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SARS-CoV-2 E Gene Variant Alters Analytical Sensitivity Characteristics of Viral Detection Using a Commercial Reverse Transcription-PCR Assay

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ABSTRACT Diagnostic assays for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are essential for patient management, infection prevention, and the public health response for coronavirus disease 2019 (COVID-19). The efficacy and reliability of these assays are of paramount importance in both tracking and controlling the spread of the virus. Real-time reverse transcription-PCR (RT-PCR) assays rely on a fixed genetic sequence for primer and probe binding. Mutations can potentially alter the accuracy of these assays and lead to unpredictable analytical performance characteristics and false-negative results. Here, we identify a G-to-U transversion (nucleotide 26372) in the SARS-CoV-2 E gene in three specimens with reduced viral detection efficiency using a widely available commercial assay. Further analysis of the public GISAID repository led to the identification of 18 additional genomes with this mutation, which reflect five independent mutational events. This work supports the use of dual-target assays to reduce the number of false-negative PCR results.

KEYWORDS COVID-19, RT-PCR, SARS-CoV-2, mutation

Coronavirus disease 2019 (COVID-19), caused by the single-stranded positive-sense RNA virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was first detected in Wuhan, Hubei Province, China, in December 2019 (1). One year later, there are over 75 million global cases of SARS-CoV-2 and over 1.6 million deaths attributed to COVID-19 (2). The first SARS-CoV-2 genome sequence was available in January, with now (as of 29 December 2020) over 270,000 SARS-CoV-2 genomic sequences publicly available via the curated Global Initiative for Sharing All Influenza Data (GISAID) repository (3).

Viral diagnostic tests are essential tools in controlling the spread of disease, and test providers should be able to rely on the accuracy of these tests. Real-time PCR is the primary tool to detect viral nucleic acid from patient specimens (4). Real-time reverse transcription-PCR (RT-PCR) was used in previous viral outbreaks, such as novel influenza A (H1N1) virus in 2009 and Zika virus in 2015, to detect the presence of virus from potentially positive patient samples (5, 6). The WHO, the CDC, academic laboratories, and commercial laboratories rapidly developed SARS-CoV-2 RT-PCR assays following the publication of the SARS-CoV-2 genome (7, 8) (see <https://www.cdc.gov/coronavirus/2019-ncov/more/scientific-brief-emerging-variant.html> and <https://www.who.int/docs/default-source/coronaviruse/whoinhouseassays.pdf>). These assays were developed without a deep understanding of one important facet of SARS-CoV-2 biology, importantly, how frequently the virus might mutate and, if so, what likely hot spots would be affected. To counteract this possibility, two parallel strategies were generally employed. The first was to target invariant regions among multiple sarbecoviruses,

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assuming that these conserved sites were less likely to affect assay performance, and the second was to simultaneously amplify two to three viral targets (9).

Multitarget assays can still produce positive results if one target fails to amplify. Some examples of dual-target assays include those deployed by the Luminex Aries, Abbott Molecular, and Cepheid GeneXpert platforms (10, 11). Another commercially available dual-target assay is the cobas SARS-CoV-2 test by Roche Diagnostics, for use on the cobas 6800/8800 systems, which targets both the E gene, which codes for the structural envelope protein, and a region in open reading frame 1ab (ORF1ab) (12, 13).

Here, we describe SARS-CoV-2 genome sequences from several patient specimens that yielded high cycle threshold (C_T) values when evaluated in the E gene compared to ORF1ab. A specific mutation, 26372G>U, within the E gene was observed in these genomes that likely reduces the E gene PCR assay's sensitivity.

MATERIALS AND METHODS

Study population. Patients suspected of having COVID-19 or with known sick contacts had mucosal sites sampled using nasopharyngeal (NP) swabs. Patient populations consisted of patients from outpatient, inpatient, or emergency department settings at various locations in the Barnes-Jewish Healthcare System in the St. Louis, MO, region. All samples were submitted to our clinical laboratory, the Barnes-Jewish Hospital Molecular Infectious Disease (BJH-MID) Laboratory, for SARS-CoV-2 testing. While multiple redundant platforms were in use to mitigate supply chain and turnaround time issues, the vast majority of these samples were routed to the high-throughput Roche cobas SARS-CoV-2 assay platform.

Roche cobas SARS-CoV-2 assay. The cobas SARS-CoV-2 assay is based on a sample-to-answer format in which RNA extraction and purification are linked to reverse transcription, PCR amplification, and detection within the instrument platform. The cobas SARS-CoV-2 assay employs three unique TaqMan probes to identify specimens that contain the targets of interest (ORF1ab, E, and internal control [IC]). Amplification curves crossing predetermined cycle threshold (C_T) values are considered positive for that target and the result was either detected or not detected based on manufacturer-provided interpretation criteria. Importantly, both ORF1ab and E gene targets do not need to be simultaneously positive for a detected result. While C_T values are provided by the instrument, neither the numerical values nor the difference in those values affects the assay interpretation. The laboratory has validated acceptable specimen types for testing on the Roche cobas platform. These are limited to nasopharyngeal and oropharyngeal swabs collected in universal transport medium or liquid Amies solution and tested within 72 h from collection. This testing began on 1 May 2020. This study was approved by the Washington University in St. Louis Institutional Review Board (approval number 202004259).

SARS-CoV-2 sequence analysis. RNA was extracted from patient specimens using Qiagen's QIAamp viral RNA extraction kit. The carrier RNA was not used in the extractions. The extracted viral RNA was used to generate next-generation sequencing libraries using Illumina's stranded total RNA preparation, ligation with Ribo Zero Plus ribosomal subtraction, according to the manufacturer's protocol. The final indexed libraries were quantified using Agilent's Bioanalyzer and pooled at equimolar concentrations. Illumina's NextSeq and MiniSeq sequencers were used to generate paired-end 150-bp reads. Adapter and low-quality read filtering of raw sequencing data was performed using fastp v0.20.1 (14). Filtered reads were aligned to the SARS-CoV-2 Wuhan reference genome (GenBank accession number [MN908947.3](https://www.ncbi.nlm.nih.gov/nuccore/MN908947.3)) using BMap v38.86 (<https://sourceforge.net/projects/bbmap/>). Mapped reads were extracted from the alignment file with SAMtools v1.10 (15) and assembled with the 6 July 2020 build of coronaSPAdes (16) to recover nearly full-length SARS-CoV-2 genomes. Alignment of recovered genomes and variant identification were performed using the nucmer component of the MUMmer package v3.23 (<http://mummer.sourceforge.net/>). Multiple-sequence analysis was performed using SnapGene software (from Insightful Science) with the Clustal Omega algorithm. Sequence data are available in Table S1 in the supplemental material.

Phylogenetic analysis. SARS-CoV-2 genomes and their variant annotations were accessed from CoV-GLUE (<http://cov-glue.cvr.gla.ac.uk/>) on 29 December 2020 as described previously (17). Phylogenetic trees were generated using Nextstrain according to the provided analysis pipeline (<https://github.com/nextstrain/ncov/>) as described previously (17). Forty-six additional genomes were randomly subsampled from the GISAID "nextfasta" sequence data file (<https://gisaid.org>) using the pipeline. Default settings were used except that the "seq_per_group" parameter was changed to "2" for the "region_global" subsampling scheme.

RESULTS

The Barnes-Jewish Hospital laboratory began testing for SARS-CoV-2 using the Roche cobas platform in May 2020. From May through the end of August, the laboratory performed 65,641 tests on this platform, with 3,401 positive results (5.2% positivity rate). A total of 3,150 specimens were reported with a C_T value for both targets, with the remainder being positive for only one target and with C_T values near the assay limit of detection. Seven SARS-CoV-2 dual-target-positive samples from different patients collected between July and August showed discordant PCR C_T values between the ORF1ab and E gene targets of the Roche cobas assay (Fig. 1). Two samples had modestly higher ORF1ab C_T values than

FIG 1 Detection of samples with discordant PCR values between E gene and ORF1a/b assays. Five samples (arrows) with E gene variants were identified by a significant deviation in the ΔC_T values between the ORF1a/b and E gene targets in the Roche cobas assay compared to other dual-target-positive samples tested from May through August 2020 ($n = 3,150$). Three of the samples (filled circles) contained a sufficient concentration of virus for further genomic characterization.

those for the E gene; however, no residual material was available for these two samples, and they were not further characterized. The other five samples had higher E gene C_T values than ORF1a/b values. While the mean difference between C_T values (ΔC_T) gradually diverges as the viral copy number decreases (higher C_T values), the average ΔC_T for the five discordant samples was significantly higher (8.21 versus 0.75 [$P < 0.0001$ by a Mann-Whitney test]) (Table 1). Repeat testing following a 1:10 dilution of 3 of 5 discordant specimens confirmed that the C_T value differences were not due to amplification errors secondary to inhibitors or unrelated assay failures. There was insufficient material remaining to retest all 5 specimens.

Next-generation sequencing of the five COVID-19-positive samples showing discordant PCR C_T values and two controls sourced from the same community site was performed. Three of the five samples with discordant C_T values yielded nearly full-length genomes (Table 1) using an assembly-based bioinformatics pipeline similar to those in previously described studies (18, 19). The other two samples, which did not yield nearly full-length genomes, had the highest C_T values and were not analyzed further. Relative to the Wuhan reference genome (GenBank accession number [MN908947.3](#)), samples USA/MO-WUSTL_D/2020 and USA/MO-WUSTL_E/2020 both had 16 single-nucleotide polymorphisms (SNPs), and all of these were shared between the two samples (Fig. 2). Sample USA/MO-WUSTL_C/2020 had 17 SNPs, with 16 being identical to the SNPs found in samples USA/MO-WUSTL_D/2020 and USA/MO-WUSTL_E/2020, along with an extra mutation in the 3' poly(A) repeat. Of the 16 mutations shared between USA/MO-WUSTL_C/2020, USA/MO-WUSTL_D/2020, and USA/MO-WUSTL_E/2020, only 1 mutation was identified in the E gene: a G-to-U transversion at position 26372. The two controls shared 4 of the 16 mutations shared among the three samples

TABLE 1 COVID-19-positive and control samples collected from BJH^a

Sample	Date collected (yr-mo)	Original C_T			1:10 dilution C_T			Length (bp)	No. of SNPs
		ORF1a/b	E	Δ	ORF1a/b	E	Δ		
A	2020-08	25.60	33.11	7.51	Not done	Not done	NA	NA	NA
B (not saved)	2020-08	29.08	38.27	9.19	Not done	Not done	NA	NA	NA
USA/MO-WUSTL_C/2020	2020-08	22.57	30.13	7.56	24.11	33.07	8.96	29,886	17
USA/MO-WUSTL_D/2020	2020-08	16.41	22.56	6.15	17.5	23.59	6.09	29,870	16
USA/MO-WUSTL_E/2020	2020-08	19.79	30.42	10.63	21.37	30.05	8.68	29,865	16
USA/MO-WUSTL-C1/2020	2020-08	22.59	22.70	0.11	Not done	Not done	Not done	29,889	16
USA/MO-WUSTL-C4/2020	2020-08	22.82	23.21	0.39	Not done	Not done	Not done	29,869	15

^aNA, not applicable.

FIG 2 Sixteen mutations shared across samples USA/MO-WUSTL_C/2020, USA/MO-WUSTL_D/2020, and USA/MO-WUSTL_E/2020. Samples USA/MO-WUSTL_C/2020, USA/MO-WUSTL_D/2020, and USA/MO-WUSTL_E/2020 shared 16 mutations present across the entire SARS-CoV-2 genome. The mutation of interest in the E gene is highlighted in orange.

with discordant C_T values, but neither control had the 26372G>U transversion or any other mutation in the E gene (Table 2) (see Fig. S1 in the supplemental material).

To determine whether this mutation is present in other SARS-CoV-2 genomes and at what frequency, we queried the publicly accessible CoV-GLUE database (<http://cov-glue.cvr.gla.ac.uk/>) on 29 December 2020 as described previously (16). This database maintains a browsable database listing variations within all SARS-CoV-2 proteins, using data from GISAID (17). Eighteen additional genomes with the same mutation in the E gene were identified from a total of 284,634 genomes at the time of the query (Fig. 3). These other genomes came from countries all around the globe, including Saudi Arabia, South Africa, England, Ireland, Denmark, and Germany. The earliest occurrence of this mutation was from a sample in Germany collected in March, while the three samples from St. Louis were collected in August. Phylogenetic analysis using Nextstrain demonstrated five independent mutational events causing the mutation of interest in the E gene, including two large clades. Both clades contain sequences from multiple continents, suggesting that the virus could be widely circulating (Fig. 4) (20).

DISCUSSION

Diagnostic RT-PCR assays have been widely used during public health emergencies involving infectious viral agents as a tool to measure the abundance and spread of the virus. These tests' reliability is paramount as false-negative results can lead to delayed or sub-optimal responses to virus spread and decisions to allocate reduced required resources to a community affected by the virus (21). These assays depend on the use of static primers targeting various regions of the SARS-CoV-2 genome. Mutations occurring at the primer and/or probe binding sites can have a deleterious effect on the RT-PCR assay's performance.

TABLE 2 Sixteen shared mutations among samples USA/MO-WUSTL_C/2020, USA/MO-WUSTL_D/2020, and USA/MO-WUSTL_E/2020^a

Position	Reference nucleotide	Alternate nucleotide	Region or gene	Predicted impact
241 ^b	C	U	5' UTR	syn
3037 ^b	C	U	ORF1ab	syn
3328	G	U	ORF1ab	Q1021H
6647	G	A	ORF1ab	A2128T
9652	G	U	ORF1ab	M3129I
10789	C	U	ORF1ab	syn
13505	G	U	ORF1ab	C4414F
14408 ^b	C	U	ORF1ab	P4715L
21292	C	U	ORF1ab	L7010F
23403 ^b	A	G	S	D614G
25049	G	U	S	D1163Y
25104	A	U	S	K1181Y
26372	G	U	E	C43F
28881	G	A	N	R203K
28882	G	A	N	syn
28883	G	C	N	G204R

^aUTR, untranslated region; syn, synonymous mutation.

^bMutations shared between discordant and control samples.

FIG 3 Multiple-sequence alignment of 21 SARS-CoV-2 genomes with the same mutation in the E gene. Twenty-one SARS-CoV-2 genomes downloaded from GISAID, labeled by their GISAID “virus name,” were aligned to the reference SARS-CoV-2 genome, highlighted in yellow. The indicated position (POS) is the position of the mutation of interest in the E gene.

Examples of mutations in the N, S, and E genes, such as a 6-nucleotide deletion (nucleotides 21765 to 21770) in the S gene in the recently identified SARS-CoV-2 B.1.1.7 variant, that alter diagnostic assay sensitivity have been reported (22, 23). S gene dropouts can occur due to mutations, including the above-mentioned 6-nucleotide mutation in the S gene of the B.1.1.7 variant. To overcome this, it is important to use assays that target multiple genes. A three-target diagnostic assay can still deliver an accurate positive result with two of the three target genes registering as positive. This type of scenario provides a further argument that assays that target more than one gene are crucial to overcoming mutations in the virus (<https://assets.thermofisher.com/TFS-Assets/GSD/Reference-Materials/69-70del-s-gene-mutation-eua-faq.pdf>). In addition, a recurring mutation at position 26340 of SARS-CoV-2, also in the E gene, is associated with E gene failure in RT-quantitative PCR (qRT-PCR) (Roche) (24). These studies, in addition to our findings, reinforce the value of and need for dual-target COVID-19 assays to avoid false-negative results.

Nearly full-length SARS-CoV-2 genomes were recovered from three COVID-19-positive samples and two controls (Table 1). The three COVID-19-positive samples were nearly identical and shared one mutation in the E gene (Table 2). Although the Roche cobas assay primer sequences are not publicly available, the mutation falls within the published WHO primers for their E gene assay (7). Coupled with the fact that both controls, sourced from the same community site, do not share the mutation in the E gene, it is very likely that the mutation in the E gene is causing an increase in the C_T value of the Roche cobas E gene assay.

A recent study genotyped over 30,000 SARS-CoV-2 genome samples and reported the prevalence of mutations in genomic regions targeted by diagnostic assays. Mutations were most prevalent in the N gene and may account for the poor performance of assays that target the N gene (25). Due to the possibility of mutations arising, dual-target assays help to minimize the risk of false-negative results should one target fail to amplify due to the presence of mutations (9). SARS-CoV-2 was still detected in all three patient samples described here due to the successful ORF1ab target amplification.

With the aid of CoV-GLUE, 18 additional genomes (21 total, including the 3 identified in this study) from around the globe were found to have the same mutation in the

FIG 4 Nextstrain phylogenetic tree. Sixty-seven genomes are shown, with genomes containing 26372G>U (21 in total) highlighted in red. Genomes with the U genotype arose independently five different times.

E gene. These viruses were in multiple clades from different continents, showing the worldwide spread of viruses with this mutation. It is impossible to know how many additional cases went undiagnosed due to the presence of mutations, such as this one, that alter the sensitivity of SARS-CoV-2 assays. Our work strongly supports the use of dual-target SARS-CoV-2 diagnostic assays.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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D.W. and L.D. carried out SARS-CoV-2 sequencing. M.A.W. participated in sample identification and curation. C.-A.D.B. and B.A.P. provided patient metadata and supervised

and guided the work in the clinical microbiology laboratory at BJH. S.T. performed bioinformatic analyses.

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