Arch Mol Biol Genet. 2022;1(2):57-65.

Archives of Molecular Biology and Genetics | Review Article

SARS-CoV-2 Mutations, Diagnosis and Their Concern

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Received date: February 21, 2022, **Accepted date:** March 23, 2022

Citation: Artik Y, Cesur NP, Laçin NT. SARS-CoV-2 Mutations, Diagnosis and Their Concern. Arch Mol Biol Genet. 2022;1(2):57-65.

Abstract

The first case is described in Wuhan city of China in December 2019 and the disease (COVID-19) continues to pose threat to global health all over the world affecting more than approximately 476 million confirmed cases and 6 million deaths. Reliable and effective methods are taken a critical role such as real-time reverse transcriptase (RT)-PCR test as a gold standard method, serological tests, and reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) method is chosen firstly. However, an actual important problem is the detection of SARS-CoV-2 mutations. Nowadays, there are many mutation types is described for the SARS-CoV-2 including D614G, N501Y, E484K, or L452R. Herein, we comprehensively review COVID-19 disease diagnosis and types of mutation from yesterday to today to contribute to the literature on the global pandemic that affects the world.

Keywords: SARS-CoV-2, Variants, Mutation, Diagnostic

Introduction

The people have been struggling with many pandemics from yesterday to today such as smallpox, Ebola, AIDS, SARS, and MERS. Nowadays, we have experienced a severe and lethal disease called coronavirus disease 2019 (COVID-19) [1]. Since the 21st century, three coronavirus family members have plagued humankind including severe acute respiratory syndrome coronavirus (SARS-CoV) in nearly 8000 cases and 800 deaths while Middle East respiratory syndrome coronavirus (MERS-CoV) causing to approximately 2500 cases and again around 800 deaths. Ultimately, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is reported [2]. SARS-CoV-2 first reported in the Wuhan city of China in December 2019 globally, as of 6:15pm CET, 25 March 2022, there have been 476.374.234 confirmed cases of COVID-19, including 6.108.976 deaths, reported to WHO. As of 26 March 2022, a total of 11.054.362.790 vaccine doses have been administered [3,4]. To curb these pandemics, early and reliable diagnosis is the actual step to control the transmission of the virus. Enzyme-linked immunosorbent test (ELISA), q-RT-PCR, polymerase chain reaction (PCR), reverse transcription loop-mediated isothermal amplification (RT-LAMP), and a variety of biosensor are employed to detect viruses.

Coronavirus disease is caused by contact of person-to-person, contaminated objects, and droplets. Moreover, direct and indirect contacts with mucous membranes in the eyes, nose, or mouth are reasons for the transmission of the virus [5]. General symptoms of the disease are termed fever, sore throat, tiredness, cough, and shortness of breath. Upper respiratory symptoms like sore throat, nasal congestion, rhinorrhea, and olfactory dysfunction can be observed. The loss of taste and smell are the additional symptom of the disease that can be emerged before other symptoms [6]. There are some patients who do not show any symptoms called "asymptomatic," while

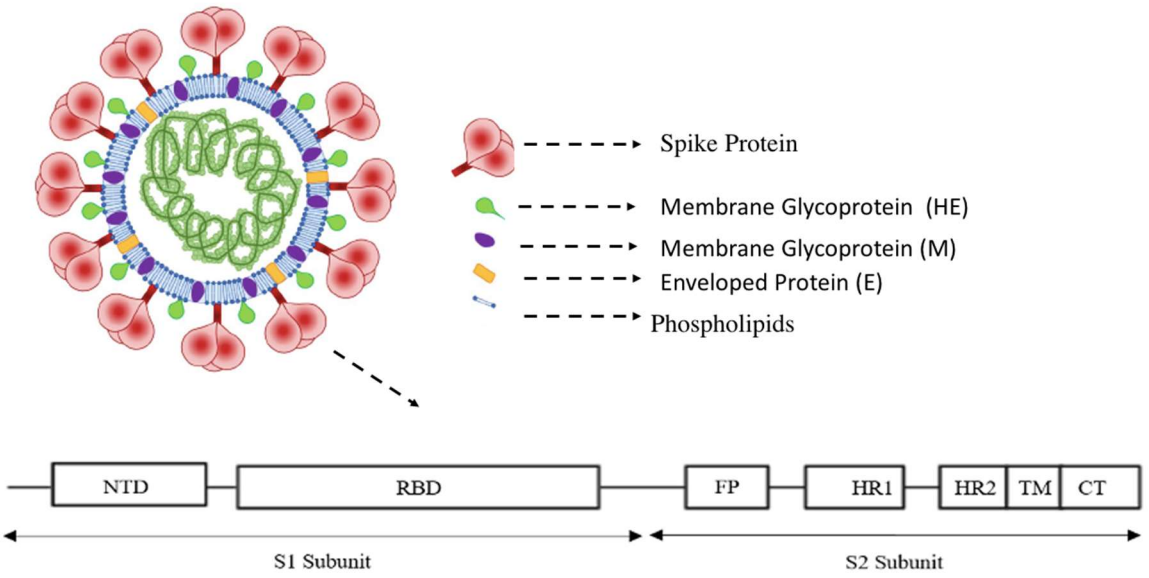

Figure 1: General Structure of SARS-CoV-2 [11]. CT: C-Terminal domain; FP: Fusion Peptide; HR1, and HR2: Heptad Repeat 1 and 2; NTD: N Terminal Domain; RBD: Receptor-Binding Domain; S: Spike protein; SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2; TM: Transmembrane.

early stages of the disease after the infection have occurred but whose symptoms have not yet developed are called "pre symptomatic" that patient can be actual reason of the disease transmission [7]. The polymerase chain reaction (PCR) is an important technique to detect the asymptomatic cases depending on their viral load representing the range of 8% to 80%. Moreover, the disease incubation period is reported as 6.4 days with recent studies [8].

The coronaviruses are one of the positively stranded enveloped RNA viruses that are categorized as beta, alpha, gamma, and delta genera coronavirus [9,10]. SARS-CoV-1, SARS-CoV-2, and MERS-CoV are beta types and only affected mammalians [11]. SARS-CoV-2 is composed of a 30 kb genome with 14 open reading frames encoded to a small membrane protein (SM), nucleocapsid protein (N), spike protein (S), and membrane glycoprotein (M) with an additional membrane glycoprotein (HE). The actual focus of the COVID-19 disease is the S protein that is the entry part of the virus into the host cells by first binding to a specific host receptor, angiotensin-converting enzyme-2 (ACE2) (Figure 1). S protein is separated into two subunits as first an amino-terminal subunit (S1) and a carboxyl-terminal subunit (S2) by host furin-like proteases when the virus-receptor is associated [12]. The host receptor recognizing part is found in the C-terminal of the S1 subunit (S1 CTD) termed receptor-binding domain (RBD). The S2 subunit includes a hydrophobic fusion loop and two heptad repeat regions (HR1 and HR2) that are taken a role in membrane charge fusion [13]. Most of the mutation is described in RBD-ACE2 interactions. The genetic sequence of SARS-CoV-2 shares similarity 79.5% of the genome with SARS-CoV and 96.2% with RaTG13, a short RNA-dependent RNA polymerase (RdRp) region originating from CoV bats [14]. However, the actual source of the virus has not been described yet. The major challenges against the control of pandemic all over the world are the mutations in SARS-CoV-2 genome and diagnosis of the disease early [15].

Diagnosis of COVID-19 Disease

The most important aspect of controlling pandemics around the world is understanding the SARS-CoV-2 genome mutations. Numerous mutations in SARS-CoV-2 have been described and controlling the virus spreading rate is obstructed by rapid and reliable detection methods. Many patients have been reported as asymptomatic which is the actual carrier of the disease in society. Thus, the first step to control the spread of virus infection is the diagnosis of the disease. From this point of view, there are three main different strategies: q-RT-PCR, serological tests, and RT-LAMP, respectively.

Reverse transcription polymerase chain reaction (RT-PCR)

Nowadays, identification and amplification of any viral genetic material are analyzed with a molecular diagnosis basis involving a gold standard method as q-RT-PCR. World health organization published many protocols including designing primers that bind to specific areas of the viral genome and subsequently amplify them. With the identification of the SARS-CoV-2 RNA and nucleocapsid genes, they are used as templates for the primer design binary hydrolysis probes for the *in vitro* qualitative detection of RNA using the q-RT-PCR technique. The mechanism of the q-RT-PCR in SARS-CoV-2

involves the isolation of RNA extracted from the upper and lower respiratory regions, then reverse transcribed into cDNA and then subjected to devices with specific software called amplification period. This procedure provides the binding of probes between the forward and reverse primers. However, during the extension phase of the PCR cycle, these probes are degraded by the 5′ nuclease activity of a reporter dye and a quencher dye that cleaves Taq polymerase. This separation produces a fluorescent signal, and with each cycle, more and more reporter dye cleavages occur, thereby increasing the fluorescence intensity. The human RNase P (RP) gene is also targeted by a set of primers and probes as a control during the procedure [16].

Different countries have studied various viral targets. For example, China relied mainly on the identification of the ORF1ab and N gene; The USA has identified three targets in the N gene of the virus; Germany focused on the RdRP, E and N gene for detection; Paris Pasteur Institute targets 2 regions within the RdRP gene; Thailand mainly used the N gene; Hong Kong has developed a diagnostic test based on the identification of the ORF1b-nsp14 and N gene; spike protein (S protein) targeted by the National Institute of Infectious Diseases of Japan [17].

Serological tests

Serological tests are blood-based test which is used to detect whether a person has an infection or not. Antibodies (IgM and IgG) are chosen for an antigen in the blood and utilized in these tests. Antigens are recognized by the immune system within the body as foreign elements and specific antibodies are created to fight the infection. Thus, antibodies have utilized a label for the detection. In the body, while IgM antibodies can be detected after 10-20 days, IgG is detected after 20 days of SARS-CoV-2 infection [18].

A specific protein termed an antibody is produced in the host cell for response to an infection. If a patient has been exposed to the pathogens as viral, or others, the immune system produces antibodies that are labeled and used by antibody tests. There are four serological assays commonly utilized in laboratories for the detection of SARS-CoV-2 infection; neutralization test, enzyme-linked immunosorbent (ELISA) qualitative detection, chemiluminescent immunoassay qualitative detection, and rapid diagnostic determinations [19].

The patient's serum consists of the antibodies that are the capability to block the replication of SARS-CoV-2 in Vero E6 cell lines. This aspect is utilized by the neutralization techniques. However, the actual problem with this technique is those specific antibodies to proteins involved in primary replication can be missed.

ELISA is the commonly chosen technique used antigen-antibody complexes. The viral-specific proteins, as S protein of SARS-CoV-2, are used for coating the plate, then loaded with a blood serum sample. If the specimens involve the antibody, an antibody-antigen complex is created which is detected by secondary antibodies labeled with fluorochrome. The color change is created or fluoresces through a chemical reaction that is marker for the detection [20].

The luminescence activity created by a chemical reaction of secondary labeled antibodies binding to the primary antigen-antibody complex is the process of the chemiluminescent or chemiluminescent immunoassay (CLIA) method which is a modified version of ELISA. The antigen-antibody complex is detected by the secondary antibody binding as ELISA and then light is created for the detection of the antibody ratio within the sample. Generally, IgG, IgM, and IgA antibodies are detected in this method [21].

The blood samples of the patients are a source of the rapid antigen tests or rapid diagnostic tests (RDT). Principally, antigen tests are worked with the lateral flow in which created antigen-antibody complexes move throughout a capillary system and are immobilized by capture antibodies producing a color change. In a lateral flow system, membranes are utilized with coating gold nanoparticles labeled antibodies, and capture specific antibodies is placed in two various lines. When the patient's sample moves through the membranes, they are associated with the Au-Ab complex and the antigens bind to create a complex. After that, the capture antibodies in the second line recognize this complex, and immobilization is created. Generally, these tests are cheap, portable, and small, and use as a nasal swab, saliva, or blood source [22].

RT-LAMP

Fast, reliable, and sensitive methods are important for the SARS-CoV-2 diagnosis, especially, RT-LAMP technique is chosen nowadays [23]. This technique is time-saving like around 30 minutes to an hour and an easily interpretable colorimetric assay requires only a heat source. Additionally, it is a fast and cost-effective method involving single-tube technology that is a creative solution to increase global testing capacity. In the RT-LAMP technique, six primers are utilized as four primers selected by combining fragments of target DNA and two additional loop primers used to amplify a particular gene region [24].

The colorimetric sensing loop-mediated isothermal amplification (RT-LAMP) method was first proposed by Zhang et al. According to the literature, the result is RT-LAMP gives similar results with q-RT-PCR. Moreover, the use of recombinase polymerase amplification (RPA) for a two-step amplification in a single tube has been utilized by Jinzhao Song, a professor at the University of Pennsylvania, in 2020 which can be used at home and clinical setups are not required as a point of care (POC) device. Such devices combine a paper-based technology with the RT-LAMP assay technique.

One of the notable features of this device is its potential to integrate quarantined and self-isolated individuals on their own with a smartphone where highly sensitive, reliable and fast results can be obtained. This can be done by collecting nasal swabs, adding RT-LAMP-specific reagents to a sheet, and visualizing a color change. Therefore, it reduces visits to hospitals for checkups, and the chance of infection spreading is significantly reduced. Nucleic acid determinations take over around 30 minutes and typically do not require the required temperature values, unlike the traditional PCR technique of RT-LAMP, a powerful amplification method performed in an isothermal environment [25]. The incubation period is around 60 to 65°C and 4 (or 6) specially designed primers, and Bst DNA polymerase is provided in a single tube. Thus, amplification and detection of genetic materials can be done in a single step. Two inner and outer primers are designed in which six different regions of the target DNA sequence are recognized. The forward inner lining (FIP) consists of the F2 region and a complementary sequence of the F1 (F1c) region, while the backward inner lining (BIP) consists of the B2 region and a complementary sequence of the B1 (B1c) region. The forward outer primer (F3) and reverse outer primer (B3) have sequences that complement the sequences of the F3c and B3c regions, respectively. These regions surround the desired amplified sequence. Primers used for any RT-LAMP assay, nucleotide base pair concentration and positions, distance between DNA regions, thermodynamics of primers, etc. should be optimized by a number of factors [26].

The RT-LAMP amplification process begins when FIP complexes with target DNA at the F2 region to form double-stranded DNA, which is in equilibrium at about 65°C. DNA polymerase with strand-changing activity then initiates DNA synthesis from the FIP and simultaneously replaces a single strand of DNA. After this initiation step, the F3 primer then binds to the complementary F3c region and replaces the FIP-complementary helix. Due to the F1c sequence in FIP, the FIP sequence can self-anneal and form a loop structure at one end of the DNA. This strand then serves as the target for BIP-initiated DNA synthesis followed by strand displacement from B3-induced DNA synthesis. This allows the other end of the single DNA strand to form a loop structure, thus resulting in a dumbbell-like DNA structure. It acts as a template for subsequent amplification. After a dumbbell-like structure is formed, exponential amplification of the dumbbell structure is initiated and DNA polymerase initiates DNA synthesis at the F1 site. FIP also hybridizes from the F2 region to the single-loop structure, and DNA synthesis of this primer causes displacement of the F1-prepared strand and self-attachment to a loop structure. Finally, self-prepared DNA synthesis is restarted from the B1 site in the new loop, reinforcing the existing template, as well as creating a new one from the displacement of the FIP-complementary strand [27].

Because DNA can be amplified 109 times in one hour, it is possible to achieve great amplification from this iterative process. To further increase amplification efficiency, RT-LAMP with six primers (loop primer) has also been developed. Loop primer RT-LAMP uses six instead of four primers, the forward and reverse loop primers (LF and LB) anneal to the regions between the F1/F2 and B1/B2 regions, respectively. Loop primers have been proven to enhance the RT-LAMP process, as amplification is fixed from increased starting points for DNA synthesis. RT-LAMP detects RNA rather than DNA sequences. Reverse transcriptase is added to the RT-LAMP mix to help convert viral RNA into complementary DNA (cDNA) to be used for amplification. This procedure has been of great help in the diagnosis of a large number of RNA viruses. To detect multiple pathogens in the same test tube, multiple RT-LAMP assays with more primers or unique fluorescent signals have been developed. All these iterations of the RT-LAMP procedure show how promising it is in the field of viral diagnostics. As a result, it has great potential in detecting SARS-CoV-2 [28].

SARS-CoV-2 Mutations

The assorted types of SARS-CoV-2 variants have been described nowadays. The first variant was reported in the United Kingdom (UK) (B.1.1.7 lineage), September 2020, and another variant (B.1.351 lineage) in October 2020 in the Republic of South Africa (RSA). It spread fastly all over the world such as South Africa [29], Brazil [30], and the United States [31]. Especially, additionally, RBD region has the other mutations such as B.1.351 lineage, the K417N, and E484K [32]. The mutation at residue 501 has significantly strengthened. In the next few months, the Delta variant, involving RBD mutation L452R and T478K, had dominantly emerged around the world and they are the most infectious variant among all variants formally reported by the WHO [33].

D614G

In late February 2020, the D614G mutation is firstly described after several months it outcompeted all ancestral viruses. The first location is the human airway cells and in the upper respiratory tracts of hamsters. This mutation additionally causes to increase in the number of spike proteins per virion and the rate of S1/S2 cleavage. In literature it has been proposed that D615G mutation has been slightly more susceptible to neutralization by mAbs [34].

N501Y

N501Y mutation is described in the RBD region of SARS-CoV-2 and N501Y, HV69- 70del, and P681H mutations have been reported in the S protein region. Humanity has been experienced P681H mutation in furin cleavage site while HV69- 70del is described in the N-terminal domain (NTD) which is described as invariants related to immune escape in immunocompromised patients. On the other side, N501Y is observed in the receptor-binding motif (RBM) of the C-terminal domain (CTD) which is described to increase its

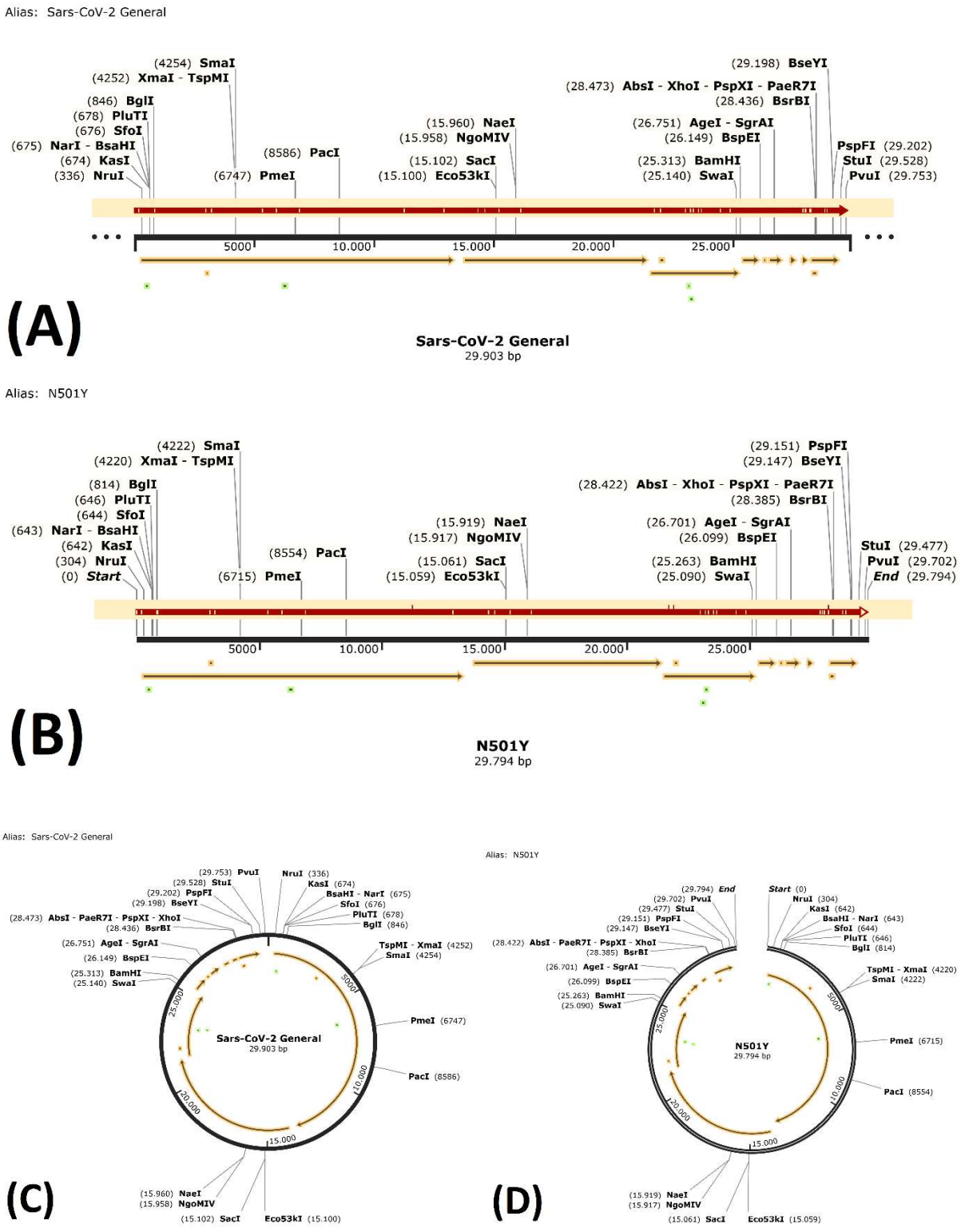

Figure 2: The comparison of NGS sequences of general SARS-CoV-2 and N501Y mutation sequence (SARS-CoV-2 is aligned through 29.903 and N501Y 29.794 base pairs (bp)) [38].

binding affinity to the human ACE2 receptor [35]. N501Y is also termed Variant of Concern 202012/01 (VOC-202012/01), and as strain B.1.1.7 is significant due to the transmission capability to increase the rate of spread of SARS-CoV-2 by 70% ratio in which 23 mutations as 13 nonsynonymous mutations (which change proteins), 4 deletions (which change proteins), and 6 synonyms (which do not change proteins) is reported in the UK as shown in Figure 2 [36]. N501Y mutation is also occurred in the receptor-binding domain and the furin cleavage site. These types of variants include the mutation of Asparagine at position 501 to Tyrosine (N501Y), which is one of the residues in the RBD-ACE2 contact area and important to enhance the binding affinity of SARS-CoV2 spike protein to ACE2, and similar findings were also reported for the D614G substitution in previous studies [37].

In addition to N501Y, lineage B.1.351 is described by the presence of five further spike amino acid substitutions (D80A, D215G, K417N, E484K and A701V) and a deletion in the NTD, Δ242–244. High numbers of B.1.351 viruses also have the spike amino acid substitutions L18F, R246I and D614G. A similar NTD deletion, Δ243–244, abolishes binding by the anti-body 4A8 [39]. The sub lineage of B.1.1.28 is firstly described in Brazil and in travelers from Brazil to Japan which is also the P.1 lineage. P.1 is characterized by the presence of several amino acid substitutions in the spike protein: L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, H655Y and T1027I [40]. N501Y does not influence the binding and neutralization of most mAbs. Additionally, it is also rarely associated with reduced susceptibility to convalescent plasma or plasma from persons receiving one of the two authorized mRNA vaccines (Pfizer/BioNTech BNT162b2 or Moderna mRNA-1273) or the Novavax NVX- CoV2373 protein subunit vaccine [41].

E484K

E484K is described in the Beta and Gamma VOCs and in the VOIs Eta (B.1.525), Iota (B.1.526)154, Theta (P.3)155,156 and Zeta (p.2). Alpha variants are also included E484K and E484Q has been reported in the Kappa VOI (B.1.617.1) [42]. 3-fold to 10-fold reduced susceptibility about 30% of plasma samples from persons immunized with one of the authorized mRNA vaccines are described in E484K variant.

Other RBD mutations

L452R mutation is found in the Delta VOC, as well as the Kappa (B.1.617.1) and Epsilon (B.1.427/9) VOIs. This mutation is caused to decline the susceptibility to several RBM class 2 mAbs, including "Bamlanivimab", but not to the other FDA EUA- approved mAbs [43]. When the L452R is compared with both D614G and N501Y mutation types, it showed that pseudo-typed viruses containing L452R were related with high effectivity to enter the lung organoid cells and lower levels compared with pseudo- typed viruses containing N501Y mutation type [44]. On the other hand, K417N/T are present in the Beta (as K417N) and Gamma (as K417T) VOCs and it is caused to reduce the ACE2 binding while N439K increases ACE2 affinity [45].

Mutations close to the S1/S2 furin cleavage site

Q675H/R, Q677H/P, N679K and P681H/R have occurred independently in many SARS- CoV-2 variants. P681H is found in the Alpha VOC and the Theta VOI, and in several additional SARS- CoV-2 variants while P681R is found in the Delta VOC and the Kappa VOI [46]. Two of these mutations are taken as an important role virus tropism by increasing S1/S2 cleavage in human airway epithelial cells [47].

Conclusion

Throughout history many pandemics has affected the entire world. Nowadays, SARS-CoV-2 is emerged and caused deathly pandemic episode. The emergence of SARS-CoV-2 variants concerning phenotypic mutations is the public health interest to control the disease. SARS-CoV-2 variants are experienced by their transmissibility, disease severity and ability to evade humoral immunity for patients. Additionally, mutations of the virus are affected by the variant types of the SARS-CoV-2. Many various variant types of SARS-CoV-2 have been described. In particular, the N501Y mutation has exhibited potential risk firstly in the United Kingdom and after a while all over the world. Additionally, S protein of the virus are highly affected to mutations including N501Y, HV69-70del, and P681H that are potential biological implications. Especially, S-protein based mutations are most likely to alter the receptor recognition properties of SARS-CoV-2. Recently, 85% of the sequenced SARS-CoV-2 genomes containing N501Y are termed VOC-202012/01 and affected in UK since September 2020. On the other hand, 1% of total sequences in South Africa contain N501Y and none of them belong to VOC-202012/01. Thus, termed as 501Y.V2 in South Africa. The genomic analyses are showed that spreading ability of 501Y is faster than the N501Y. SARS-Cov-2 includes other two Alpha variants (B.1.1.7, United Kingdom, and B.1.1.7 with the additional E484K mutation), the Beta variant (B.1.351, South Africa), and the Gamma variant (P.1, Brazil). On the other side, the most important consequence of emergent SARS-CoV-2 mutations is that their impact on vaccine efficacy. The main reason for focusing on 3 different mutations (D614G, N501Y, E484K) in this research article is because SARS-CoV-2 which occurs in the early stages of the pandemic is associated with the genetic evolution process. D614G emerging as a globally dominant variant has been associated with high infectiousness [48]. When we evaluated the public health impacts of these mutations, they were recognized as variants of concern (VOC). VOCs are associated with enhanced infectiousness or virulence, decreased neutralization by natural infection or vaccination-derived antibodies, the ability to evade detection, or a reduction in therapeutics or vaccination efficacy [49]. Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1) and Delta (B.1.617.2)

variants have mutations in RBD and NTD. A second reason for focusing on these mutations (D614G, N501Y, E484K) is that of these VOC variants, the N501Y mutation in RBD is common to all variants except the Delta variant, which results in increased spike affinity. It increases viral binding from protein to ACE 2 receptors and subsequent entry into host cells. Along with NBD, RBD serves as the predominant neutralization target and facilitates antibody production in response to antisera or vaccines [50]. It has been described that a single mutation of N501Y alone increases the affinity between RBD and ACE2 approximately ten times greater than the ancestral strain (N501-RBD) [51]. Finally, the delta and omicron variants, which emerged with the genetic evolution of these most influential mutations (D614G, N501Y, E484K), were defined by WHO as the fourth and fifth VOCs, respectively. VOI variants [Epsilon (B.1.427 and B.1.429); Zeta (P.2); Eta (B.1.525); Theta (P.3); Iota (B.1.526); Kappa (B.1.617.1); Lambda (C.37) and Mu (B.1.621)]; are defined as variants with specific genetic markers associated with changes that can cause enhanced transmissibility or virulence. The main reason why VOCs, unlike VOI variants, are so important for public health is that the genetic evolution of these 3 different mutations (D614G, N501Y, E484K) regions in VOC variants has undergone a drastic change in stopping the spread of SARS-CoV-2. continues to threaten to reverse the significant progress made so far [49]. Therefore, the extraordinary pace of vaccine development against COVID-19, and the review of ongoing mass vaccination efforts around the world, have led us to present this research study specifically, relying on the preliminary view that it can be resolved by focusing on these mutation sites. Some mutations in variant types of the SARS-CoV-2 are also taken a role in levels of neutralizing mAbs elicited by the mRNA vaccines. Thus, the mutations in SARS-CoV-2 are not only important to understand the disease severity, transmission capacity or evolutionary process of the virus but also important for getting significant information about the vaccine efficiency.

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