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Unique truncated and non-synonymous mutations in functional domains of ORF3a SARS-CoV-2

Jeanne Elvia Christian*, Hartiyowidi Yuliawuri
Calvin Institute of Technology, Jakarta, Indonesia

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ABSTRACT

Previous studies showed that mutations in the SARS-CoV-2 ORF3a protein can influence viral pathogenesis. Therefore, it is necessary to observe mutations, especially in the functional domain of the protein. We observed the presence of mutations in the ORF3a protein by analyzing 5,131 samples from the GISAID database since it was first discovered in March 2020 until November 2021. The sequence was aligned using Clustal Omega Multiple Sequence Alignment from EMBL-EBI and analyzed using BioEdit version 7.2.5 software using reference sequences NC045512. Samples having the letter N were omitted from the analysis. The effect of point mutations on proteins was analyzed using the Protein Variation Effect Analyzer (PROVEAN) v1.1.3 software. The functional domains of the ORF3a protein were visualized using RasWin software. We identified 312 mutations in the SARS-CoV-2 ORF3a protein. In addition, from 5,131 samples, 915 samples were found to be truncated in the C-terminal region of the protein. These non-synonymous mutations data in functional domains and truncated sequences indicate that amino acid changes in the ORF3a protein require further studies to determine the effect of viral pathogenicity in humans.

ABSTRACT

Beberapa penelitian menunjukkan bahwa mutasi pada protein ORF3a SARS-CoV-2 dapat memengaruhi patogenesis virus. Oleh karena itu, pengamatan mutasi terutama pada domain fungsional protein perlu dilakukan. Kami mengamati keberadaan mutasi pada protein ORF3a dengan menganalisis 5,131 sampel dari database GISAID sejak virus SARS-CoV-2 pertama kali ditemukan pada bulan Maret 2020 hingga November 2021. Sekuens disejajarkan menggunakan *Clustal Omega Multiple Sequence Alignment from EMBL-EBI and analyzed using BioEdit version 7.2.5 software* menggunakan sekuens referensi NC045512. Sampel yang memiliki huruf N dihilangkan dari analisis. Efek mutasi titik pada protein dianalisis menggunakan *Protein Variation Effect Analyzer* (PROVEAN) v1.1.3 piranti lunak daring. Domain fungsional protein ORF3a divisualisasi menggunakan piranti lunak RasWin. Kami mengidentifikasi 312 mutasi pada protein ORF3a SARS-CoV-2. Selain itu, dari 5,131 sampel ditemukan sebanyak 915 sampel mengalami pemotongan pada daerah C-terminal protein. Data mutasi tidak identik pada domain fungsional dan sekuen terpotong ini menunjukkan bahwa perubahan asam amino pada protein ORF3a memerlukan penelitian yang lebih lanjut untuk menentukan efek patogenisitas virus pada manusia.

Keywords:
domains;
mutations;
ORF3a;
SARS-CoV-2;
truncated

INTRODUCTION

The disease caused by SARS-CoV-2 is officially termed as COVID-19. The virus has a positive sense, single-stranded RNA virus, enveloped belonging to the genus Beta coronavirus in the family of Coronaviridae. The genome consists of 4 structural proteins (spike, envelope, membrane, and nucleocapsid), 16 non-structural proteins (nsp1-16) and 7 accessory proteins.[1,2]

ORF3a is the largest accessory protein of SARS-CoV-2 and has 275 amino acids in lengths. It is only present in SARS-CoV and SARS-CoV-2, and not found

*corresponding author: jeanne.christian@calvin.ac.id

in another Beta coronavirus.[3,4] Some analysis suggest that this protein might be derived originally from the M gene in the CoV lineage.[5,6] It has transmembrane proteins of the viroporin family that form ion channels in the host membrane and inhibit IFN-**α** signaling. So, it may be implicated in inducing apoptosis and virus release.[7,8] Some researchers showed that ORF3a protein has a role in cytokine storm by up-regulating fibrinogen secretion. SARS-CoV-2 ORF3a possesses six domains and interacts with the membrane (M) and envelope (E) protein during viral assembly. The presence of ORF3a protein is essential for viral reproduction when E protein is absent. It contains a PDZ-binding motif at C-termini (amino acid 209-264) which plays a role in viral pathogenesis.[4,9] Besides that, this accessory protein was found to be important in severity of COVID-19 and had the contribution to post-COVID conditions. Its mutations may be also correlated with mutations in the spike protein and could potentially affect the function of ORF3a.[1,4,10,11]

Based on some research, the ORF3a protein is indeed not conserved.[5,12] Several amino acid changes in ORF3a protein were likely associated with the characteristic's alteration of the virus variant. Previous Variants of Concerns (VOCs) like Beta (lineage B.1.351) and Gamma (lineage P.1) had amino acid changes in Q57H and S253P respectively. While the current VOCs like Delta (lineage B.1.617.2) and Omicron (lineage BA.1, BA.1.1, BA.2) had amino acid changes in S26L and T223I respectively. The previous Variants of Interest/VOIs (lineage B.1.427/1.429, P.2, B.1.525, P.3, B.1.526, B.1.617.1, C.37, B.1.621) had amino acid changes in S26L, P42L, Q57H, and V256del.[13] The variant that correlates with the designated VOC or VOI will be reclassified continuously through the assessment of global public health significance.

From our previous study, we found non-synonymous mutations in ORF3a SARS-CoV-2 protein and we focused on the highest frequency of the mutations from 3,791 samples. Those highest mutations did not occur in the functional domain.[14,15] So, the purpose of this study is to look for other non-synonymous mutations, which were found in Indonesia samples that occur in the functional domains of ORF3a SARS-CoV-2. Furthermore, we analyze the unique truncated protein of ORF3a SARS-CoV-2. This data can contribute to enhancing our understanding about the diversity of this virus and how these mutations could affect its functional role in viral pathogenesis.

MATERIAL AND METHODS

ORF3a sequences data retrieval

From our previous study, a total of 3,751 SARS-CoV-2 Indonesia samples retrieved from GISAID from March 2nd 2020 until July, 31st 2021 were analyzed and we found 203 non-synonymous mutations. We added more data and analyzed 1,380 Indonesia samples from August, 1st 2021 until November, 30th 2021. The data were retrieved from GISAID.[16] We aligned using Clustal Omega Multiple Sequence Alignment from EMBL-EBI (www.ebi.ac.uk/Tools/msa/ clustalo/) and analyzed using BioEdit version 7.2.5 software. The reference sequence was taken from GISAID database with isolate hCoV-19/Wuhan/WIV04/2019 (EPI_ISL_4021 24). We excluded the samples containing N letters due to inaccurate reading of amino acid.

Determine the biological effect prediction of ORF3 non-synonymous mutations

We predicted the protein sequences to Protein Variation Effect Analyzer (PROVEAN) v1.1.3. software online tools.[17] The software predicts if the amino acid substitution has an impact on the biological function of a protein with -2.5 as cut off value. Above -2.5 indicates that the substitution had no effect or neutral.

Protein 3D visualization

We used RasWin v2.7.5.2 software to visualize the six domains in ORF3a SARS-CoV-2. As the model, the 6XDC in PDB format was used.

RESULT

ORF3a sequences data retrieval

We analyzed 5,131 ORF3a SARS-CoV-2 from the GISAID database. Based on data alignment, we found total 312 non-synonymous mutations, and they were scattered in all six functional domains of ORF3a protein. Domain I, III, IV, and V proteins almost had mutations in their amino acid sequences. Whereas domain II and VI proteins had mutations in all amino acid sequences. The highest total frequency of mutations was found in VI domain with 83 non-synonymous mutations (TABLE 1).

TABLE 1. Non-synonymous mutations of ORF3a SRS-CoV-2 and their effect prediction based on PROVEAN Score.

Domains of ORF3a SARS-CoV-2	ORF3a amino acid locations	ORF3a amino acids		Total Frequency of mutations	PROVEAN Score	Variation effect on protein based on PROVEAN
		Wild type	Non-synonymous mutations			
I	1	M	N/A	0	N/A	N/A
	2	D	Y	1	-8.581	Deleterious
	3	L	N/A	0	N/A	N/A
	4	F	S	1	-7.257	Deleterious
	5	M	I	1	-1.257	Neutral
			V	11	-1.581	Neutral
	6	R	N/A	0	N/A	N/A
	7	I	N/A	0	N/A	N/A
	8	F	N/A	0	N/A	N/A
	9	T	K	1	-4.276	Deleterious
	10	I	N/A	0	N/A	N/A
	11	G	N/A	0	N/A	N/A
	12	T	I	1	-0.781	Neutral
	13	V	L	10	-1.648	Neutral
			A	1	-3.914	Deleterious
	14	T	I	4	-4.61	Deleterious
			N	1	-1.286	Neutral
	15	L	F	4	-1.314	Neutral
II	36	P	T	1	-5.924	Deleterious
	37	I	T	4	-0.305	Neutral
	38	Q	H	2	-2.286	Neutral
			K	4	-2.629	Deleterious
			R	2	-2.629	Deleterious
	39	A	T	3	-0.962	Neutral
			S	1	-1.638	Neutral
	40	S	L	9	-2.971	Deleterious
			P	1	0.276	Neutral

TABLE 1. Non-synonymous mutations of ORF3a SRS-CoV-2 and their effect prediction based on PROVEAN Score (cont.)

Domains of ORF3a SARS-CoV-2	ORF3a amino acid locations	ORF3a amino acids		Total Frequency of mutations	PROVEAN Score	Variation effect on protein based on PROVEAN
		Wild type	Non-synonymous mutations			
III	91	Y	N/A	0	N/A	N/A
	93	S	N/A	0	N/A	N/A
	109	Y	N/A	0	N/A	N/A
	127	L	F	2	-1.981	Neutral
	128	W	G	1	-7.419	Deleterious
			C	1	-7.419	Deleterious
			L	1	-7.752	Deleterious
	129	L	F	10	-3.829	Deleterious
	130	C	F	1	-7.79	Deleterious
	131	W	C	36	-7.752	Deleterious
			S	2	-8.733	Deleterious
			L	2	-6.752	Deleterious
	132	K	N/A	0	N/A	N/A
	133	C	S	1	-9.81	Deleterious
IV	141	Y	N/A	0	N/A	N/A
	14	D	H	8	-6.771	Deleterious
			Y	2	-8.733	Deleterious
	143	A	S	3	0.724	Neutral
			V	1	-2.59	Deleterious
	144	N	S	8	-3.571	Deleterious
			D	1	-1.571	Neutral
	145	Y	S	1	-5.495	Deleterious
	146	F	C	1	-7.848	Deleterious
	147	L	V	1	2.943	Neutral
			F	3	-1.962	Neutral
	148	C	N/A	0	N/A	N/A
	149	W	C	7	-9.752	Deleterious
			L	1	-9.419	Deleterious
V	160	Y	N/A	0	N/A	N/A
	161	N	S	4	-3.571	Deleterious
	162	S	N/A	0	N/A	N/A
	163	V	L	1	-1.238	Neutral
VI	171	S	L	83	-2.238	Neutral
			P	1	-2.419	Neutral
	172	G	C	46	-6.752	Deleterious
			V	3	-6.762	Deleterious
			R	3	-6.114	Deleterious
			D	2	-5.133	Deleterious
	173	D	G	12	-4.867	Deleterious

Out of 5,131 samples, we found 915 samples of ORF3a SARS-CoV-2 were truncated in C-termini. The amino acid position 212-218 were mutated and from 219-275 were deleted in these samples (FIGURE 1). Furthermore, we analyzed the lineage of these truncated samples and see if those samples were included in the Variants of Concerns (VOCs) based on WHO (FIGURE 2).

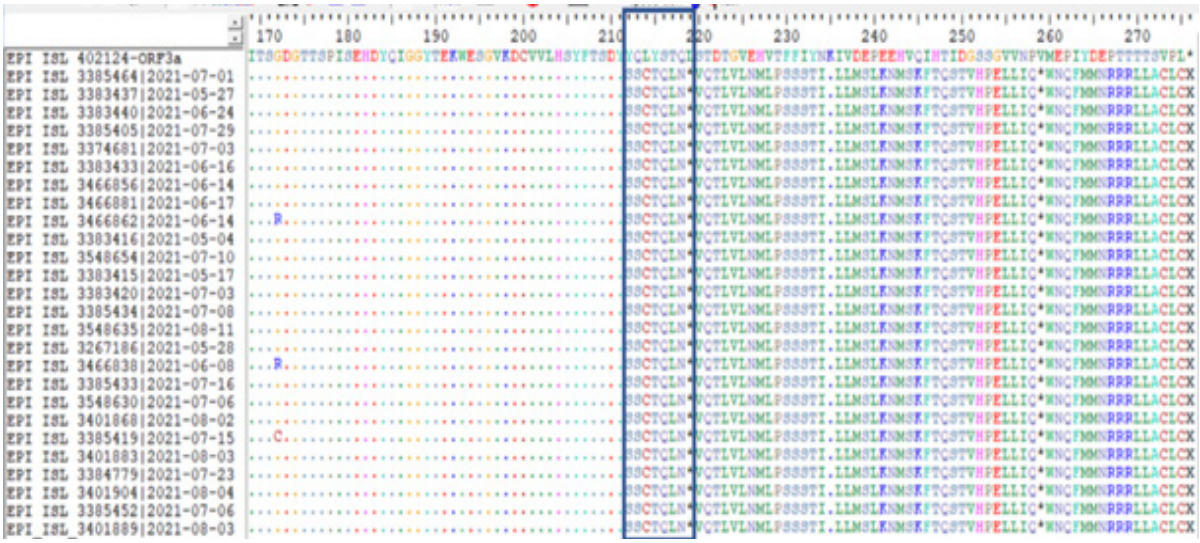

FIGURE 1. ORF3a SARS-CoV-2 truncated samples. Blue box indicates C-termini sequence was mutated and truncated protein at amino acid position 212-219. The dot indicates the similar amino acid compare with the reference sequence.

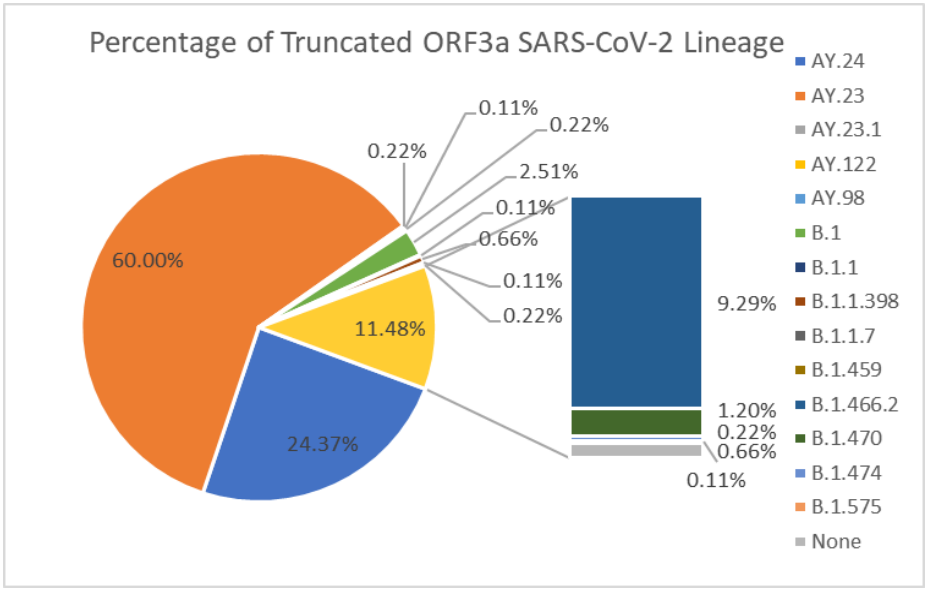

FIGURE 2. Lineage's percentage of ORF3a SARS-CoV-2 truncated samples in Indonesia.

The biological effect prediction of ORF3 non-synonymous mutations

Based on PROVEAN prediction, the non-synonymous mutations that found in six functional domains of ORF3a protein had neutral or deleterious effects. There were 32 deleterious effects and 19 neutral effects in six functional domains (TABLE 1).

Protein 3D visualization

The 3D visualization highlights the location of six functional domains in ORF3a proteins. Domain II, III, IV are located in alpha helices, while domain I and VI are located in loops and turns. The domain V could not be shown in this visualization (FIGURE 3).

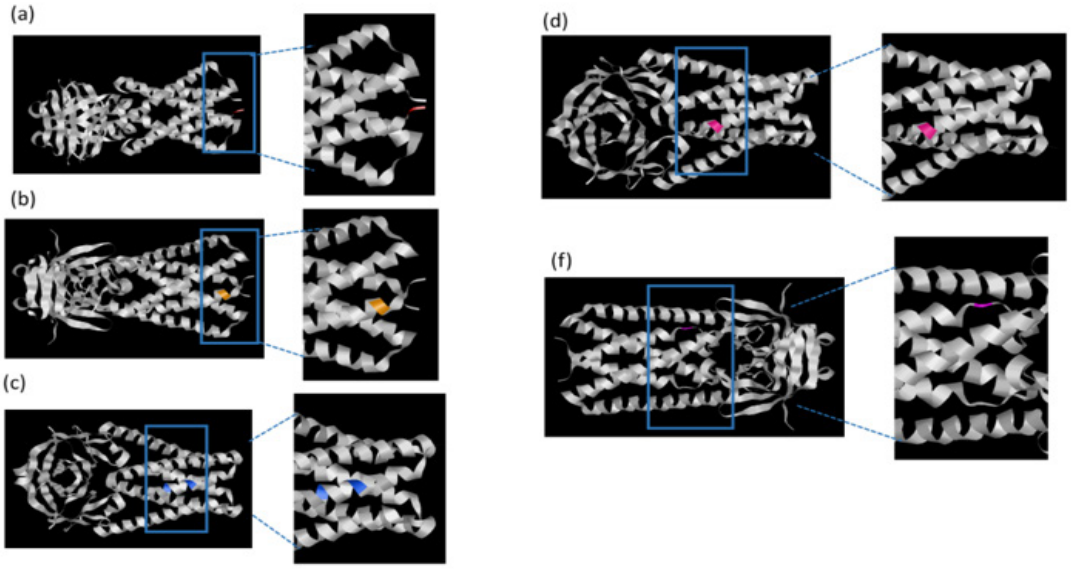

FIGURE 3. 3D Visualization of ORF3a SARS-CoV-2 functional domains: (a) domain I (amino acid 1-15), (b) domain II (amino acid 36-40), (c) domain III (amino acid 91, 93, 109, 127-133), (d) domain IV (amino acid 141-149), (e) domain V (amino acid 160-163, FIGURE not shown), (f) domain VI (amino acid 171-173).

DISCUSSION

We found a number of mutations in all six ORF3a protein domains (TABLE 1). Some mutations in the ORF3a domain show deleterious mutations based on Provean scoring. Even though deleterious mutations usually occur in nature, they tend to be fewer than neutral mutations. Mutation in structural protein might increase the stability of the protein, on contrary in non-structural protein might decrease its stability.[18]

Domain I (amino acids 1-15) is the N-terminal region of the signal peptide that plays a role in localizing the subcellular protein ORF3a SARS-CoV-2 (FIGURE 3).[1,9] In this area, a number of mutations in the amino acids were found. The D2Y, F4S, T9del, T9K mutations were deleterious which may eliminate the function of the signal peptide. The V13L mutation is neutral, but the valine → arginine mutation at position 13 (V13R) is deleterious, even though it does not change the nature of the protein, but it might change the protein's function. Likewise, T14I is neutral, while T14N is deleterious.

Domain II (amino acids 36-40) has a TRAF-3 (TNF receptor-associated factor 3) binding motif. The presence of this

domain can activate inflammatory Nf-kB and NLRP3 by promoting TRAF-3-mediated ubiquitination.[1,19] P36T and S40L mutations were found which were deleterious. There were 3 types of mutations in amino acid position 38, where glutamine (Q) → lysine (K) and glutamine (Q) → arginine (R) are deleterious by changing the polarity of the amino acid to become positively charged. However, the change in the amino acid glutamine (Q) → histidine (H) does not change the nature of the amino acid even though the amino acid at that position becomes positively charged as well.

Domain III functions as the SARS-CoV-2 viroporin. The ion channel activity was carried out in the amino acid domain 93-133.[8] The *in vitro* study showed the amino acid 70-133 in Domain III was responsible for increasing the suppressor of cytokine signaling 1 (SOC 1). As a result of this up-regulation, the JAK-STAT signaling activation was inhibited and therefore led to the JAK2 degradation. This study was conducted with HEK293T transfected with ORF3a plasmid to determine SOCS 1 in mRNA and protein levels.[20] Position 128 mutation of tryptophan (W) → glutamine (G) changes the amino acid from non-polar to polar and deleterious. Changes in tryptophan (W) → leucine (L) or cysteine (C) are also deleterious although they do not change the properties of amino acids. Likewise, mutations at position 131 of tryptophan (W) → serine (S) change the amino acid to be polar and deleterious. Mutation in W131C and W131L that do not change the amino acid properties are also deleterious, where it is possible that these changes alter the protein's function. In addition, mutations L129F, C130F, C133S are also deleterious. Based on study, the C133S mutations in Domain III will reduce the level apoptosis.[4] The W131C mutations were the second highest frequency of mutations with deleterious mutations in our study. These amino acid changes might facilitate the process of tetramerization to form ion channels and support the infectivity of the virus.[21,22]

Domain IV regulates viral uptake and trafficking of proteins to the plasma membrane or intracellular membrane.[1,3] The mutation of position 143 of alanine (A) → serine (S) which changes the polarity of the amino acid is neutral, while the change of alanine (A) → valine (V) is deleterious although it does not change the polarity of the amino acid. Mutation of position 144 of asparagine (N) → serine (S) is deleterious, but not for changes in asparagine (N) → aspartic acid (D) although this change makes polar amino acids negatively charged. Position 142 mutations of aspartic acid (D) → histidine (H) and tyrosine (Y) are deleterious and change amino acids into positively charged polar and neutral charged polar. The Y145S, F146C, W149C, and W149L mutations were deleterious although they did not change the amino acid polarity. The 147 position changes of leucine (L) → valine (V) and phenylalanine (F) were neutral. These mutations in C148Y and A143S may increase the infectivity rate, even though these amino acid changes were neutral based on Provean score.[23]

Domain V is responsible for Golgi to plasma membrane transport, and mutations in this site made ORF3a protein to be aggregated. It has a bulky hydrophobic residue YNSV, 160-163.[3,9,23] Mutations at position 161 of asparagine (N) → serine (S) and position 163 of valine (V) → leusine (L) are deleterious although they do not change the polarity of amino acids.[3]

Domain VI is a di-acidic peptide that has an SGD motif that is not conserved in SARS-CoV-2.[1,5] In these samples, the three amino acids were found to be mutated. Changes at position 171 of serine (S) → leucine (L) and proline (P) are neutral. Position 172 experienced with four kinds of changes, and the most changes from

glycine (G) → cysteine (C), then from glycine (G) → valine (V) followed by arginine (R), and the least from glycine (G) → aspartic acid (D). Position 173 undergoes a deleterious change from aspartic acid (D) → glycine (G). The highest frequency of mutations with deleterious mutations in our study was G172C with 46 frequencies of mutations. This effect mutation in SGD motif is unknown until now. The other mutations in SGD motif in Domain VI, S171L mutations show the highest frequency mutations with neutral mutations based on our data. More offer the G172V mutations in Domain VI in extracellular domain, could stabilize β-barrel to decrease the local flexibility.[24]

Domains V and VI are not conserved in ORF3a. These motifs are specific adaptations of the ORF3a family and they do not play a role in the structural integrity of the fold.[5] Another study showed that C terminus Domain III-VI were necessary for blocking autophagy. Using constructed truncated several proteins, in N-terminal, transmembrane, and C-terminal regions, they determine the function of autophagy inhibition. The N-terminal regions or Domain I and II did not show the function in blocking autophagy and had no influence in ORF3a localization.[25]

ORF3a-like viroporins contain two types of domains. A transmembrane domain (TM) in the position 33-141 and a cytosolic domain (CD) in position 145-237. We found 915 samples were truncated in the C-termini amino acid 220-275 C-termini (FIGURE 1).[26,27] Based on the lineage of truncated samples, we found the most lineage of SARS-CoV-2 was AY.23 (60%), but it does not belong to Variants of Concerns (FIGURE 2). Lineage B.1.1.7 that was found in one of 915 (0.11%) samples belonged to Alpha variants that designated previous Variants of Concern by WHO. Lineage B.1.466.2 (9.29%) was former Variants of Concerns, but had been reclassified by WHO because the variants are no longer circulating and impact significantly in public health.[13]

The wild type ORF3a protein is predicted to have six B cell epitopes which are located in six locations. This truncated position resulted in the loss of B cell epitope corresponding to 219-225, 237-243, 251-256 and 261-273 amino acid residues.[10] One study showed that the effect of mutation in ORF3a had a destabilizing effect for the protein.[18] The impact of the truncated proteins toward pathogenicity of virus needs further study. Other limitation in this study is we only used one software to predict the mutated proteins. Furthermore, the study needs to compare the prediction with other various tools to see the impact and functionality of the mutated proteins.

CONCLUSION

This study indicates 312 non-synonymous mutations in functional domains of ORF3a protein and showing their probable effects on protein. We also found 915 from 5,131 samples in Indonesia that truncated in the C-terminal of ORF3a protein. The data obtained here need validation to better understand the implications of these mutations on the function of ORF3a SARS-CoV-2 protein.

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