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Research Article

Conserved SARS-CoV-2 Viral Peptides as Potential Prophylactic and Therapeutic Targets

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ABSTRACT

The ongoing evolution of SARS-CoV-2, especially the emergence of heavily mutated variants like Omicron and its sub-lineages, has resulted in antigenic drift that diminishes the effectiveness of current first-generation vaccines, diagnostic tests, and treatments. This study employed a comprehensive immuno-informatics approach to identify highly conserved protein sequences from SARS-CoV-2 isolates reported in India. 1,33,154 complete protein sequences retrieved from the GISAID database between September 2021 and March 2023 were analysed. The analysis revealed a total of 62,94,995 mutations, which include 66,861 unique mutations. Sequences comprising at least eight consecutive amino acids with mutation frequencies below 0.1% were considered conserved regions. This analysis identified 270 conserved sequences across both structural and non-structural proteins. Of these, 73 sequences were found to be antigenic and non-allergenic and were mapped onto their respective crystal structure of proteins to evaluate their functional relevance. Many conserved sequences overlapped with the known functionally significant epitopes conserved across SARS-CoV-2 variants, underscoring their importance. The identified conserved sequences offer valuable targets for developing variant-resilient peptide-based diagnostics, monoclonal antibody therapeutics, and multi-epitope peptide vaccines. This study provides a curated collection of conserved SARS-CoV-2 protein regions identified from Indian clinical isolates and emphasises their potential for diagnostic and therapeutic applications. These findings may contribute to developing universal, variant-proof strategies for SARS-CoV-2 detection, prevention, and treatment.

1. Introduction

The rapid emergence of SARS-CoV-2 variants has challenged the efficacy of first-generation vaccines. For instance, the heavily mutated Omicron variant evades many neutralising antibodies from prior infection or vaccination [1]. This immune escape has led to breakthrough infections and prompted the rollout of variant-updated booster vaccines (e.g. bivalent BA.4/5 and monovalent XBB.1.5 boosters) to restore protection [2]. Emerging COVID-19 vaccines increasingly focus on conserved viral epitopes to achieve broader protection. Strategies include multi-epitope vaccines incorporating conserved B- and T-cell epitopes across the SARS-CoV-2 proteome [3].

The SARS-CoV-2 genome is approximately 30,000 nucleotides long and encodes 29 proteins, with four structural proteins, 16 non-structural proteins (NSPs), and nine accessory proteins [4]. Recent studies revealed that including peptides from internal proteins like nucleocapsid (N) and membrane (M) alongside Spike (S) elicit robust $CD4^+$ and $CD8^+$ T-cell responses to complement neutralising antibodies. These peptides are also reported to induce potent and long-lasting B- and T-cell immunity in clinical trials, suggesting that they confer broad protection against diverse variants [5–8]. T-cell-mediated immunity has proven more resilient to viral variation; studies show that most T-cell epitopes remain unchanged across variants [9]. Notably, >95% of $CD8^+$ T-cell epitopes identified in the original strain are still conserved in Omicron [1]. This preservation of conserved peptide targets helps maintain T-cell recognition and protection against severe disease despite the antigenic drift of SARS-CoV-2 [1]. These reports underscore why next-generation vaccines are pivoting to include conserved elements to confer immunity that endures against current and future variants [9].

Computational immunology has been instrumental in pinpointing conserved SARS-CoV-2 peptide targets. Immuno-informatics leverages the growing genomic data from SARS-CoV-2 variants and related coronaviruses to map regions of high sequence conservation. These conserved epitopes are often functionally important; mutation-intolerant regions are attractive targets for the broad-spectrum vaccine and serve as potential regions for the design of diagnostic and therapeutic approaches. In silico tools also predict the binding of these epitopes to the common human MHC alleles, ensuring broad population coverage [9]. By integrating sequence alignment, structural modelling, and epitope prediction algorithms, researchers can efficiently prioritise viral peptides that remain conserved across variants for inclusion in universal vaccine candidates. This computational screening has unveiled key immunodominant regions likely to induce cross-protective immunity, guiding experimental vaccine design and speeding the development of variant-proof immunotherapies. [10].

Many studies in silico were on the spike protein, and a small subset of SARS-CoV-2 proteins [8]. The primary objective of this study is to identify conserved and immunogenic regions across the SARS-CoV-2 genome using an immunoinformatic approach. The secondary aim of this study is to determine the allergenicity of the conserved epitopes to screen out the best possible, reliable candidates for broad-spectrum vaccines and monoclonal antibody therapy targets.

2. Materials and Methods

2.1. Identification of conserved regions in the SARS-CoV-2 genome

One lakh thirty-three thousand one hundred and fifty-four (1,33,154) SARS-CoV-2 genome sequences from Indian patients deposited from 1st September 2021 to 31st March 2023 were collected from the GISAID database. The total number of mutations was obtained from analysing data through the R package. R libraries like seqinR (4.2.8) (29), protr (1.6.2) (30) and Biostrings (2.58.0) (31) were used to analyse this data. Regions with a minimum of 8 amino acid length that showed no mutation were considered conserved sequences. The reference genome was obtained from the NCBI accession number: NC_045512.2. The frequency of the mutations in any given position was also calculated. Nearly every single position had a mutation, and no conserved regions could be found. To account for that, we then assumed that mutations with less than 0.1% of the maximum frequency of a protein were rare in the population or sequencing errors.

The frequency of mutations for every single position (for each protein) is calculated, and the maximum frequency is identified. 0.1% of this maximum frequency was set as the cut-off, and everything below this was assumed to be zero. The presence or absence of a mutation at any given position was set to binary values, 1 (presence of mutation) and 0 (absence of mutation). Regions with more than eight consecutive zeroes were identified, and their corresponding protein sequence was determined (Figure 1).

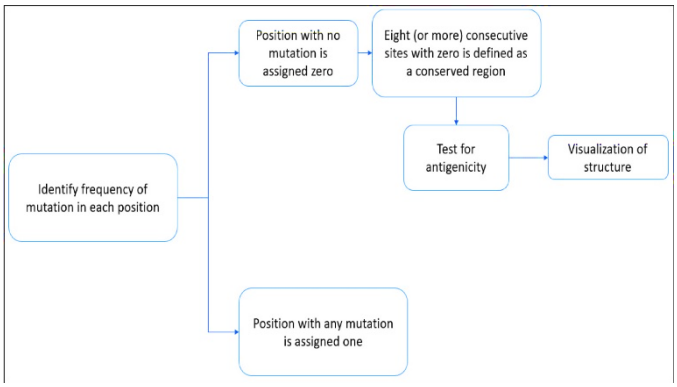


Fig 1. Graphical representation of the analysis workflow

2.2. Immunogenicity and protein modelling

Antigenicity and allergenicity of the identified conserved regions were determined as follows. VaxiJen tool was used to identify immunoprotective sequences based on identifying such sequences from bacterial, viral, tumour, fungi and parasite antigens and calculating their auto-cross covariance values [11]. The threshold for viral models was 0.4 (default value); sequences that scored equal to or below 0.4 were considered non-antigenic, and those that scored above were considered antigenic. The Immune Epitope Database (IEDB) tools were used to find possible T-cell and B-cell epitopes in conserved protein sequences. From the input sequence, it identifies the string most likely to have immunogenicity and assigns a score of likelihood [12]. The NetCTL 1.2 tool was used to identify CTL epitopes in the amino acid sequences of SARS-CoV-2. It supports 12 MHC class 1 supertypes [13]. The allergenicity of the amino acid sequences was identified using the AllerTOP tool. Only those sequences with no allergenic effects were used for further analysis [14]. The conserved antigenic and non-allergenic epitopes were then mapped onto the respective crystal structure of the proteins as follows. Experimentally determined structures of non-structural proteins (NSPs) and structural proteins of SARS-CoV-2 were retrieved from the RSCB Protein Data Bank (PDB) [15]. The structure of conserved peptide sequences was visualised in PyMOL v2.4.1 [16]. The structures of NSP-4 and NSP-6 were predicted using the Alphafold2 v2.1.1, which uses a machine learning approach for modelling even without homologous structures [17]. The best model was selected from the five predicted structures based on the predicted local distance difference test (PLDDT) score. Further, the model was evaluated using the PROCHECK [18] and ERRAT [19] modules of the SAVES v6.0 server to validate the stereochemistry and overall quality factors. The loop regions of the predicted models were refined using the ModLoop server [18] and validated using the SAVES server. Modelled structures were analysed using PyMOL.

3. Results and Discussion

The analysis revealed 62,94,995 total mutations, including 66,861 unique mutations across the SARS-CoV-2 genome in the Indian strains (Table 1). When data was analysed directly, no conserved regions were detected. Then, an algorithm was employed, assuming that the mutations below a certain "level" would be zero. Several trials were conducted, with cut-off numbers, namely "below 5 (whole number)", "below 10 (whole number)", "0.1% of highest frequency", and "1% of highest frequency" across multiple clades and lineages during the preliminary stage.

Table 1. Number of conserved, antigenic and non-allergenic sequences in each of the SARS-CoV-2 proteins.

S.No	Protein	Length of the protein (aa)	No. of conserved sequences	No. of antigenic and non-allergenic sequences
1	Spike	1273	49	11
2	Envelope	75	2	1
3	Membrane	222	7	4
4	Nucleocapsid	419	14	2
5	NSP-1	180	7	2
6	NSP-2	638	0	0
7	NSP-3	1945	59	21
8	NSP-4	500	20	6
9	NSP-5	306	11	2
10	NSP-6	290	9	4
11	NSP-7	83	0	0
12	NSP-8	198	1	0
13	NSP-9	113	0	0
14	NSP-10	139	0	0
15	NSP-12	932	31	6
16	NSP-13	601	24	6
17	NSP-14	527	22	6
18	NSP-15	346	14	2
19	NSP-16	298	0	0

It was observed that "0.1% of highest frequency" gave a reasonable number of conserved sequences. Therefore, the algorithm used was that all sequences comprising at least eight consecutive amino acids with mutation frequencies above 0.1% were classified as conserved regions in this study (Supplementary Table 1).

SARS-CoV-2 viral proteins were then analysed for conserved sequences to identify areas of potential clinical significance. The analysis focused on identifying highly conserved regions across different variants to ensure broad applicability. A total of 270 conserved sequences were identified throughout the SARS-CoV-2 genome (Supplementary Table 1). These sequences were then assessed for their antigenicity and allergenic properties. Among these, 73 antigenic and non-allergenic residues were identified throughout the proteome (Table 2, Supplementary Table 1, Supplementary Table 2). These findings provide insights into the stability and evolutionary conservation, suggesting their probable crucial role in developing robust detection assays with targeted therapeutic and preventive interventions.

In the present study, conserved peptide regions are identified from the target proteins involved in the viral RNA synthesis and replication, viral invasion via structural proteins, and act as virulence effectors. The conserved, antigenic, and non-allergenic residues identified in each SARS-CoV-2 protein were subsequently mapped onto corresponding crystal structures and predicted homology models. The functional significance of each residue is detailed below.

Table 2. Number of mutations and unique mutations observed across the SARS-CoV-2 genome

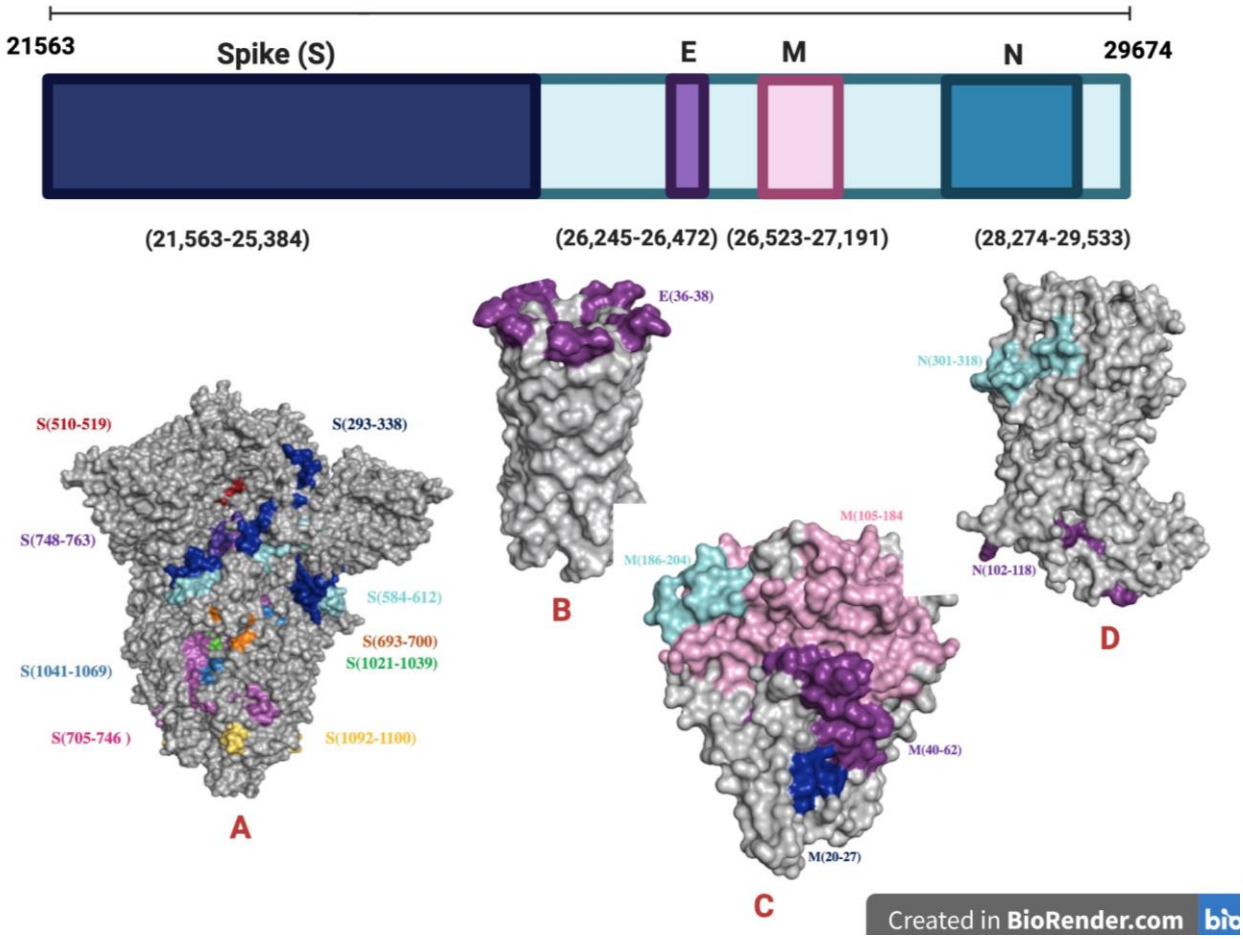
SNo	Protein	Length (AA)	Total No. of mutations	No. of Unique mutations	AA Position with most mutations and the mutation	Frequency of highest mutation
1	Spike	1273	2875012	12929	D614G	131707
2	Envelope	75	108563	481	T9I	87662
3	Membrane	222	237591	1801	A63T	88148
4	Nucleocapsid	419	679718	3811	P13L	86041
5	NSP1	180	94446	1177	S135R	76750
6	NSP2	638	45316	4556	P129L	1581
7	NSP3	1945	458711	18134	G489S	79123
8	NSP4	500	428822	4556	T492I	119745
9	NSP5	306	117695	2131	P132H	89191
10	NSP6	290	262791	2080	G107del	57094
11	NSP7	83	1625	317	M75I	74
12	NSP8	198	20068	926	N118S	12832
13	NSP9	113	3826	380	T35I	375
14	NSP10	139	6023	610	M122V	1209
15	NSP12	932	224130	5698	P323L	129979
16	NSP13	601	168140	3740	R392C	80369
17	NSP14	527	153419	3371	I42V	86910
18	NSP15	346	92205	2562	T112I	73896
19	NSP16	298	11520	2092	K160R	800

3.1. Spike Protein:

Our analysis revealed that the Spike (S) protein, which is 1273 amino acids long, contains 49 conserved regions (Table 2, Supplementary Table 1). Of these, 11 were found to be antigenic and non-allergenic peptides (Table 2, Supplementary Table 1, Figure 2A). Identifying conserved regions within the S protein is pivotal for developing broad-spectrum antivirals and vaccines. Despite the high mutation rate observed in various S protein domains, certain regions remain highly conserved, underscoring their functional importance. The N-terminal conserved domain (NTD) peptide (293–338) is implicated in host-cell recognition and has been identified as a target for neutralising antibodies [19]. Mutations in this region can contribute to immune evasion; however, conserved epitopes within the NTD may serve as viable targets for vaccine development [19].

The S protein's Receptor Binding Domain (RBD) is critical for binding to the ACE2 receptor, facilitating viral entry into host cells [20]. While the RBD is subject to frequent mutations, specific conserved sequences within this domain are essential for maintaining its structural integrity and function. One of the identified conserved peptides is mapped (510–519) in the RBD. Also, previous study has found peptide (510–519) as a potential target for binding the S2P26 antiviral peptide and can restrict from forming interaction with ACE2 receptor [21]. Notably, the N501Y mutation has strengthened the binding affinity between

Fig 2. Mapping of immunogenic conserved peptide residues onto SARS-CoV-2 structural protein homology models.
Antigenic and non-allergenic residues identified were mapped onto Spike (A), Envelope (B), Membrane (C), and Nucleocapsid (D) homology models. Residues mapped on the surface of the homology models are depicted



the spike protein and ACE2 receptor, enhancing viral infectivity [20]. The H519N mutation significantly decreases SARS-CoV-2 replication in human lung epithelial cells and reduces infectivity in pseudotyped virus [22]. Targeting these conserved regions could lead to the development of broad-spectrum neutralising antibodies [23]. The fusion peptide of the spike (S) protein plays a critical role in mediating the fusion between the viral and host cell membranes, an essential step in viral entry [24]. The high conservation of fusion peptide sequences across various coronavirus strains suggests their potential as universal vaccine targets. Peptide-based fusion inhibitors derived from these conserved sequences have demonstrated efficacy against emerging coronaviruses [25]. We have identified conserved regions (693–700, 705–746, 748–763) in this region, suggesting these peptides' functional significance. S protein's heptad repeat (HR) regions are integral to the conformational changes required for membrane fusion and viral entry [26]. Mimetic proteins structurally imitating the HR1 region in a trimeric coiled-coil conformation showed potential in inhibiting SARS-CoV-2 infection *in vitro* [27]. Our analysis revealed three conserved peptides (1021–1039, 1041–1069, 1092–1100) in this region. Targeting these conserved regions within the S protein is a strategic approach to developing effective therapeutics and vaccines across multiple coronavirus strains. Focusing on these regions makes it possible to design interventions that maintain efficacy even as the virus undergoes mutations in other regions.

3..2. Envelope Protein:

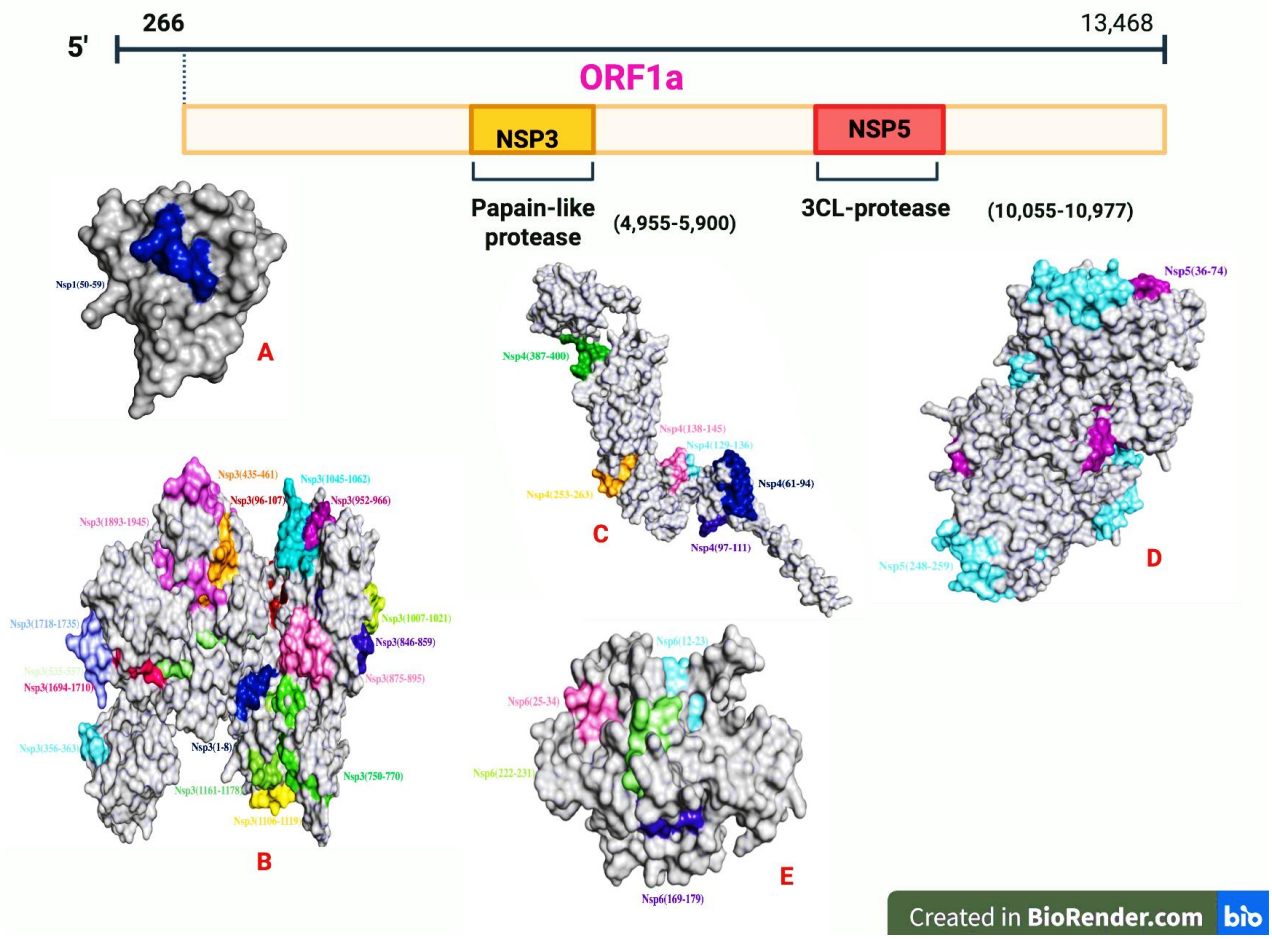
The SARS-CoV-2 envelope (E) protein, comprising 75 amino acids, plays a crucial role in virus assembly, budding, and pathogenesis. The conserved antigenic and non-allergenic sequence (residues 36–48) is located within the hydrophobic transmembrane domain (TMD) of the E protein, which is essential for anchoring it to the viral envelope (Figure 2B, Table 2) [28]. Structural studies have shown that the E protein's TMD forms a pentameric helical bundle, creating a cation-selective ion channel critical for viral pathogenicity, highlighting its role as a potential target for antiviral drugs [28,29]. This underscores the TMD's potential as a therapeutic intervention target for hindering virus assembly and release. Targeting this region could lead to the design of inhibitors that disrupt E protein function, thereby mitigating SARS-CoV-2 infectivity and pathogenicity.

3.3. Membrane Protein:

The SARS-CoV-2 membrane (M) protein, comprising 222 amino acids, plays a vital role in viral assembly, morphogenesis, and pathogenesis [30]. It is the most abundant structural component of the virus and interacts with other essential proteins, such as S and E proteins, to regulate viral budding and genome encapsulation [30]. Four of the seven conserved regions observed in the current study were found to be antigenic and non-allergenic (Figure 2C, Table 2). The transmembrane location of the conserved region (residues 20-27) could contribute to its anchoring within the viral envelope and is essential for membrane curvature during viral budding [31].

Fig 3. Mapping of immunogenic conserved peptide residues onto SARS-CoV-2 non-structural protein (located on the Orf1-a of the SARS-CoV-2 genome) homology models.

Antigenic and non-allergenic residues identified were mapped onto NSP-1 (A), NSP-3 (B), NSP-4 (C), NSP-5 (D), and NSP-6 (E) homology models. Residues mapped on the surface of the homology models are depicted



Hydrophobic interactions within this region facilitate lipid bilayer integration, ensuring the stability of the viral structure [32]. Another conserved region (residues 40–62) is known to be critical for protein-protein interactions involved in viral assembly [30]. Studies suggest that mutations in this domain disrupt virion formation, producing non-infectious viral particles [33].

The conserved region (residues 105–184) is known to interact with the nucleocapsid (N) protein, which plays a key role in encapsulating the viral RNA genome [33]. Disrupting this region has been shown to impair viral replication and assembly, making it a key target for antiviral drug development [33]. The host-cell interaction domain region (residues 186–206) plays a fundamental role in binding to host cell membranes and is involved in the budding process of new virions [33]. Computational docking studies suggest that peptides mimicking this domain could act as viral assembly inhibitors, preventing new virions from exiting the host cell [31]. Given its role in viral structure and function, the M protein is a key therapeutic intervention target. Conserved sequences within the M protein offer promising opportunities for developing antiviral agents that can disrupt viral assembly and budding [31]. In addition, targeting the M protein interactions with S and E proteins could prevent the formation of fully functional virions, reducing viral spread within infected individuals [34]. Moreover, the immunogenic properties of the M protein suggest its potential use in vaccine formulations. The conserved nature of the M protein ensures broad-spectrum vaccine effectiveness, even against emerging SARS-CoV-2 variants.

3.4. Nucleocapsid Protein:

The nucleocapsid (N) protein of SARS-CoV-2, a crucial structural component, is involved in RNA binding, genome packaging, and viral replication [35]. Comprising 419 amino acids, the N protein exhibited 14 conserved regions, with two areas being antigenic and non-allergenic, underscoring its evolutionary stability across different variants. This conservation suggests a crucial functional significance, making the N protein a prime target for diagnostic and therapeutic applications. Both the conserved antigenic residues (residues 102–118 and residues 301–318) (Figure 2D, Table 2) are particularly noteworthy due to their potential involvement in RNA binding and protein-protein interactions [36]. The first conserved peptide sequence (residues 102–118) falls within a highly structured region, which may contribute to the protein's ability to interact with viral and host components [36]. Similarly, the second conserved peptide sequence (residues 301–318) is positioned within a flexible domain that may facilitate dynamic conformational changes essential for nucleocapsid function [36,37].

The high conservation of these regions across SARS-CoV-2 variants suggests their indispensable role in viral assembly and propagation. The N protein is highly immunogenic so that these

conserved sequences may serve as potential epitopes for monoclonal antibody development and vaccine design [38]. Additionally, the involvement of the identified conserved sequences in viral RNA replication processes suggests that targeting them with small molecules or peptide inhibitors could disrupt viral replication, offering a promising therapeutic avenue [39]. The N protein remains a primary target for rapid antigen detection tests in diagnostic applications due to its abundance during infection. Monoclonal antibodies directed against the identified conserved regions could enhance the sensitivity and specificity of lateral flow assays, aiding in early and reliable SARS-CoV-2 detection [40]. In conclusion, the remarkable conservation of these two regions within the SARS-CoV-2 N protein highlights their functional importance in viral pathogenesis. Future studies should focus on elucidating their structural and functional roles in greater detail, paving the way for improved antiviral strategies and diagnostic tools.

3.5. Non-structural protein 1:

NSP-1 is a key virulence factor in suppressing host immune responses and facilitating viral replication [41]. Of the seven conserved regions in the NSP-1, two peptides were found to be antigenic and non-allergenic. One of the peptides (residues 50–59) was mapped onto the surface of the NSP-1 (Figure 3A, Table 2). This region (residues 50–59) within the N-terminal domain (NTD) of SARS-CoV-2 NSP-1 may play a role in host immune suppression and viral replication. Structurally, this region forms part of the β2–β3 loop, contributing to the electrostatic surface properties of the NTD and stabilising its interactions with host factors [42]. The acidic patch formed by residues E55 and E57 is located adjacent to the α1-helix and may play a role in the selective binding of viral RNA, while simultaneously inhibiting host mRNA translation [41]. Functionally, NSP-1 inhibits host gene expression by blocking translation and suppressing mRNA transport [43]. Mutational studies indicated that alterations in the 50–59 loop impair NSP-1's ability to suppress host defences, leading to attenuation of viral replication [44]. Deletion or mutation of residues in this region has been linked to reduced cytotoxicity and loss of host shutoff function, suggesting its importance in NSP-1's virulence mechanism [45]. Additionally, attenuated viral strains with targeted mutations in this region may serve as candidates for live-attenuated vaccine development [44]. This conserved region presents a promising therapeutic target for antiviral strategies and vaccine design by enabling immune evasion and viral replication.

3.6. Non-structural protein 3:

The NSP-3 of SARS-CoV-2 is reported to play a role in viral replication and the modulation of host immune responses [46]. In our study, NSP-3 is the protein identified with the maximum number of conserved residues with antigenic significance (N=59, N=21, respectively) (Table 2, Figure 3B). Other researchers analysed several key amino acid sequences within NSP-3 for their structural and functional significance in viral pathogenesis [47]. The N-terminal region, consisting of residues 1–8, plays a crucial role in the early stages of viral replication and is also associated with ubiquitin-like domain 1. This domain has been implicated in protein-protein interactions that may modulate host immune responses [48]. Similarly, residues 96–107 are located within the SARS-Unique Domain (SUD), a region unique to SARS-related coronaviruses, which interacts with host cell proteins and may influence viral replication efficiency [46]. The papain-like protease (PLPro) domain, containing residues 356–363, plays a pivotal role in cleaving the viral polyprotein and has de-ubiquitinating functions that help SARS-CoV-2 evade immune detection [49]. Structural studies have highlighted the significance of PLPro in counteracting host antiviral responses, making it a key therapeutic target [50].

Additionally, the macrodomain 1 of NSP-3, which includes residues 435–461, has been identified as essential in interfering with the host's ADP-ribosylation signalling. This domain is vital for viral replication and immune evasion, with structural analyses emphasising its potential as a drug target [51]. The transmembrane regions of NSP-3, represented by residues 535–557 and 846–859, contribute to double-membrane vesicles (DMVs), which provide a protected niche for viral RNA synthesis [52]. These domains interact with NSP-4 and NSP-6, highlighting their role in viral replication complex formation. The SUD contains multiple functionally relevant sequences, residues 740–748 and 750–770, which are implicated in host-pathogen interactions and may influence the efficiency of viral replication [53]. Studies suggest that SUD enhances viral pathogenicity through interactions with cellular proteins, further supporting its significance in SARS-CoV-2 infection dynamics. Further downstream, residues 1844–1866 and 1893–1945 are located in regions that may contribute to NSP-3's overall structural stability and interactions with other viral and host components[46]. Their functional implications warrant further investigation, particularly in viral replication and immune evasion mechanisms. The comprehensive analysis of these NSP-3 sequences provides valuable insights into their functional roles in SARS-CoV-2 pathogenesis. Given their involvement in viral replication, immune modulation, and host interactions, these sequences are potential targets for antiviral drug development. Further structural and biochemical studies must elucidate their full mechanistic contributions and therapeutic potential.

3.7. Non-structural protein 4:

SARS-CoV-2 non-structural protein 4 (NSP-4) is crucial for membrane remodelling during viral replication, interacting with NSP-3 to promote the formation of double-membrane vesicles (DMVs) [54,55]. Our analysis revealed 20 conserved residues, with 6 having antigenic potential (Table 2, Figure 3C). These six conserved motifs in NSP-4, including residues 61–94, 97–111, 129–136, 138–145, 253–263, 387–400, have been shown to possess functional importance [56]. Structurally, these conserved sequences map to key domains, including luminal loops involved in membrane curvature (61–145, 253–263), transmembrane helices forming the DMV-spanning pore (387–400), and the cytosolic C-terminal tail critical for replication complex assembly (493–500) [57,58]. Notably, N-glycosylation at Asn131 and multiple disulfide bonds within luminal loops suggest an additional layer of regulation influencing NSP-4 stability and interaction with NSP-3 [59]. Despite NSP-4's high conservation, the T492I mutation, which emerged in Delta and Omicron variants, enhanced viral replication by increasing 3CLpro processing efficiency [60]. This highlights how even minor mutations in NSP-4 can impact viral fitness. Given its critical role in DMV formation, NSP-4 represents an attractive antiviral target, particularly disrupting the NSP–3–NSP–4 interface or DMV-spanning pore [61]. Future studies should explore small molecules or antibodies targeting NSP4, given its low mutation rate compared to other viral proteins, making it a viable broad-spectrum target across coronaviruses.

3.8. Non-structural protein 5:

The NSP-5 main protease (Mpro) of SARS-CoV-2 plays a pivotal role in viral replication by cleaving polyproteins (pp1a/pp1ab) into functional non-structural proteins [62]. The enzyme is highly conserved, with key sequence segments 36–74 and 248–259 demonstrating strong purifying selection across SARS-CoV-2 variants and related coronaviruses [56]. Interestingly, in our study of the 11 conserved residues identified, these two have antigenic potential (Table 2, Figure 3D). The 36–74 region contributes to the catalytic domain (domain I) and harbours His41, a critical residue forming the His41–Cys145 catalytic dyad essential for substrate cleavage and viral replication [63]. The previous study has reported anti-asthmatic drug montelukast binds with NSP-5 protein with low affinity, involving His41 forming a hydrophobic pocket to accommodate the drug molecule [64]. This segment also defines the substrate-binding pocket and interacts with known inhibitors such as Paxlovid (nirmatrelvir) [65]. The 248–259 region within domain III is crucial for dimerisation, an essential step in NSP-5 activation [66]. Disrupting this interface destabilises the enzyme, leading to a loss of function and viral replication inhibition [67].

Despite the emergence of SARS-CoV-2 variants of concern, NSP-5 remains highly conserved, with minimal mutations in its active site or dimerisation interface [68]. Mutational studies confirm that substitutions in these regions impair viral fitness, highlighting their functional importance [69]. Given the stability of these sequences, Mpro remains a prime antiviral target. Covalent inhibitors like nirmatrelvir and peptidomimetics have been designed to irreversibly bind the active site, effectively blocking viral replication [65]. Additionally, allosteric inhibitors targeting the dimerisation interface have shown promise, offering an alternative antiviral approach [70]. Given these segments' extreme conservation and functional indispensability, targeting NSP5 with direct-acting antivirals provides a robust therapeutic strategy against SARS-CoV-2 and future coronavirus outbreaks [62]. Future research should focus on dimer interface inhibitors to complement existing active-site-targeting drugs, ensuring broad-spectrum efficacy against coronaviruses.

3.9. Non-structural protein 6:

The NSP-6 of SARS-CoV-2 promotes the virus's ability to remodel host cell membranes, facilitating DMVs essential for viral RNA replication. This function is conserved across coronaviruses, underscoring the importance of specific sequences within NSP-6 [71]. We identified nine conserved residues in this protein, with 4 exhibiting antigenic potential (Table 2, Figure 3E). The sequences with residues 12–23 and residues 25–34 are located within the transmembrane domains of NSP-6, which are highly conserved among SARS-CoV-2 variants and related coronaviruses, including SARS-CoV and MERS-CoV. This conservation suggests a fundamental role in membrane association and DMV formation [72]. Similarly, the sequences with residues 169–179 and residues 222–231 are preserved across various coronavirus species, indicating their critical role in viral replication [73]. The transmembrane regions encompassing residues 12–34 are integral to NSP-6's ability to anchor to the endoplasmic reticulum membrane. This anchorage is vital for inducing autophagosome formation and subsequent DMV development in viral replication [74].

The structural conservation of these sequences ensures the integrity required for proper membrane curvature and vesicle formation. Residues 169–179 and 222–231 are implicated in protein-protein interactions within the viral replication complex, facilitating the coordination necessary for efficient RNA synthesis [73]. Mutations within these conserved regions can significantly impact NSP-6 function. For instance, alterations in the transmembrane domains may disrupt membrane association, hindering DMV formation and attenuating viral replication [75]. Targeting these conserved sequences with antiviral agents could impair NSP-6's function, offering a potential therapeutic strategy [35]. Compounds that disrupt NSP-6's membrane interactions or their role in autophagosome formation could effectively inhibit viral replication [71]. The conserved sequences within NSP-6 are integral to modifying host cell membranes for viral replication. Their preservation across coronavirus species highlights their essential role and presents opportunities for targeted antiviral interventions. Given these sequences' high conservation and functional importance, NSP-6 remains a viable antiviral target, particularly for strategies that disrupt its interactions with host cell membranes and interfere with DMV formation [35].

3.10. Non-structural protein 12:

The SARS-CoV-2 NSP-12 functions as the RNA-dependent RNA polymerase (RdRp), a crucial enzyme for viral genome replication [76]. We identified 31 conserved residues in this protein, with 6 exhibiting antigenic potential (Table 2, Figure 4A). These six conserved sequence regions (residues 186–226, 294–322, 401–462, 464–486, 672–693, and 720–738) have been shown to play essential roles in RdRp function, structural stability, and antiviral drug interactions. Residues 186–226 of NSP-12 facilitate nucleotidyl transfer reactions necessary for RNA synthesis and viral RNA capping [77]. Key residues within this region participate in ATP and GTP binding, making it an attractive antiviral target [78] Region 294–322 contains a highly conserved Cys/His-rich zinc-binding motif stabilising NSP-12 by coordinating Zn^{2+} ions [48]. Structural studies indicate that disrupting these zinc-coordinating residues impairs polymerase activity [79]. Polymerase core regions with residues 401–486, 672–693, 720–738 form key subdomains essential for RNA synthesis. The fingers domain 401–486 contributes to RNA binding and cofactor interactions, particularly with NSP-8, which enhances processivity [76]. The palm domain 672–693 contains motif B, a flexible loop that adjusts during nucleotide incorporation, facilitating efficient RNA synthesis [80].

The 720–738 region lies adjacent to the active site and stabilises the polymerase's catalytic domain [81]. The high conservation of these sequences makes them ideal targets for antiviral drugs. Remdesivir, a nucleotide analogue, binds to the polymerase active site and disrupts RNA synthesis by causing delayed chain termination [56]. Structural studies have shown that remdesivir interacts with conserved residues in the fingers and palm domains, stabilising an inactive polymerase complex [82]. The studies also revealed that remdesivir forms an interaction with the RNA-binding channel of RdRp involving Asn497, Arg569, and Asp684 to form polar contact and Leu576, Ala685, and Tyr687 to form a hydrophobic pocket [64]. This report aligns with our findings that interacting residues Asp684, Ala685, and Tyr687 belong to the predicted conserved peptide 672–693. Molnupiravir and favipiravir also exploit conserved residues to induce lethal mutagenesis [83]. These regions remain highly conserved across SARS-CoV, MERS-CoV, and other coronaviruses, indicating strong evolutionary constraints [84]. However, mutations such as P323L (interface domain) and G671S (motif B loop) have emerged in SARS-CoV-2 variants, potentially enhancing viral replication efficiency [85]. Nevertheless, remdesivir resistance mutations (e.g., F480L,

V557L) remain rare due to the functional constraints on these conserved regions [83] Overall, the conserved sequences in NSP-12 are essential for viral replication and represent key targets for developing antiviral drugs. Their limited mutational tolerance reinforces their potential as drug-binding sites, highlighting the importance of continued structural and functional studies to optimise therapeutic strategies.

3.11. Non-structural protein 13:

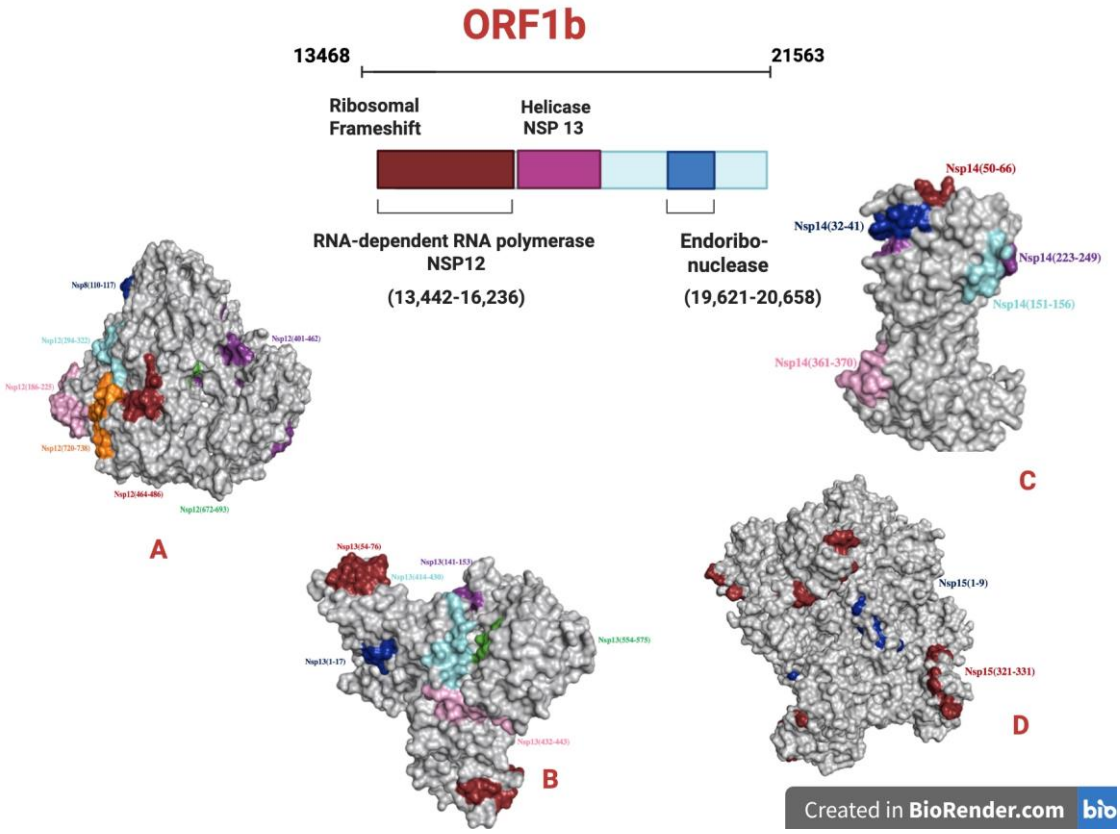
The SARS-CoV-2 NSP-13 is a highly conserved helicase that plays a critical role in viral replication by unwinding RNA and hydrolysing ATP [86]. We identified 24 conserved residues in this protein, with 6 exhibiting antigenic potential (Table 2, Figure 4B). These conserved sequences in NSP-13, including residues 1-17, 54-76, 141-153, 414-430, 432-443, and 554-575, correspond to essential functional motifs required for enzymatic activity, structural integrity, and interaction with other viral components. The zinc-binding domain (ZBD), comprising the 1–17 and 54–76 sequences, coordinates Zn^{2+} or Fe–S clusters necessary for NSP-13 stability and RNA unwinding. Studies have shown that mutations in these cysteine-rich motifs disrupt metal coordination and abolish enzymatic function [87]. The stalk domain (141–153) supports the helicase core and facilitates interactions with NSP-12 and NSP-8 in the replication-transcription complex [88]. The helicase core, including 414–430 (RecA1 domain), 432–443 (RecA2 domain), and 554–575 (motif VI), contains essential ATP-binding and RNA-binding motifs. Motif VI, particularly Arg567 within the 554–575 segment, functions as an "arginine finger," crucial for ATP hydrolysis and energy transduction [89]. Structural studies have revealed that these conserved sequences undergo conformational changes upon ATP binding, facilitating RNA unwinding [86]. NSP13 is a promising antiviral target due to its high conservation across coronaviruses. Small-molecule inhibitors such as bismuth complexes, flavonoids (myricetin, scutellarein), and SSYA10-001 have been shown to target these conserved motifs, disrupting ATPase and helicase activity [90]. Additionally, the ZBD is a druggable site, as bismuth-based compounds destabilise its zinc-finger motifs, effectively inhibiting the helicase [91]. Comparative sequence analysis across SARS-CoV, MERS-CoV, and other coronaviruses highlights the strong evolutionary conservation of these motifs, underscoring their indispensable role in viral replication, making them attractive targets for therapeutics and vaccines.

3.12. Non-structural protein 14:

The NSP-14 of SARS-CoV-2 is integral to the virus's replication fidelity and immune evasion strategies [92,93]. Our sequence analysis has identified 22 conserved regions within NSP-14, including six antigenic residues: 32–41, 50–66, 126–139, 145–156, 223–249, and 361–370 (Table 2, Figure 4C). The conservation of these sequences across various SARS-CoV-2 isolates suggests their critical role in maintaining the structural integrity and enzymatic functions of NSP-14 [94]. Notably, NSP-14 of SARS-CoV and SARS-CoV-2 share over 95% amino acid sequence similarity, underscoring their evolutionary conservation and potential as a therapeutic target [95]. The ExoN activity of NSP-

Fig 4. Mapping of immunogenic conserved peptide residues onto SARS-CoV-2 non-structural protein (located on the Orf1-b of the SARS-CoV-2 genome) homology models.

Antigenic and non-allergenic residues identified were mapped onto NSP-12 (A), NSP-13 (B), NSP-14 (C), and NSP-15 (D) homology models. Residues mapped on the surface of the homology models are depicted.



14 is essential for correcting errors during RNA synthesis, thereby reducing the mutation rate and contributing to the stability of the viral genome [96]. Additionally, the N7-MTase domain's role in mRNA capping protects viral RNA from host immune responses, facilitates efficient translation, and ensures viral proliferation [97]. The crystal structure of SARS-CoV-2 NSP-14 demonstrates that the ExoN domain's enzymatic activity is metal ion-dependent, preferably utilising Mg^{2+}, and that the N7-MTase domain harbours a conserved DxG motif for S-adenosyl-L-methionine binding, characteristic of coronaviruses [94].

3.13. Non-structural protein 15:

The SARS-CoV-2 NSP-15 is an endoribonuclease highly conserved among coronaviruses and plays a crucial role in viral RNA processing and immune evasion [98]. It cleaves viral RNA at uridine sites, preventing the accumulation of immunostimulatory dsRNA, thereby helping the virus evade host immune responses [99]. We identified 14 conserved residues in this protein, with 2 exhibiting antigenic potential (Table 2, Figure 4D). The conserved residues 1–9 and 321–336 are functionally significant as they contribute to NSP-15's hexameric structure and catalytic activity, making them potential antiviral drug targets ([100]. The peptide with residues 1-9 at the N-terminus is part of the oligomerisation domain, essential for forming the active hexameric complex [98]. Mutations in this region impair hexamerization and lead to loss of enzymatic activity, suggesting its importance in structural integrity [101] The conservation of this motif across coronaviruses highlights its critical role in viral replication and potential as a drug target [99].

The peptide with residues 321-336 in the C-terminal region is located near the active site of NSP-15 and is highly conserved across beta-coronaviruses [100]. It contributes to substrate recognition and enzymatic function, ensuring efficient RNA cleavage [100]. Structural studies show that this sequence forms part of the uridine-binding pocket, crucial for its RNA endonuclease activity [102, 103]. Mutations in this region disrupt catalytic efficiency, accumulating dsRNA and increasing host immune activation [99]. Viruses lacking a functional NSP-15 enzyme show reduced replication efficiency and are more susceptible to host immune responses. The conserved residues 1–9 and 321–336 in SARS-CoV-2 NSP-15 are structurally and functionally crucial. They contribute to enzyme oligomerisation, substrate recognition, and RNA processing, ensuring viral replication and immune evasion. Their high conservation makes them ideal targets for antiviral drug development.

4. Conclusion

Our findings provide insights into the stability and evolutionary conservation of SARS-CoV-2 protein sequences, suggesting their probable crucial role in developing robust detection assays and targeted therapeutic and preventive interventions. These conserved sequences within SARS-CoV-2 are integral for viral replication and immune evasion. Mutations within these sequences are rare due to their functional constraints, and the reports of experimental mutagenesis studies confirm that disruptions in these regions would lead to loss of viral replication efficiency, making them attractive therapeutic and preventive targets for broad-spectrum SARS-CoV-2 variants. Future research may focus on developing inhibitors that exploit these conserved sites to disrupt viral replication, effectively providing effective therapeutic and prophylactic strategies against SARS-CoV-2 and related coronaviruses.

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Declarations

Ethics approval and consent to participate: Not applicable

Availability of data and material:

The datasets used/analysed during the current study are available in the NISAID repository, https://gisaid.org

Competing interests:

The authors declare that they have no competing interests

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