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Severe Acute Respiratory Syndrome Coronavirus 2: The Emergence of Important Genetic Variants and Testing Options for Clinical Laboratories

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Abstract
Monitoring the spread of emerging severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants relies on rapid genetic testing of the viral genome. The sequencing method commonly called next-generation sequencing can identify virus variants. At times, for target-specific mutation detection, reverse transcriptase polymerase chain reaction is used to identify specific variants. The Centers for Disease Control and Prevention’s national SARS-CoV-2 Strain Surveillance Program is a comprehensive, population-based U.S. surveillance system to monitor SARS-CoV-2 genes, identifying emerging SARS-CoV-2 variants to determine implications for coronavirus disease 2019 (COVID-19) diagnostics, therapy, and vaccines. This review describes the main viral variants of concern and their potential impacts and briefly describes testing strategies.

Introduction
A novel coronavirus was identified as the causative pathogen of the coronavirus disease 2019 (COVID-19) outbreak in December 2019. This virus was named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and is responsible for causing the respiratory illness associated with COVID-19. The virus spread worldwide rapidly, and was officially designated as a pandemic by the World Health Organization (WHO) on 11 March 11, 2020 [1]. Outbreaks of SARS-CoV-2 infection continue to occur worldwide, and the viral genome continues to evolve [2]. At this point in the COVID-19 pandemic, multiple SARS-CoV-2 variants are circulating globally [3]. Extensive mutations in the viral spike protein, responsible for binding to the host receptor angiotensin-converting enzyme 2 (ACE2), raise concerns that current vaccines, therapeutic monoclonal antibody therapies, and other future therapeutic options might be rendered ineffective. Further, viral mutations may alter diagnostic test accuracy. Thus, genomic surveillance and rapid identification of new mutations and variants are essential. Worldwide sequencing efforts aim to characterize the molecular evolution of the virus and the geographical spread. Importantly, restrictive countermeasures, personal hygiene, face masking, and physical distancing remain relevant in fighting the virus [4].

Viral Genome Mutations
Given time and viral reproduction, genetic mutations occur in the genomes of all known human viruses. For example, mutations occur rapidly in common viruses such as influenza virus, human immunodeficiency virus, hepatitis C virus, and SARS-CoV-2. These RNA viruses reproduce quickly inside a host and have high mutation rates; one or more mutations can occur per genome per round of replication. The high mutation rate contributes to the virus’s ability to adapt to changes in its environment quickly. Specifically, certain mutations may provide a selective
advantage through increased binding to host cells or reduced binding to neutralizing antibodies.

Because of the degenerate genetic code (i.e., multiple codons corresponding to the same amino acid), many mutations impact only the RNA sequence and not the viral proteins. These are known as “silent mutations” because the amino acid coded for, and the resulting protein, are not altered by the RNA sequence change. With little or no ability to change the viral proteins or the virus’s behavior, no substantial clinical impact occurs because of silent mutations. However, these mutations would still be capable of altering the performance of diagnostic tests if the primer or probe target site is altered. In other cases, genetic mutations can cause changes when they alter the amino acid sequence of viral proteins resulting in phenotypic changes in the virus. Genetic mutations can result in amino acid changes (“substitutions”), truncations, or loss of viral proteins; all of which have implications for the virus phenotype and some of which create variants with increased capacity to cause human harm (Table 1).

Rarely, a viral mutation can help the virus survive, perhaps by evading the host immune system after a natural infection or after a vaccination, by enhancing viral transmission or impacting host interactions. If mutations are advantageous to the virus for survival or virus reproduction, the associated mutations are said to have a selective advantage, and they become more frequent as viral replication continues. The process, called natural selection, indicates that mutations can be positively selected for if they confer a fitness advantage for the virus. Viral evolution results in emergence of viral quasispecies, defined as a group of genetically-related viral strains competing within an environment. As such, it is common to have multiple variants of the same virus spreading simultaneously.

The mutation rate of a virus is the probability that a change in genetic information passes to the next generation. In viruses, a generation is often defined as a cell infection cycle, including attachment and invasion of the host cell, viral gene expression and replication, encapsidation, and release of infectious viral particles from the infected cell. Higher mutation rates lead to higher genetic diversity. Mutations can occur during replication but are not restricted to it; mutations can also arise with editing errors of the genome or spontaneous nucleic acid damage. There are many types of viral mutations [5]. Genetic changes within a virus include, but are not limited to, the following nucleic acid changes, deletions, insertions, substitutions, and rearrangement, listed in Table 1.

The common genes in the SARS-CoV-2 genome are the ORF1a and 1b proteins, the spike protein (S), the envelope protein (E), the matrix protein (M), and the nucleocapsid protein (N), depicted in Fig. 1. These genes play specific roles in viral replication, pathogenicity, and transmission. Some are the targets for vaccine development and laboratory testing. Many molecular diagnostic tests specifically target one or more of the genes, and assay accuracy could be potentially impacted by genetic variants of SARS-CoV-2 [6].

Over the course of the past year, thousands of mutations occurred within the SARS-CoV-2 genome. As the virus traveled across the globe, mutations occurred in nucleotides within the viral genetic code. Nucleotides are the building blocks of all genomes; for the SARS-CoV-2 RNA genome the nucleotides are adenine (A), uracil (U), guanine (G), and cytosine (C). SARS-CoV-2 has a single stranded viral genome composed of 29,903 nucleotides, 11 coding regions (aka genes), and 12 potential gene products (e.g., proteins like the spike protein) [7].

Errors occur more often in the coronavirus genome than in eukaryotic cells. RNA viruses, including SARS-CoV-2 rely on an error-prone RNA-dependent RNA polymerases to facilitate virus infection, replication, and adaptation [8]. The mutation rate of SARS-CoV-2 is approximately 1 × 10⁻³, or 1:1000 substitutions per site per year [9]. This is similar but slightly slower than the mutation rate of influenza A (2.6 × 10⁻³ substitutions/site/year) and HIV (1.3-3.5 × 10⁻³ substitutions/site/year). Due to robust replication rates, combined with a relative high mutation rate,

| Mutation     | Definition                                                                 |
|--------------|---------------------------------------------------------------------------|
| Deletions    | One or more nucleic acids are removed from the viral genome.              |
| Insertions   | One or more nucleic acids are added from the viral genome.                |
| Substitutions| One or more nucleic acids are interchanged within the viral genome.       |
| Recombination| Sections of viral genomes are exchanged.                                  |

Figure 1. Important genes found in the SARS-CoV-2 viral genome include those that are transcribed and translated by human cell machinery to create the ORF1a and 1b viral proteins, and the spike (S), envelope (E), matrix (M), and nucleocapsid (N) proteins.
the accumulation of mutations in the viral genome occurs quickly if transmission and shedding remain unchecked [8]. The risk of mutations increases with long-term shedding of SARS-CoV-2, associated with certain immunocompromised illnesses. In vivo, substantial viral evolution occurred with the continuous turnover of dominant viral variants [10]. Factors driving SARS-CoV-2 variant emergence vary. To understand the risk they pose, it is essential to consider the factors driving the mutations.

For SARS-CoV-2, multiple pathways may have led to the development of novel variants [11]. The most likely selective pressure is defined as changes that improve intrinsic viral fitness, either via direct replication of the virus within a host or in host-to-host transmission. Over the past year, the virus had the opportunity to mutate in humans and animals, resulting in altered replication and transmission. One notable example is the D614G substitution in the virus spike protein, which enhances viral binding to the host receptor (ACE2) for host cell entry and ultimately improves SARS-CoV-2 infection in the upper respiratory tract [11-13]. This advantage allowed the D614G quasispecies to rapidly become the dominant variant in the world, now accounting for over 90% of all sequenced SARS-CoV-2 viruses [14]. Similar adaption has been observed for both SARS-CoV-2 and influenza virus strains in the past [15, 16].

A SARS-CoV-2 variant may also incorporate mutations that alter interactions with over 300 other host cell proteins [17]. Substitutions in essential viral proteins may alter control of the host translational machinery, disrupt membrane/vesicle trafficking, or influence epigenetic regulation in the host cell [17]. Much attention has been placed on variant mutations affecting the viral spike protein because of its central role in binding and entry into host cells (discussed below); however, mutations may also confer a selective advantage through alteration of the host immune response [18].

The adaptive immune response is a third selective pressure, which can induce the generation of mutations that permit viral immune escape. The spike protein is a primary target for host immunity after natural infection and after vaccination. Antibodies that target key regions of the spike glycoprotein can prevent binding/entry and disrupt infection. However, the prevalence of SARS-CoV-2-specific immune memory at the population level may not be sufficient to generate variants driven primarily by escape from antibodies [19].

Finally, variant mutations may result in no significant advantage or disadvantage for viral infection or transmission. Such mutations may result from genetic drift, including “genetic hitchhiking”, which is the fixation of a mutation that occurs with an advantageous mutation, or as a result of founder effects, when a lineage is introduced into a new geographic region, carrying chance mutations that are rarely beneficial. These changes may have been incorporated early on during SARS-CoV-2 spread and were subsequently maintained through the resulting daughter lineages. Alternatively, heterogeneity can also be influenced by super-spreading and impact genetic drift on variant frequencies [20].

**SARS-CoV-2 variants**

When a set of commonly shared mutations are identified in a group of viruses that make them sufficiently different from the “parent” they are designated as a new virus strain, whether the mutations cause observable differences in virus behavior or not. The term “variant” is the best term to use when indicating that a known virus that has developed a specific group of mutations, which cause the variant to behave differently than the original (aka wild-type) strain. For example, when new SARS-CoV-2 strains develop significant genetic diversity from the wild-type or cause the virus to become more transmissible, pathogenic, resistant to treatment, or able to evade vaccines or diagnostic testing strategies, a new variant is defined and named. New variants may cause serious challenges to providers, epidemiologists, and laboratorians.

Unique SARS-CoV-2 variants are grouped and defined as strains that contain a common set of codon (amino acid) mutations, denoted using the single letter corresponding to the “wild-type” amino acid, followed by the genomic location, and then the single letter alternative “mutant” amino acid, e.g. N501Y. The same mutation or group of mutations can be present in multiple viral variants, creating an overlap of independent mutations and variants.

**Major variants of SARS-CoV-2**

Scientists identify circulating viral variants based on genetic sequence data, link them to epidemiological and biological events, and track them [7]. Scientists rank the threat of the virus and categorize the threats, according to the risk. Most variants do not impose risk and are not informative for national and state public health actions. Categories include a variant of interest (VOI), one that requires further investigations; a variant of concern (VOC), one that may demonstrate increased risk or potential for increased risk in vitro but lacks clinical evidence to support increased risk; or a variant of high consequence (VOHC), the highest threat level [21]. VOHCs have strong evidence that prevention and medical countermeasures will not be as effective as they were for previously circulating strains. A VOHC impact might include a demonstrated failure of diagnostics tests to identify the virus variant, a reduction in vaccine effectiveness, an unusually high number of vaccine breakthrough cases, or low vaccine protection against severe disease. In other words, VOHCs could have reduced susceptibility to multiple therapeutics, lead to increased disease severity, increased hospitalizations, or evasion of diagnostic test methods. As of date of writing, no SARS-CoV-2 variant is currently classified as a VOHC. Identification of a new VOHC would trigger the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) to create new strategies to prevent or contain the transmission, and, if needed, recommendations to update treatments and vaccines. Several new variants emerged in late 2020. The CDC lists common variants of concern, discussed below, with World Health Organization (WHO) designation in parenthesis [21-23].
The first estimated emergence of the B.1.1.7 variant was in the United Kingdom (UK), during September 2020. Now, the variant exists in numerous countries worldwide, including the United States (US), where it was identified at the end of December 2020 [24]. The variant is associated with increased transmissibility (i.e., more efficient and rapid transmission) and increased risk of death when compared to other strains [25].

The variant has a mutation in the receptor-binding domain (RBD) of the spike protein at position 501, where the amino acid asparagine (N) was replaced with tyrosine (Y). The shorthand for this mutation is N501Y. Another mutation is the deletion at position 69/70, which likely led to a conformational change in the spike protein (P681H) near the S1/S2 furin cleavage site (a gene region known for high variability in coronaviruses). Although the deletion occurred spontaneously in several strains, no reports have found evidence to suggest that the mutation has any impact on disease severity or vaccine efficacy [21].

B.1.351 lineage (20H/501Y.V2)

The B.1.351 variant emerged independently of B.1.1.7, but the variant shares some mutations with the UK strain. B.1.351 was first identified in South Africa in samples dating back to the beginning of October 2020, and found in Zambia in late December 2020, where it has already become the predominant variant in that country. Cases have since been detected outside of South Africa, including the U.S. at the end of January 2021. This variant has multiple mutations in the spike protein, including K417N, E484K, and N501Y. Unlike the B.1.1.7 lineage, the variant does not contain a deletion at position 69/70 [26]. Currently, there is no evidence to suggest that this variant has any impact on disease severity. Evidence indicates that one of the spike protein mutations, E484K, may affect neutralization by some polyclonal and monoclonal antibodies and may reduce efficacy of natural and vaccine-induced neutralizing antibodies. Importantly, the clinical impact of these findings is still unclear.

P1 lineage (20J/501Y.V3)

A variant of SARS-CoV-2 (known as P1) was first identified in four travelers from Brazil who were tested at an airport in Japan [27]. The variant has 17 unique mutations, including three (K417T, E484K, and N501Y) in the RBD region of the spike protein. Surveillance detected the variant in the U.S. at the end of January 2021. The P1 variant represents a branch from the B.1.1.28 lineage. Evidence suggests that some of the mutations in the P1 variant may affect its transmissibility and antigenic profile. The mutations may affect the ability of antibodies to recognize and neutralize the virus. A cluster of cases in the city of Manaus, Brazil identified the P1 variant in 42% of the specimens sequenced from late December 2020. In October 2020, approximately 75% of the city’s population was infected with SARS-CoV2, and the surge continued for months. The variant’s emergence and association with a higher viral density raised concerns about a potential increase in transmissibility and a propensity for re-infection.

B.1.427 and B.1.429

Two coronavirus strains originating in California (B.1.427 and B.1.429) are now officially characterized as VOCs [28]. The strains may be 20% more transmissible than common strains. Some COVID-19 treatments (e.g., Eli Lilly and Company, bamlanivimab) may not work well against the variants. Vaccines are still effective against both strains.

Continued adaptation of the zoonoses continues to support genetic variability

Recently, SARS-CoV-2 infections has been reported in cats, dogs, tigers, and lions [29]. SARS-CoV-2 illness affected minks severely, and zoonotic transfer with a variant SARS-CoV-2 strain evidenced in Denmark, Netherlands, USA, and Spain, suggesting animal-to-human and animal-to-animal transmission within mink farms [29]. Furthermore, experimental studies documented the susceptibility of different animal species to SARS-CoV-2, such as mice, golden hamsters, cats, ferrets, non-human primates, and tree shrews [29].

Known Impacts of SARS-CoV-2 Variants

Increased mortality and transmissibility

The SARS-CoV-2 B.1.1.7 variant has been linked to significantly increased transmissibility and may also be linked to an increased death rate [30,31]. Specifically, one study demonstrated an increased risk of death by 28 days post infection when the B.1.1.7 VOC infections were compared to non-VOC infections (hazard ratio: 1.67; 95% confidence interval [CI]: 1.34 to 2.09; p < 0.0001), which was also associated with age and specific comorbidities [32]. Likewise, Challa et al. compared circulating SARS-CoV-2 variants in a matched cohort study. Among community-based COVID-19 testing centers with 54,906 matched pairs of participants who tested positive for SARS-CoV-2, the mortality hazard ratio associated with B.1.1.7 was 1.64 (95% CI 1.32 to 2.04) compared with previously circulating strains, and COVID-19 related deaths increased from 2.5 to 4.1 per 1000 detected cases [33].

Reinfection

Harrington et al. published a case report describing re-infection in an individual from South India characterized by whole genome sequencing (WGS) of the viruses isolated from both episodes [34]. The analysis identified an immune escape variant, N440K, in the spike protein in both episodes of infection. The variant was also found in a case of reinfection previously reported in a healthcare worker from North India [35]. Others reported similar severe re-infections with the South African SARS-CoV-2 variant B.1.351 and the Brazilian variant P1, both of which carry the E484K mutation [36, 37]. More recently, Kustin et al. suggested increased infection rates attributable to the B.1.351 variant among individuals who were fully vaccinated (i.e., received two vaccine doses) [38].

Eluding the immune response

Callaway et al. showed that COVID variants could elude immune responses [39]. Specifically, independent lineages of SARS-CoV-2 including: the UK, B.1.1.7; South Africa, B.1.351; and Brazil, P1 variants have multiple changes in the immunodominant spike
protein that facilitates viral cell entry via the ACE2 receptor. Mutations in the receptor recognition site on the spike protein are of great concern for their potential for immune escape. In a structure-function analysis of B.1.351 the RBD mutations provided tighter ACE2 binding and widespread escape from monoclonal antibody neutralization largely driven by E484K, although K417N and N501Y act together against some important antibody classes [40]. The neutralizing antibody responses of the California variant following natural infection or mRNA vaccination using pseudoviruses expressing the wildtype or the B.1.427/B.1.429 S protein were examined. Plasma from vaccinated or convalescent individuals exhibited neutralizing titers, which were reduced 3 to 6-fold against the B.1.427/B.1.429 variant relative to wildtype pseudoviruses. The RBD L452R mutation reduced or abolished the neutralizing activity of variants. A complete loss of B.1.427/B.1.429 neutralization for a panel of monoclonal antibodies targeting the N-terminal domain (NTD) was observed due to a large structural rearrangement of the NTD antigenic supersite involving an S13I-mediated shift of the signal peptide cleavage site [41].

Enhanced replication
Grabowski, et al. estimated that the replicative advantage of the B.1.1.7 variant is in the range of 1.83 to 2.18 (95% CI, 1.71 to 2.40) for England in November 2020, and in the range of 1.65 to 1.72 (95% CI, 1.46 to 2.04) in Wales, Scotland, Denmark, and the U.S. As this VOC spreads globally, these mutants may hinder the efficiency of existing vaccines and expand in response to the increasing after-infection or vaccine-induced seroprevalence [42]. Kidd et al. showed B.1.1.7 is associated with significantly higher viral loads in samples indicating the strains may show higher infectivity and rapidity of spread [43].

Next Generation Sequencing of Variants
Monitoring the spread of emerging SARS-CoV-2 variants in the U.S. relies on rapid molecular characterization of most, if not all, of the viral genome. This characterization is best accomplished using a sequencing method, commonly called next-generation sequencing (NGS) [44]. NGS is a laboratory method that can identify the full genomic sequence of a virus, or other pathogens [45], which can then be reported for the purposes of surveillance and epidemiology [46]. Sequence analysis may be accomplished by NGS, targeted sequencing of a portion of the genome (e.g., spike protein), or RT-PCR tests that target a specific mutation(s). Such testing is usually conducted by public health laboratories or large genomic sequencing centers, which can perform high-throughput sequencing on positive clinical specimens among the infected population. These tests may also be conducted by a hospital or other accredited reference laboratory, with results that are not typically reported to the patient and healthcare provider [47]. The CDC's national SARS-CoV-2 Strain Surveillance program is population-based, comprehensive program to monitor the evolution of the SARS-CoV-2 genome and identify emerging SARS-CoV-2 variants [48]. It aims to determine variant implications for COVID-19 diagnostics, therapy, vaccines, and public health interventions [21].

Currently, sequencing of SARS-CoV-2 is used primarily for public health applications. Identifying the circulating and prevailing variants of SARS-CoV-2 that may be associated with increased infectivity, transmission, or severity of infection in a given community or geographic region is essential for resource planning and effective mitigative measures to reduce the risk of infection [49]. Surveillance for specific mutations in the viral genomic sequences of circulating variants is also necessary to identify potential problems (e.g., false-negative test results) with diagnostic nucleic acid and antigen assays used to detect SARS-CoV-2 [6].

Identifying SARS-CoV-2 variants in clinical specimens serves two primary purposes, public health, and possible future clinical care. Within the public health domain, NGS is commonly used for WGS of viral samples and provides an unbiased analysis of the specific circulating viral in each population. WGS results help monitor changes in the viral genome as the pandemic progresses, to track the spread of variant strains locally, regionally, nationally, and globally. WGS is also used in outbreak investigations and studies of viral transmission within communities, health care facilities, schools, and other workplaces. WGS can identify the rapid emergence or introduction of a specific variant associated with increased transmissibility, reduced vaccine efficacy, or severe disease. The sequencing results are not routinely reported to the patients or healthcare providers for direct patient care purposes; however, public health laboratories coordinate sequencing efforts for public health purposes.

In the clinical domain, analyzing SARS-CoV-2 sequences could improve the care of an individual patient, if VOHCS emerge. Mutations continue to emerge independently or in conjunction with other mutations and appear to be associated with increased transmissibility, disease severity, or the potential for vaccine failure. Examples include B.1.1.7 or B.1.526; however, a given mutation may not be present in all viruses within a specific variant type [21]. Currently, there is no definitive evidence that directly links a given mutation to poor outcomes, significantly reduced efficacy of SARS-CoV-2 therapies, or vaccine coverage [50]. Although not routinely performed or reimbursed, the potential for direct medical care of a COVID-19 patients exist by sequencing of the SARS-CoV-2 variant present in the positive clinical specimens. Such testing could identify variants and specific spike protein codon substitutions that could directly impact the affected patients' ongoing medical management in the future in several dimensions. First, NGS could distinguish between persistent infection with the same viral strain and re-infection with a new viral strain. This strategy would help design interventions for infection prevention or identify re-infection in a patient with repeatedly positive SARS-CoV-2 results. Second, NGS could detect and identify specific viral spike protein gene mutations in certain variants that are potentially resistant or less susceptible to neutralizing antibodies or monoclonal antibodies in patients who are not responding to such therapy for COVID-19 [51,52]. Finally, NGS could detect and identify viral gene substitutions in specific variants that are potentially resistant or less susceptible to vaccine-induced S-protein neutralizing
antibodies in individuals who develop COVID-19 after successful vaccination [53].

**Clinical samples can be used for NGS sequencing to identify variants**

NGS sequencing for variants is not currently intended for the diagnosis of infection and testing to identify which viral variant is present in a patient's specimen is not routinely needed. Variant identification via genetic sequencing provides infection prevention or epidemiology results upon which policy and infection prevention decisions can be made. Clinical specimens used for strain identification should optimally be the same clinical specimens that are strongly positive for SARSCoV-2 by target-based detection method [54]. For NGS, variant identification can only occur for positive specimens placed in viral transport medium (e.g., universal transport medium, viral transport medium, or possible saline) and cannot be performed from dry swabs. Likewise, pooled samples cannot be used for NGS analysis. If specific tests methods use transport medium that interferes with sequencing, they could also not be used for variant identification. Currently, no matter the method used, viral strain identification is most useful for epidemiology purposes.

While some routine molecular test methods can be more sensitive for viral detection than NGS and target specific mutation assays for VOCs are available, it is full genome sequencing that is required to identify novel mutations in strongly positive samples. When genetic variants might be suspected, testing positive samples might be warranted. Some examples of those situations are listed below:

- Fully vaccinated patients who develop a new laboratory-confirmed COVID-19 infection with known strains or with new strain variants. NGS could be useful to identify newly emerging variants for which vaccines might be ineffective. Of note, NGS sequencing of hospitalized patient samples who were fully vaccinated may be a way to enrich the likelihood of identifying new viral variants via genetic sequencing.
- Reinfection with SARS-CoV-2 does occur and can occur with the same strain, a different strain, or with multiple strains. Therefore, NGS may help to assess the viral complete genetic sequence for public health purposes.
- As more treatment options become available, prospective sequencing may identify viruses with possible treatment failure and assist selection of the most effective therapy.

**Targeted Molecular Methods to Screen for Commonly Known Variants**

Detecting a virus with a target-specific method like RT-PCR or transcription mediated amplification (TMA), loop-mediated amplification (LAMP), or CRISPR-Cas methods is not the same as full viral strain identification, which occurs by performing complete NGS genetic sequence analysis. But some of the high throughput molecular methods, like RT-PCR or Mass Array are specifically designed as variant screening methods, as described below.

Non-commercial or laboratory developed variant screening methods have been reported. Annavajhala et al, created a target-specific RT-PCR assays to survey novel variants in New York City for two mutations, E484K and N501Y. Phylogenetic analyses of sequences in the database further reveal that this B.1.526 variant is scattered in the Northeast of the U.S. [55]. Its unique set of spike mutations may also pose an antigenic challenge for current interventions. Bedotto et al. also created a target-specific RT-PCR screen for mutations within the spike RBD [56]. In France, a viral variant named Marseille-4, harbors a S477N substitution in this RBD. Marseille-4 variant strains identified using NGS tested positive using the Marseille-4 specific quantitative PCR, whereas all 32 cDNA samples from other variants tested negative [56]. Such an approach allows the rapid real-time surveillance of SARS-CoV-2 variants.

In addition to RT-PCR to screen for SARS-CoV-2 variants, Agena Bioscience developed the MassARRAY® SARS-CoV-2 Variant Panel (RUO), the MassARRAY SARS-CoV-2 Panel (EUA, CE-IVD), and the MassARRAY SARS-CoV-2/Flu Panel (RUO). These panels enable the detection of SARS-CoV-2, dominant SARS-CoV-2 variants, and influenza virus RNA.

For any target-specific method, the reliability of targeted molecular methods could still be affected by SARS-CoV-2 genetic variability, because genomic evolution could cause primer and probe binding errors in regions targeted by the diagnostic probes. RT-PCR can fail, and vigilance of routine test methods is warranted [57].

Luckily the *in silico* analysis of the most recently described virus variants, showed that genetic variability should have minimal or no effect on the sensitivity of most existing diagnostic protocols SARS-CoV-2 genome detection [58]. However, given the continuous emergence of new variants, the situation should be monitored continuously, and molecular testing protocols including multiple targets are preferred to mitigate the chances that mutations could affect more than one target.

**Summary**

Since all viruses mutate, it is common to find multiple strains (lineages or variants) of the same virus spreading simultaneously, and this is true of the COVID-19 pandemic. Unique SARS-CoV-2 variants are grouped and defined as those strains containing a common set of genetic mutations. Emerging SARS-CoV-2 variants can be problematic if one or more of the independent mutations result in changes that make the virus more pathogenic, resistant to treatment, able to escape vaccines, or able to evade diagnostic tests. RT-PCR and MassArray are methods that can identify SARS-CoV-2 variants. NGS can identify existing and new variants.

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