A study on non-synonymous mutational patterns in structural proteins of SARS-CoV-2

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Abstract

SARS-CoV-2 is mutating and creating divergent variants across the world. An in-depth investigation of the amino acid substitution in the genomic signature of SARS-CoV-2 proteins is highly essential for understanding its host adaptation and infection biology. A total of 9587 SARS-CoV-2 structural protein sequences collected from 49 different countries are used to characterize protein-wise variants, substitution pattern (type and location), and major substitution changes. The majority of the substitutions are distinct, occurred mostly in a particular location, and leads to a change in amino acid’s biochemical properties. In terms of mutational changes, Envelope (E) and Membrane (M) proteins are relatively stable than Nucleocapsid (N) and Spike (S) proteins. Several co-occurrence substitutions are observed, particularly in S and N proteins. Substitution specific to active sub-domains reveals that Heptapeptide Repeat, Fusion peptides, Transmembrane in S protein, and N-terminal and C-terminal domains in N protein are remarkably mutated, and also found few deleterious mutations in these domains.

Keywords: Mutation, Amino acid substitution, Structural proteins, Biochemical properties, Functional sub-domains.

1. Introduction

The outbreak of novel Severe Acute Respiratory Syndrome Corona Virus (SARS-CoV-2), causing the disease COVID-19. With the passage of time a good number of variants are reported so far across the globe. Though the rate of mutation in SARS-CoV-2 observed to be relatively low, even then the variants of SARS-CoV-2 are differed by a number of mutations in their genome. Mutations in the nucleotide sequence can prevent binding PCR primers to target sequences \[1\]. Different biological properties such as pathogenicity, tissue tropism or host range can happen diversely in closely related variants too \[2, 3\]. Couple of studies reveal that mutation can trigger enzyme motion in dihydrofolate reductase \[4\], impact on three-dimensional structure, stability and redox potential \[5\], hydrophobic effect \[6\], functional diversity in the proteins. The same may be observed even in the structurally similar proteins \[7, 8, 9\].

The genetic diversity in SARS-CoV-2 may impact on molecular functionality of the protein \[10\]. Mutation and sequence variant analysis is crucial for understanding the disease pathogenesis and structural changes in the SARS-CoV-2 proteins, which ultimately helps in designing more stable small molecules that may binds viral proteins.

Mutation is the key factor that trigger a virus to switch hosts \[11\]. SARS-CoV-2 genome shows different degree of similarity with other coronaviruses. SARS-CoV-2 genome is highly

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similar with SARS-related coronaviruses[1] derived from Pangolin [12] or Bat [13, 14]. A minor
dissimilarity [15] might lead to the variation in functionality of SARS-CoV-2 protein with
other class of coronaviruses. Scientists observing variants in novel coronavirus strains due to
mutation, insertion or deletion reported from different geographical regions [16,17,18].

At least 26 protein-coding genes are available in each SARS-CoV-2 genome that encodes
mainly three classes of proteins, such as nonstructural (Nsp1-Nsp16), structural (Spike glycoprotein-
S, Envelope-E, Membrane-M and Nucleocapsid - N), and several accessory protein chains
[19, 20]. The two-third of the complete genome is at the 5’ site that encoding the nonstruc-
tural proteins, and one-third are at the 3’ side which encodes structural and accessory proteins
[19]. SARS-CoV-2 proteins play a diverse functional role. Mutations in structural proteins can
alter various functionality of the virus, more importantly protein from this class initiate host
jumping mechanism. For example, the envelope protein promotes viral assembly and release
[21]. The Spike protein responsible for the occurrence of spikes on the viral surface that binds
to host receptors [21] and it is mainly responsible for receptor recognition, cell attachment, and
fusion during viral infection [22,23,24]. The Nucleocapsid protein capable of self-association
through a C-terminal [25, 26] and it activates the expression of cyclooxygenase-2 [27]. The
roles of Membrane protein include promoting membrane fusion, regulating viral replication,
packing genomic RNA into viral particles, interaction with other proteins [28,29,30]. Both
the spike and nucleocapsid proteins are in the main target for antibody detection [31]. Many
of the sequence variability features of structural proteins are still unknown and need to be
investigated thoroughly.

Studies on three severe class of coronaviruses have shown that hydrophobic interaction
in receptor binding motif for SARS-CoV and SARS-CoV-2 could allow the spike protein for
zoonotic transmission [32]. Further, insertion of an extra charged amino acid residue in SARS-
CoV-2 receptor-binding domain, creates a larger hydrophobic surface that underlies the higher
binding affinity of SARS-CoV-2 to ACE2 compared to SARS-COV [32] and that might allow
more flexibility for zoonotic transmission. In comparison to Bat and Pangolin coronavirus,
SARS-CoV-2 sequence seems to possess specific modifications and characteristics than other
SARS-CoV viruses [33]. For instance, in case of homologous coronavirus proteins, positively
charged amino acid (Arg69) replaces negatively and neutrally charged Glu or Gln residues.
Moreover, a deletion specific to SARS-CoV-2 proteins flanks this position. In a different stud-
ies have highlighted the impact of the non-synonymous substitutions, that change biochemical
properties of amino acids, are highly crucial for protein stability, binding with receptors, sub-
strate specificity, affinity of amino acid change [34,35,36,37,38,39]. Therefore, amino acid
insertion, deletion and even substitution playing significant role towards attaching with recep-
tor proteins that could impact on the binding affinity of SARS-CoV-2 protein. Several other
studies on SARS-CoV-2 have shown that a mutation can trigger protein structure alteration,
dynamics and function while binding with human receptor protein (ACE2) [40,41,42,43].
Therefore, these studies are important to understand the clinical presentation and spread of
the disease, and also useful for antiviral drug design [44,45].

In this study, we focus on sequence variability of worldwide SARS-CoV-2 genome, particu-
larly four structural proteins. For each protein, we identify unique variants. Then we identify
and localize the amino acid substitution in each variant. Substitutions are then quantified and
categorised according to their type and physico-chemical properties of amino acids. We then
report country-specific unique variant and substitution type in each structural protein.

[1] https://www.ecohealthalliance.org/2020/01/
2. Materials and methods

To the best of our knowledge no prior research analyse different SARS-CoV-2 strains collected from 49 different countries across the global to understand the worldwide sequence variation due to mutation. In this section we report the sequence used and various exploratory analysis performed on the sequences.

2.1. Sequence dataset

We collect around 9,587 SARS-CoV-2 complete genomic (nucleotide) sequences of length \( \approx 28 \text{kb} \) from NCBI database reported till 15th July 2020. We exclude sequences with undetermined character present within the sequence for our analysis, and obtain 9058, 8954, 8271 and 7009 number of sequences for Spike (S), Envelope (E), Membrane (M), Nucleocapsid (N) protein, respectively. Collected sequences are then grouped based on similarity to isolate unique variants for further analysis.

2.2. Multiple sequence alignment

The unique list of sequences (one representative from each group) are aligned using multiple sequence aligner (MSA) to observe possible insertion or deletion in amino acid residues. We select first reported sequence from Wuhan city of China (2019-nCoV/USA-WA1/2020, Accession number: MN985325/NC_045512), E-protein (Accession number: QHO60596.1); M-protein (Accession number: QHO60597.1); N-protein (Accession number: QHO60601.1); S-protein (Accession number: QHO60594.1) as a reference sequence. We use MEGAX [46] for MSA. We observe few sequences with indels in Spike and Nucleocapsid proteins, which we separately analyze as reported in Supplementary-A (Figure S1 and S2), and all the remaining sequences are considered for subsequent analysis.

2.3. Amino acid substitution identification

The amino acid substitution (or non-synonymous mutation) is a common phenomenon in virus genome and the rate of substitution is mainly depends on the protein’s expression level, functional category, metabolic costs, hydrophobicity and electrostatic, physicochemical properties, annotated active or binding site [47, 48, 49, 39].

To understand and observe any pattern during amino acid substitutions in the collected strains, we compare each and every aligned sequences with the reference sequence and report position-wise substitutions. In our study, we try to identify all possible substitutions for twenty (20) amino acids \((20 \times 20 - 20 = 380)\) without considering synonymous substitutions and categorizing them based on similar substitution patterns.

2.4. Investigating the change in biochemical properties

Amino acid substitutions due to non-synonymous mutation may change the biochemical properties of the target proteins. We categorize observed amino acid substitutions based on the change in the chemical properties. Here, we consider two kinds of broad groupings based on the biochemical properties of amino acid. One is eight chemical sub-groups based on side-chain structure [50], and the other is three Hydropathy classes of amino acids [51]. The sub-groups in each category are as follows:

- **Side-Chain based classes:** According to this grouping 20 amino acids are clustered as Acidic (D, E), Basic (R, H, K), Aromatic (F, W, Y), Aliphatic (A, G, I, L, V), Cyclic (P), Sulfur (C, M), Hydroxyl (S, T), and Amide (N, Q).

- **Hydropathy based classes:** Three such groups include Hydrophobic (A, C, I, L, M, F, W, V), Neutral (G, H, P, S, T, Y) and Hydrophilic (R, N, D, Q, E, K).

Our major goal is to highlight what kind of biochemical properties are majorly changed (quantitatively) due to substitution.
2.5. Functional domains of SARS-CoV-2 structural proteins

The SARS-CoV-2 structural protein (particularly S and N) encompasses several sub-domains responsible for a specific functional activities. Few of them are:

- **Transmembrane (TM)** is a stretches of amino acids responsible for viral entry [52, 53].
- **Heptapeptide Repeat 1-2 (HR1, HR2)** are responsible for virus fusion [54].
- **Receptor-Binding domain (RBD)** is mainly responsible for binding of the virus to the receptor protein [53].
- **N-terminal (NTD) and C-terminal domain (CTD)** are two main RNA binding domains in SARS-CoV-2 N protein [26]. Both of them function as a receptor-binding entity. CTD recognizes the receptor and NTD engages the receptor [55].
- **Fusion peptides (FP)** are created fusing using two or more genes playing different functional roles. The FP play an important role in fusion of viral envelope with host cellular membranes [56].

The typical length of SARS-CoV-2 spike (S) protein domain is 1273 amino acids. Primarily it consists of three units: a) a signal peptide (amino acids 1–13) located at the N-terminus; b) the S1 subunit (14–685 residues), which is consisting of N-terminal domain (14–305 residues) and a receptor-binding domain (RBD, 319–541 residues); and c) the S2 subunit (686–1273 residues), which is consisting of the fusion peptide (FP) (788–806 residues), heptapeptide repeat sequence 1 (HR1) (912–984 residues), HR2 (1163–1213 residues), Transmembrane (TM) domain (1213–1237 residues), and cytoplasm domain (1237–1273 residues) [57].

Usually SARS-CoV-2 nucleocapsid (N) protein domain consists of 419 amino acids. SARS-CoV-2 N protein contains two distinct RNA-binding domains: the N-terminal domain (NTD, 44-179 residues) and the C-terminal domain (CTD, 247-363 residues) [58]. These two domains are linked by a poorly structured linkage region (LKR), and N-tail and C-tail domain at the beginning and end of the protein domain.

Identification of substitutions, particularly in functional domains, might help understand the virulence power of SARS-CoV-2. Therefore, we try to highlight the substitutions in different functional domains in the next section.

2.6. Software tools and programming

For predicting whether a mutation is deleterious or neutral, we use software tool PROVEAN [2]. PROVEAN (Protein Variation Effect Analyzer), a web server software tool, is used to predict any non-synonymous amino acid substitution or indel impacts on the biological function of a protein [59]. We utilize a recently developed mutation simulation tool [60] which generates fine-grained simulated random mutations in any genome. We use Python scripting 3.7 for quantitative analysis.

3. Results and Discussion

3.1. Grouping identical variants

At first, similar sequences are grouped for each structural protein. We observe 24, 51, 258, 364 number of groups (distinct variants) for E, M, N and S protein, respectively (Figure 1(a)). The distinct variants are represented as \(v_1, v_2, \ldots, v_k\) (\(k\) is the number of distinct variants).

[http://provean.jcvi.org/index.php](http://provean.jcvi.org/index.php)
observed in each protein category). If we consider the group size for different proteins, we observe that more than 95% of the samples from E and M proteins sharing two groups and 85% samples from N and S proteins distributed in two groups (Figure 1(c)). We observe a maximum variation in S protein followed by N, M and E proteins. It further reveals interesting fact that in terms of mutational changes E and M proteins are relatively stable than N and S proteins. A country-wise variants and its count (sample frequency) for each structural protein is listed in Supplementary-A (Table S1).

While studying the number of substitutions (or substituted positions) occurs in each variant, interestingly we observe maximum of seven (07) substituted positions. We represent number of substituted positions by numbering, for example, one substituted positions by 1p, two substituted positions by 2p, and so on. Out of which single substitution occurring most commonly, whereas seven number of substitutions are rarely occurring in different candidate proteins (Figure 1(b)). In case of E and M protein, we observe only single amino acid substitution position in each variant. For N protein, number of substitutions varies from 1p to 4p numbers, whereas for S protein, count varies from 1p to 7p.

Figure 1: The quantification of observed variant and non-synonymous substitutions positions in each SARS-CoV-2 structural protein. (a) collected sample with observed distinct variants; (b) Variant frequency with number of substituted positions; (c) Percentage of sample frequency for the top two variant class (v1 and v2).

3.2. Substitution patterns and locations

In this section, we first try to investigate the substitution patterns in comparison to our reference sequence. All possible substitutions (source to target amino acid) are shown in a 2D matrix representation in Figure 2. In general, there could be $20 \times 20 - 20 = 380$ possible unique non-synonymous substitutions considering twenty amino acids. The value in each cell of the matrix depicts the count for a particular substitution from a source (row) to target amino acid (column) occurs in different locations in different proteins, we consider.

Irrespective of variant and position of substitutions, we can observe from the Figure 3 a total 316, 217, 50, 23 number of amino acid substitutions in S, N, M, E protein, respectively. To understand how much random the location of substitutions in different proteins, we observe a variable number of substitution locations such as 261 ($\approx 21\%$ of sequence length) for S, 167 ($\approx 40\%$ of sequence length) for N, 45 ($\approx 20\%$ of sequence length) for M, and 19 ($\approx 25\%$ of sequence length) for E protein. Interesting, these findings show that mutation location in the N protein is most random and difficult to localize or predict its site of alternation. M protein relatively most stable in such scenario.
Figure 2: The figure shows amino acid substitution by position count. The amino acid substitution type is shown by a 2D matrix relation. In figure, a substitution type, say $P > Q$, indicates left amino acid $P$ (y-axis, left) (source) to substitution right amino acid $Q$ (x-axis, top) (target), and the cell value in the matrix indicates observed number of substitution positions for that particular substitution type (from Figure 4).

It is evident from our analysis that a total 91 types of substitutions are observed in S proteins. Similarly, for N, M and E proteins total 74, 33, and 19 types of substitutions are observed. A position-wise substitutions in each proteins is shown in Figure 4. A number of substitutions in different proteins showing multiple target amino acids in the same location. For example, in case of N proteins, four target amino acids (K, M, S and G) as substitutions are found in position 203 distributed across 36 variants (Supplementary-B).

The top few substitutions (based on sample frequency) are observed common in the majority of the countries in different SARS-CoV-2 structural proteins (Figure 3), which are $P > L(71)$ in E protein; $T > M(175)$ in M protein; $R > K(203)$, $G > R(204)$ and $S > L(194)$ in N protein; $D > G(614)$ and $L > F(54)$ in S protein. Several substitutions are observed, mostly specific to in different countries, with sample frequency 3 or more as depicted in the Figure 5. The complete list of substitutions patterns observed in different countries (with sample frequency $\geq 1$) can be seen from Supplementary-C.

3.3. Trend of substitutions by change in amino acids

Amino acid substitution changes the linear organization of peptide bond, and hence some changes can lead to abnormal functionality and disrupting its structure. So, we focus on amino acid substitution by different types of amino acid change looking into substitutions in different positions. Majority of substitutions are observed distinct and occurring mostly in particular...
position (Figure 2). We observe maximum substitutions for $A > V$, which occurs in 19 different positions followed by $A > S$ (17 different position) and $L > F$ (13 different positions) in S protein. In case of N protein, we observe maximum substitution for $T > I$, which occurs in 15 different positions followed by $P > S$ (12 different position) and $P > L$, $A > S$ and $D > Y$ (11 different positions). In case of M protein, we observe maximum substitution for $A > S$ (15 different positions), and we do not observe more than two substitutions in any particular position in case of E protein.

Towards understanding amino acid change by substitutions type, we calculate the percentage by observing all substitutions occurring in all positions (discussed above). There are two categories of substitution we consider, one to many and many to one fixing a particular source or target amino acid. For both the categories, the total substitution count, the number of distinct substitution type (from Figure 2), and their percentages for each category of proteins are calculated. The top 5 substitutions are reported in Table 1. Majority of the amino acid changes from $L$ to other in both the envelope (E) protein ($\approx 17\%$, 3 distinct types) and membrane (M) protein ($\approx 20\%$, 4 distinct types). In case of nucleocapsid (N) protein, substitutions occur for $S$ to other ($\approx 13\%$, 10 distinct types). For spike (S) protein, we observe $A$ to other change is the maximum ($\approx 14\%$, 5 distinct types). However, maximum distinct substitution are observed in $S$ to others (10 distinct types).

We also observe several co-occurrence substitutions in Spike (S) and Nucleocapsid (N) proteins (Table 2). For example, dominant substitution $D > G$ (614) in S protein is co-occurred with $L > F$ (54) in 53 samples and $L > F$ (5) in 39 samples. Similarly, in N protein, dominant substitution $R > K$ (203) is co-occurred with $G > R$ (204) in 766 samples.

3.4. Trend of substitutions by changing chemical properties of amino acids

A non-synonymous substitution that alters the amino acid, in turn alter the biochemical properties of the amino acid. In this section, we categorise the substitution type by observing their chemical properties of amino acids. We consider two kinds of biochemical properties, eight chemical properties of amino acids and three Hydropathy class of amino acid as discussed in Section 2.4.

In the case of side-chain structure change (using eight chemical properties of amino acids), most of the substitutions change its chemical properties. We see 77 out of 91 ($\approx 85\%$), 61 out of 74 ($\approx 82\%$), 24 out of 33 ($\approx 73\%$), 15 out of 19 ($\approx 83\%$) of substitutions in S, N,
Figure 4: The figure shows observed amino acid substitution by position and type, which are obtained from across all the variants and shown by each structural protein shaded at the top (S, N, M, E).

In case of E and M proteins, majority of the substitutions lead to change in chemical properties from Aromatic to Aliphatic, Aliphatic to Hydroxyl-containing for M protein, Hydroxyl-containing to Aliphatic for N protein, Aliphatic to Aromatic and Hydroxyl-containing for S protein (Figure 6(a)).

In case of more than 50% substitutions (except E protein), we observe a change of one Hydropathy class to another. We observe 50 out of 91 (≈55%), 46 out of 74 (≈62%), 17 out of 33 (≈52%), 8 out of 18 (≈44%) of substitutions in S, N, M, and E protein, respectively, that changes the Hydropathy class. The significant change in Hydropathy classes from Hydrophilic to Hydrophobic (S, N and E protein), and Hydrophobic to Hydrophilic (M protein) (Figure 6(b)).
3.5. Substitution in different functional sub-domains of structural proteins

We try to highlight and quantify the substitutions in various functional sub-domains of two structural proteins, S and N. We report domain specific substitutions in S and N proteins in Table 3.

Observed substitutions are mostly unique. In a particular location not more than two substitutions are observed. In case of S protein, we observe maximum substitutions in HR2 and FP domains (≈ 22% locations) followed by Transmembrane (TM) domain (≈ 17% locations).
Table 1: The table shows top 5 substitution type and associative quantitative information for two categories. For a substitution \( X > Y \), in the first category, \( X \) is fixed and \( Y \) can be any amino acid, and in the second category, \( X \) is any amino acid and \( Y \) is a fixed.

| Protein | AA Count | Distinct | Percentage |
|---------|----------|----------|------------|
| E       | L 4      | 3        | 17.39      |
| E       | E 3      | 3        | 13.04      |
| E       | V 3      | 2        | 13.04      |
| E       | A 3      | 2        | 13.04      |
| M       | L 10     | 4        | 20.00      |
| M       | A 10     | 4        | 20.00      |
| M       | V 5      | 4        | 10.00      |
| M       | D 4      | 3        | 8.00       |
| M       | R 3      | 2        | 6.00       |
| N       | S 30     | 10       | 13.82      |
| N       | P 29     | 5        | 13.36      |
| N       | R 23     | 9        | 10.60      |
| N       | D 22     | 5        | 10.14      |
| N       | A 22     | 4        | 10.14      |
| S       | A 43     | 5        | 13.61      |
| S       | S 29     | 10       | 9.18       |
| S       | T 28     | 6        | 8.86       |
| S       | V 24     | 5        | 7.59       |
| S       | Q 21     | 6        | 6.65       |

Although receptor binding domain in S protein is equally important for viral entry to host [53], we observe comparatively few substitutions (≈ 12% locations) in this domain. We then predict whether the mutations/substitutions in functional domains are deleterious or neutral. We find the dominant substitutions that are co-occurred with other substitutions (with sample frequency 5 or more) in Spike(S) and Nucleocapsid (N) protein.

Table 2: The dominant substitutions that are co-occurred with other substitutions (with sample frequency 5 or more) in Spike(S) and Nucleocapsid (N) protein.

| Protein | Substitution Co-occurred substitution with Substitution with (a) Sample frequency |
|---------|-----------------------------------------------|-------------------------------|
| S       | D>G(614) L>F(54)                           | 53                            |
| S       | L>F(5)                                      | 39                            |
| S       | S>N(477)                                   | 37                            |
| S       | H>Y(146)                                   | 21                            |
| S       | S>L(221)                                   | 17                            |
| S       | P>L(681)                                   | 16                            |
| S       | D>G(253)                                   | 14                            |
| S       | N>Y(501)                                   | 13                            |
| S       | Q>H(677)                                   | 13                            |
| S       | T>H(572)                                   | 11                            |
| S       | E>D(583)                                   | 11                            |
| S       | Q>H(14)                                    | 8                             |
| S       | R>M(78)                                    | 8                             |
| S       | V>L(308)                                   | 8                             |
| S       | R>K(403)                                   | 8                             |
| S       | A>D(845)                                   | 7                             |
| S       | P>L(1263)                                  | 7                             |
| S       | F>L(220)                                   | 6                             |
| S       | D>H(138)                                   | 6                             |
| S       | H>Y(149)                                   | 6                             |
| S       | S>F(939)                                   | 6                             |
| S       | T>H(859)                                   | 6                             |
| N       | N>K(203)                                   | 8                             |
| N       | G>R(204)                                   | 106                           |
| N       | G>R(204);T>H(292)                          | 16                            |
| N       | G>R(204);Q>H(229)                          | 10                            |
| N       | G>R(204);T>I(205)                          | 5                             |
| N       | A>G(208)                                   | 6                             |
| N       | E>V(62)                                    | 6                             |
| N       | P>L(13)                                    | 28                            |
eleven (11) deleterious mutations in functional domain of S protein (*Supplementary-A (Table S2)*), which are distributed in RBD domain (*C379F, V382E*); FP domain (*F797C*); HR1 domain (*A924V, S929I, A930V, D936Y, S939F, S939Y, S940F*); HR2 domain (*L1203F*). From our study we may assume that SARS-CoV-2 favours mutations in HR1 and FP to become more virulent by making the mechanism of host cell membrane fusion and entry to host cell more improved.

In case of N protein, we observe substitutions in both the NTD and CTD domains (~30% locations). We observe few substitutions in NTD and CTD domain are related to charged amino acids. This may leads to issues in RNA packaging in the virus [61]. Although the molecular mechanism of SARS-CoV-2 N protein is yet to explore thoroughly [62]. There are total of twenty (25) deleterious mutations are observed, 13 in CTD domain and 12 in NTD domain (*Supplementary-A (Table S3)*).

### 3.6. Comparison of real vs. simulated mutations

Various simulation tools are developed assuming certain probabilistic distributions followed during genetic mutations. It may be interesting to verify whether mutations in SARS-CoV-2 follow similar distributions or not. We use very recently developed mutation simulation tool [60] which generates fine-grained simulated random mutations in any genome. We consider reference sequence (*Wuhan-Hu-1*) and mutation rate (per base) (Table 4) and use ARGS mode as defined in [60] to generate simulated sequences.
Table 3: Quantification of non-synonymous amino acid substitutions observed in different functional domains of S and N protein.

| Protein Domain | Substitution (freq) | # mutated position | Domain (% | Overall (%) |
|----------------|---------------------|--------------------|-----------|-------------|
| S RBD          | Q314K(1), Q314L(1), Q341R(1), T342L(1), T323H(2), P348S(1), T345S(1), A348S(1), A348T(1), N345K(1), V367F(4), C379F(1), V382L(1), P384L(1), T387P(1), R408I(1), L441I(1), R457K(1), E471Q(1), G476S(2), S477N(1), V483A(2), V483F(1), G485R(2), S494P(1), N501Y(1), H519Q(2), A520S(2), A522S(1), A522V(1), K529E(1) | 25 | 12.55 | 10.72 |
| HR1            | L922F(1), A924V(1), S929I(1), A930V(1), I931V(1), D936Y(1), S939F(2), S939Y(1), S940F(1), L981F(1) | 9 | 12.85 | 3.44 |
| HR2            | D1163G(1), D1168H(1), V1176F(1), K1181R(1), N1187K(1), N1187Y(1), K1191N(2), N1192T(1), E1195Q(1), Q1201K(1), L1203F(1), K1205N(1) | 11 | 22.0 | 4.21 |
| FP             | Y789D(1), T791I(5), K795Q(1), F797C(1) | 4 | 22.22 | 1.53 |
| TM             | G1219C(1), G1219V(1), V1228L(2), V1230L(1), M1237I(1), M1237T(1) | 4 | 22.2 | 1.53 |
| N NTD          | P46S(1), G60R(1), E62V(2), D63N(1), P67T(1), P67S(1), Q70H(1), S79T(1), D81Y(1), Q83R(1), R89I(1), A90S(1), R92S(1), R95L(1), G97S(1), S105N(1), A119V(2), A119S(1), G120R(1), P122L(1), A125T(1), D128Y(1), D128H(1), H145Y(1), I146F(1), P151L(1), P151S(1), A152S(3), N154Y(1), A155V(1), A156S(3), I157T(1), Q163K(1), Q163R(1), T165I(1), T166I(1), L167F(1), P168Q(1), K169R(1) | 40 | 29.6 | 23.95 |
| CTD            | T247I(1), T247A(1), K249R(1), S250F(1), A252S(1), S255F(1), S255A(1), Q260L(1), Q260E(1), V270L(2), T271E(2), T282L(2), G284E(1), Q289H(1), Q294L(1), Q294R(1), V295L(2), V295Q(1), K298E(1), V298L(2), V298Q(1), K299Q(1), Q303L(1), Q303R(1), V307F(1), V307L(1), V307S(1), Q312L(1), Q312R(1), L314F(1), L314I(1), L314V(1), V315L(1), V315S(1), W316L(1), W316S(1), W316F(1), W316E(1), W316Y(1), W316C(1), W316Q(1), K317F(1), D340C(1), K342N(1), P344S(1), D348Y(4), D348H(1), K361H(1), T362I(2) | 37 | 31.89 | 22.15 |

The mutation rate is the number of mutations per genetic unit at (gene, base) per unit time (year, million years, or generation) \[63\]. We calculate mutation rate (\(\mu\)) based on number of mutations and distinct variants for each structural protein, which is calculated as follows:\[3\]

\[
\mu = \frac{m}{t},
\]

where, \(m\) is the total number of mutations in \(k\) number of variants each of length \(l\). In case of per base and per gene mutation rate, \(t = k \times l\), and \(t = k\), respectively. The per base mutation rate is relatively high for \(E\) protein followed by \(M\), \(N\), \(S\), whereas mutation rate per gene is high for \(S\) and \(N\) genes (Table 4).

Table 4: The mutation rate (per base and per gene) in four SARS-CoV-2 structural proteins. For calculating mutation rate, we considered mutation (non-synonymous substitutions) observed in all the variants of respective protein.

| Gene | # variant | # mutation | Length (nucleotide) | Mutation rate (per base) | Mutation rate (per gene) |
|------|-----------|------------|--------------------|--------------------------|--------------------------|
| E    | 23        | 23         | 228                | 0.004386                 | 1.000195                 |
| M    | 50        | 45         | 669                | 0.000195                 | 1.000195                 |
| S    | 69        | 367        | 1200               | 0.001147                 | 1.444882                 |

A total of five set of distinct simulated sequences (with non-synonymous mutations) are generated. Each set is consisting of equal number of sequences as we have real and distinct mutated sequences (or variant) for each structural protein of SARS-CoV-2 (Table 4).

We compare two sets by the Jaccard index, also known as the Jaccard similarity coefficient \[64\]. It is used to calculate similarity and diversity of sample sets. Given two sets of real and simulated mutations/substitutions, \(R_m\) and \(S_m\), respectively, it can be calculated as follows:

\[
J(R_m, S_m) = \frac{|R_m \cap S_m|}{|R_m \cup S_m|} = \frac{|R_m \cap S_m|}{|R_m| + |S_m| - |R_m \cap S_m|}.
\]

\[3\]http://www.biology.arizona.edu/evolution/working/act/mutation/population.html
The value of \( J(R_m, S_m) \) lies between 0 and 1, i.e. \( 0 \leq J(R_m, S_m) \leq 1 \). The higher value of \( J(R_m, S_m) \) indicates more similarities between the sample sets.

We report in Figure 7 the similarity scores for each protein. It can be observed that the Jaccard similarity for Nucleocapsid (N) and Spike (S) proteins substitutions is approximately 0.6 and 0.5, respectively. However, Envelope (E) and Membrane (M) proteins show low similarity. The low similarity score further reflects that the mutations are non-uniform (as ARG5 mode assumes uniform distribution) i.e. probability of mutations is not same in all positions.

![Comparison of real and simulated substitutions. The Jaccard similarity score are shown for six set of compared simulated substitutions (sim1,sim2,...,sim5 and sim1-5) with the real substitution set.](image)

4. Conclusion

In this work, we performed a sequence analysis on the structural proteins of SARS-CoV-2, reported from different parts of the world. All the unique sequences are grouped, and the variations are analyzed based on Wuhan-Hu-1 SARS-CoV-2 sequence (as reference). We highlighted various commonly and rarely occurring amino acids substitution in four structural proteins. We reported location-wise amino acid substitution patterns in the structural proteins. The change in biochemical groupings as a result of substitutions are also reported for the candidate variants. We even highlighted the above variations specific to any particular country.

Although we observed much more unique variants in S protein than N protein, in terms of substitutions changes, N protein is much vulnerable as it shows the substitution in 40% positions. The majority of substitutions type for all four proteins are observed distinct, and they occur mostly in a particular location. The substitution type \( A > V \) is very common for S, N, and M protein. We have highlighted few dominant substitutions that are co-occurred with several substitutions, particularly in S and N proteins. We further noted that the majority of the substitutions lead chemical group change from Aromatic and Aliphatic, Aliphatic and Hydroxyl-containing, and from Hydrophilic to Hydrophobic. A few substitutions in functional domains of S and N proteins are also highlighted found to be deleterious.

The overall study summarizes diversity amongst viruses sequenced early in the pandemic. We believe that the current findings will help better understand the disease pathogenesis of COVID-19 followed by suitable and relatively stable small molecule identification that may bind susceptible structural proteins like Spike (S) protein that is frequently changing.
Author contributions

J.K.D. and S.R. conceived and designed the research. J.K.D. collected, performed and analyzed the data. J.K.D. wrote the original manuscript. S.R. reviewed and edited. All authors read and approved the final manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

Data availability statement

All the relevant data are presented withing the supplementary files.

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