32853546
69. MacLean OA, Orton RJ, Singer JB, Robertson DL. No evidence for distinct types in the evolution of SARS-CoV-2. *Virus Evolution*. 2020; 6(1). <https://doi.org/10.1093/ve/veaa034> PMID: 32817804
70. Jaroszewski L, Iyer M, Alisoltani A, Sedova M, Godzik A. The interplay of SARS-CoV-2 evolution and constraints imposed by the structure and functionality of its proteins. *PLoS Comput Biol*. 2021; 17(7):e1009147. Epub 2021/07/09. <https://doi.org/10.1371/journal.pcbi.1009147> PMID: 34237054.
71. Lo Presti A, Rezza G, Stefanelli P. Selective pressure on SARS-CoV-2 protein coding genes and glycosylation site prediction. *Heliyon*. 2020; 6(9):e05001. <https://doi.org/10.1016/j.heliyon.2020.e05001> PMID: 32984566
72. Weissman D, Alameh MG, de Silva T, Collini P, Hornsby H, Brown R, et al. D614G Spike Mutation Increases SARS CoV-2 Susceptibility to Neutralization. *Cell Host Microbe*. 2020; 29(1):23–31.e4. Epub 2020/12/12. <https://doi.org/10.1016/j.chom.2020.11.012> PMID: 33306985
73. Mansbach RA, Chakraborty S, Nguyen K, Montefiori DC, Korber B, Gnanakaran S. The SARS-CoV-2 Spike variant D614G favors an open conformational state. *Science Advances*. 2021; 7(16):eabf3671. <https://doi.org/10.1126/sciadv.abf3671> PMID: 33863729
74. Plante JA, Liu Y, Liu J, Xia H, Johnson BA, Lokugamage KG, et al. Spike mutation D614G alters SARS-CoV-2 fitness. *Nature*. 2020; 592(7852):116–21. <https://doi.org/10.1038/s41586-020-2895-3> PMID: 33106671
75. Gong YN, Tsao KC, Hsiao MJ, Huang CG, Huang PN, Huang PW, et al. SARS-CoV-2 genomic surveillance in Taiwan revealed novel ORF8-deletion mutant and clade possibly associated with infections in Middle East. *Emerg Microbes Infect*. 2020; 9(1):1457–66. Epub 2020/06/17. <https://doi.org/10.1080/22221751.2020.1782271> PMID: 32543353

76. Slezak T, Kuczmarski T, Ott L, Torres C, Medeiros D, Smith J, et al. Comparative genomics tools applied to bioterrorism defence. *Briefings in Bioinformatics*. 2003; 4(2):133–49. <https://doi.org/10.1093/bib/4.2.133> PMID: 12846395
77. Tang JW, Toovey OTR, Harvey KN, Hui DDS. Introduction of the South African SARS-CoV-2 variant 501Y.V2 into the UK. *The Journal of infection*. 2021; 82(4):e8–e10. Epub 2021/01/17. <https://doi.org/10.1016/j.jinf.2021.01.007> PMID: 33472093
78. La Rosa G, Mancini P, Bonanno Ferraro G, Veneri C, Iaconelli M, Lucentini L, et al. Rapid screening for SARS-CoV-2 variants of concern in clinical and environmental samples using nested RT-PCR assays targeting key mutations of the spike protein. *Water Res*. 2021; 197:117104. Epub 2021/04/16. <https://doi.org/10.1016/j.watres.2021.117104> PMID: 33857895
79. Vandenberg O, Martiny D, Rochas O, van Belkum A, Kozlakidis Z. Considerations for diagnostic COVID-19 tests. *Nat Rev Microbiol*. 2021; 19(3):171–83. Epub 2020/10/16. <https://doi.org/10.1038/s41579-020-00461-z> PMID: 33057203 selling diagnostic tests for infectious diseases. bioMérieux had no part in the design and writing of this work. Where an author is identified as a member of the personnel of the International Agency for Research on Cancer, WHO, the authors alone are responsible for the views expressed in this article and the views do not necessarily represent the decisions, policy or views of the International Agency for Research on Cancer, WHO. O.V., D.M. and Z.K. declare no competing interests.
80. Kralik P, Ricchi M. A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. *Front Microbiol*. 2017; 8:108. Epub 2017/02/18. <https://doi.org/10.3389/fmicb.2017.00108> PMID: 28210243.