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LETTER

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A novel diagnostic test to screen SARS-CoV-2 variants containing E484K and N501Y mutations

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ABSTRACT

Spike protein mutations E484K and N501Y carried by SARS-CoV-2 variants have been associated with concerning changes of the virus, including resistance to neutralizing antibodies and increased transmissibility. While the concerning variants are fast spreading in various geographical areas, identification and monitoring of these variants are lagging far behind, due in large part to the slow speed and insufficient capacity of viral sequencing. In response to the unmet need for a fast and efficient screening tool, we developed a single-tube duplex molecular assay for rapid and simultaneous identification of E484K and N501Y mutations from nasopharyngeal swab (NS) samples within 2.5 h from sample preparation to report. Using this tool, we screened a total of 1135 clinical NS samples collected from COVID patients at 8 hospitals within the Hackensack Meridian Health network in New Jersey between late December 2020 and March 2021. Our data revealed dramatic increases in the frequencies of both E484K and N501Y over time, underscoring the need for continuous epidemiological monitoring.

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The COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has plagued human society causing immeasurable losses in an unprecedented way. In the circumstances, many monoclonal and polyclonal antibodies have been approved for clinical use and several vaccines have been licenced with different mechanisms including mRNA vaccines and viral vectored vaccines being rolled out for population vaccination in various countries and geographical areas. Opposed to these encouraging progress and facts, this virus has quickly adapted to various pressures from antiviral therapy and host immunity and evolved independently into several SARS-CoV-2 variants of concern (VOC). These variants, including B.1.1.7 (a.k.a. 501Y.V1), B.1.351(a.k.a. 501Y.V2), and P.1(a.k.a. 501Y.V3) variants, are concerning because they either resist neutralizing antibody and possibly reduce vaccine efficacy or show increased transmissibility, via making some key mutations in the spike protein [1–5]. Studies have shown that currently observed resistance to neutralizing antibodies is largely associated with the E484K mutation [1,2,5–7]. Previously, E484K was only harboured by B.1.351 and P1 variants. The most recent report found that the E484K has been successfully incorporated into some isolates of the B.1.1.7 variant [8]. Moreover, the new variant of interest discovered in New York (B.1.526) also carries the E484K mutation, and alarmingly, fast-spreading over the past two months [9,10]. Another key spike mutation, N501Y, present in all three VOCs is considered to enhance the binding between spike and the ACE2 receptor in human cells, thus contributing to increased transmissibility and possibly virulence as well [11–13]. As we continue to understand the impact of these variants on the mode of the ongoing pandemic, efficient and continuous monitoring of these variants is critical to the implementation of fast and effective countermeasures to eventually defeat this devastating disease. The closer this genotyping can happen to the testing, the quicker the data can be used and actioned. To this end, we developed a novel molecular diagnostic assay capable of identifying the signature mutations within 2.5 h from sample

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preparation to report and used this tool to screen clinical nasopharyngeal swabs (NS) collected from COVID patients at Hackensack Meridian Health (HMH) network hospitals from late December 2020 to March 2021.

This novel genotyping is based on the thermal dynamic difference of molecular beacon (MB) binding with a perfectly complementary target or mismatch target. To generate single-stranded target DNA for the MB probe, an asymmetric reverse transcription (RT)-PCR assay was developed to amplify the mutation hotspot region covering both 484 and 501 codons of the S gene (supplemental material). Upon completion of thermal cycling, a melting curve analysis is performed to characterize dissociation between the single-stranded DNA product and two differentially labelled MB probes, to enable simultaneous genotyping at both loci. Owing to the probe design, the wildtype (WT) template is expected to generate a higher melting temperature (*Tm*) than that of the mutated genotype at a corresponding locus. As shown in Figure 1, E484 WT is featured for a *Tm* at 54.85 ± 0.19°C, ~ 5°C higher than the *Tm* of E484K (49.81 ± 0.07°C). Similarly, the signature *Tm* for N501 WT was 59.97 ± 0.09°C, higher than 54.78 ± 0.12°C for N501Y. In a blinded fashion, this test correctly genotyped RNA samples extracted from six different reference viral strains, including one WT (SARS-CoV-2 USA WA1/2020), two B.1.1.7 variants (SARS-CoV-2 hCoV-19/USA/CA_CDC_5574/2020 and SARS-CoV-2 hCoV-19/England/204820464/2020), and two B.1.351 variants (SARS-CoV-2 hCoV-19/South Africa/KRISP-EC-K005321/2020 and SARS-CoV-2 hCoV-19/South Africa/KRISP-K005325/2020) purchased from BEI resources, and one E484K variant isolate recently obtained from our network hospital. The analytical sensitivity of the assay was evaluated against 10-fold serial dilutions of RNA prepared from each of the reference viral strains. The assay can reliably identify as low as 200 copies of 484WT, 200 copies of E484K, 20 copies of 501WT, and 200 copies of N501Y per reaction, respectively.

It should be noted that this genotyping assay is intended for a secondary test upon primary COVID diagnosis to facilitate epidemiological and/or clinical practices. Therefore, we next accessed SARS-CoV-2 positive NS specimens through the HMH institutional Biorepository under an HMH-IRB approved protocol Pro2018-1022, to screen the signature mutations. To verify the specificity of the assay, we accessed eight SARS-CoV-2 negative NS samples and employed genotyping. All eight samples remained unamplified throughout the test and no false-positive genotyping signal was observed. This study also accessed de-identified data for time and location of sample collection through the HMH-IRB approved protocol, Pro2020-0342. A total of 1135 samples collected between late December 2020 and March 2021 from eight HMH hospitals with a cycle of threshold (*Ct*) value < 37 in the SARS-CoV-2 N2 RT-PCR test [14] were subjected to the spike mutation screening.

To speed up the screening procedure, we adopted an extraction-free sample process method by heat inactivating a 50 µl aliquot of swab specimen in the presence of proteinase K at 95°C for 5 min [15], prior to genotyping test. As a result, 960 and 971 samples yielded identifiable signals for 484 and 501 sites, respectively. The proportion of E484K was 17.2% (165/960), and it was 30.6% (297/971) for N501Y. There were six samples carrying both E484K and N501Y, and the remaining samples flagged as mutants only carry one of the two signature mutations. In addition, we discovered a new genotype at the 501-probe binding site from 12 samples (12/971, 1.2%), which thereafter was confirmed to be a N501T (AAT > ACT) mutation in subsequent sequencing. The melting profile of N501T is markedly different from that of WT and N501Y, with a signature *Tm* of 56.41 ± 0.15°C. We also happened to capture one sample eliciting a distinct 484 *Tm* at 48.88°C ~ 1°C lower than E484K and ~6°C lower than 484WT.

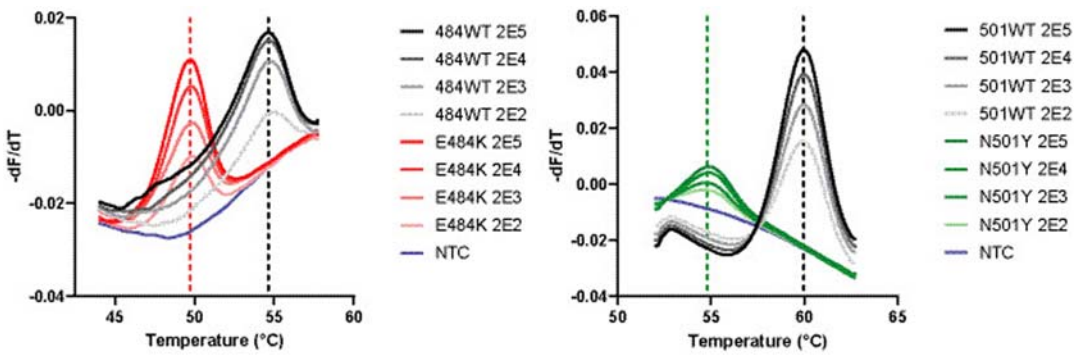

Figure 1. Melting profiles for E484WT and E484K (left panel), and those for N501WT and N501Y (right panel). For each genotype, 10-fold serial RNA dilutions containing 2×10^5–200 genome equivalents/reaction were tested. Dashed lines indicate the *Tm* value of corresponding genotype. NTC is the abbreviation for no template control.

Amplicon sequencing verified that this sample carries an E484Q (GAA > CAA) mutation. Dramatic increases in both E484K and N501Y prevalence over time were observed (Figure S1). E484K climbed up swiftly from none in December ($n = 28$) and 3.2% (3/95) in January to 12.0% (29/242) in February and 22.4% (133/595) in March. The N501Y followed the same trend with even higher speed, rising sharply from < 4% in December and January to 11.2% (28/251) in February and 44.9% (267/595) in March. All six samples carrying dual mutations of E484K and N501Y were from March. We also analysed genotype distributions in different hospitals in February and March (Figure S2). While E484K occurrence in each hospital was very similar, N501Y varied between hospitals in a wide range from 20.2% to 47.1% yet lack of obvious concentration in a certain geographic area.

While our screening efforts were continuing with newly collected March samples, we performed whole-genome sequencing (WGS) with a panel of 74 samples from earlier months representing different genotypes flagged by this screening tool, including 24 E484K, 25 N501Y, 5 N501T, and 20 WT at 484 and 501 loci. Within this WGS confirmed panel, our assay achieved 100% sensitivity and specificity for both 484 and 501 genotyping. However, we acknowledge that these diagnostic parameters were biased towards samples with successful genotyping results, as those that failed to amplify in our genotyping assay were not subjected to WGS. Were those "genotyping-negative" samples subjected to WGS and returned with qualified results, sensitivities of the genotyping diagnosis are likely to drop although the exact level of decrease is yet to be determined. Genomic analysis (Figure S3) showed that the majority of the E484K cases ($n = 19$) fell within the B.1.526 lineage, a recent clone emerged from New York, and the rest belong to clade 20C B.1 lineage ($n = 2$), and clade 20B under R.1 ($n = 2$) and B.1.1.309 lineage ($n = 1$), respectively. All N501Y cases except one are members of B.1.1.7 lineage.

Currently, WGS is being used as the main tool for epidemiological monitoring of SARS-CoV-2 variants. However, the relatively long turn-around-time for WGS and the demand for bioinformatic expertize for data analysis makes identification of concerning variants lagged far behind laboratory COVID diagnosis. Clearly, there is an unmet need for a faster and simpler screening tool that can be used in a high-throughput fashion to increase the capacity of SARS-CoV-2 variant detection in real-time. Herein, we demonstrate that a novel and easy molecular diagnostic assay can be used as a convenient tool for large-scale SARS-CoV-2 variant screening, thus, to enable highly efficient epidemiological monitoring. Not only the assay is proved to be highly accurate in our clinical screening, notably, it is also sensitive to new mutations within the probe binding site. This has been well exemplified by our discovery of the N501T and E484Q mutations from the clinical specimens. While the virus is continuously evolving, we cannot completely rule out the possibility that certain untested/new mutation within the probe binding site may generate a melting profile similar to one of the target mutations tested, if by any chance the mutation causes thermal dynamic change very close to one of those tested, hence compromising the diagnostic performance for defined signature mutation. However, owing to the nature of the assay design, any mutation potentially occurred within the probe binding region would result in a *Tm* shift from that of the WT. This feature ensures the capability of the assay discriminating mutation from the WT, a key asset that holds up the value of the screening. To our knowledge, this is the first report of using a novel screening tool to identify key mutation harbouring SARS-CoV-2 variants in NJ hospitals. Our data revealed dramatic increases in the frequencies of both E484K and N501Y over time, underscoring the need for continuous epidemiological monitoring.

A couple of limitations of this study need to be noted. Firstly, this novel screening assay is slightly less sensitive than the widely used RT-PCR COVID diagnostic assay. Therefore, samples with very low viral load (e.g. N2 $Ct > 35$) may not yield identifiable signals in this genotyping test. Efforts towards improving the sensitivity of the test are ongoing. Secondly, the current assay only aims at picking up mutations at 484 and 501 loci. It is our intention to keep expanding and updating this test by adding new mutations associated with important phenotypic change, to better respond to the fast-evolving situation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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