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The role of conserved arginine and proline residues in enterovirus VP1 protein

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Abstract *Background:* High diversity of VP1 protein among enteroviruses has been a barrier in developing universally effective antiviral drugs. To maintain structure stability during evolution, several residues of VP1 protein of enteroviruses are conserved. Therefore, investigation of highly conserved residues in VP1 protein may provide information for antiviral drug candidates against enteroviruses.
Methods: To identify highly conserved amino acid sequences of the VP1 in *enterovirus* genus, the Consurf and CABS-flex 2.0 web software were applied. Through the combination with secondary structure information, we focused on conserved amino acids of VP1 property analysis.
Results: Most conserved residues of VP1 were in the interior and interacted with VP2, VP3 and VP4 capsid proteins. Structure of EV-A71 (PDB code 4AED) showed conserved residues were at hydrophobic pocket and close to the junction between the loop and β-barrel. Interestingly, arginine was the most common conserved residue of VP1. Proline was the second most common conserved residue and was found in the loop and β-barrel intersection areas. VP1 protein flexibility was associated with the secondary structure. Conserved residues of VP1 in β-barrel showed significantly low flexibility.

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Conclusion: Through large scale sequence analysis, we identified the amino acid distribution and location of conserved residues in VP1. This knowledge can be extrapolated for the *Enterovirus* genus and may contribute to developing the potential compound as an anti-enteroviral agent.

Background

Enterovirus genus belongs to the Picornaviridae family, contains a non-enveloped, single, positive-stranded RNA genome and is the most diversified small RNA virus family. *Enterovirus* genus consists of fifteen species and seven of them are important human pathogens.[1] Enterovirus A71 (EV-A71) is one of the most pathogenic enterovirus, due to this serotype being highly associated with severe diseases including poliomyelitis-like paralysis, brainstem encephalitis, fatal cardiorespiratory failure, pulmonary edema, and even death.[2] Not only EV-A71 is important clinically, but rhinoviruses are also common pathogens in acute lower respiratory tract infection and asthma exacerbations.[3] EV-A71 vaccines have been available recently and have demonstrated to be effective for children less than 5 years old.[4] However, because there is no universal specific epitope, these vaccines cannot induce cross protective neutralizing antibodies against different types of enterovirus.[5] Therefore, further strategies of universal antiviral development for enterovirus has become more and more important.

Small molecule anti-enterovirus drugs, like capsid binder and viral protease inhibitors, used to treat EV-A71 infections have been developed and clinically evaluated. However, they failed due to limited efficacy or toxicity issues.[6] The diverse viral structures and frequent mutation among enteroviruses may be one of the barriers for drug development. Previous study using bioinformatics to analyze the degree of conservation of nucleoprotein sequence among influenza A virus subtypes identified conserved binding sites which may be utilized as potential drug targets.[7] Additionally, systematic analyses of highly conserved regions in dengue virus suggest potential long-term target for dengue treatment.[8] Those examples hint that the conserved residues might play roles in enteroviruses. However, studies on the highly conserved residues of enteroviruses on viral properties were rarely reported.

VP1 is the most exposed protein, involved in the constitution of the canyon on the surfaces of *Picornaviruses*, and potentially implicates for pathogenesis.[9] A hydrophobic pocket in the canyon within the VP1 protein is believed to be the attachment site to host receptors. This pocket region is the most frequently considered therapeutic target. Chen et al. observed that targeting at the inside of the "pocket" could be a broad-spectrum inhibitor for human enteroviruses.[10] VP1 also plays a pivotal role in the life cycle of EV-A71, and its main functional roles include being involved in viral assembly, maturation and uncoating.

Mutations on RNA viruses could adapt to the environment more easily. Conserved residues in the RNA virus are important for viral biological function and structure stabilization. With massive enterovirus genomes and protein structure information being completed and uploaded to the database, it is possible to have a clearer investigation about the conserved determinants in the viral genome. Defining the role of conserved residues of VP1 may provide the target for the universal anti-enteroviral agent. In this study, we identified conserved residues in *enterovirus* genus. Using a large scale of VP1 sequences, ConSurf and CABS-flex 2.0 web software were applied for conserved residue property investigation.

Methods

Identification of the conserved residues of VP1

A total of 500 sequences of enteroviruses were obtained from the UniRef90 database and aligned against reference sequences (PDB code 4AED) for homology search (Table S1). The conserved sequences of protein structures of the *enterovirus* genus were analyzed by the Consurf server.[11—13] The known EV-A71 structure of PDB code 4AED was used as an alignment target and chain A of the structure was applied for the analysis with homologs by ConSurf. The default parameters were chosen to select 500 sequences for the analysis. MAFFT was used as the alignment method to build the Multiple Sequence Alignment (MSA), and the calculation method used was the Bayesian method. Evolutionary substitution model also chooses the best default model. The sequence data and final alignment results were downloaded for further analysis.

Structural visualization according to conservation score with NGL viewer

Fig. 1 shows the NGL viewer.[14] The viewer display options were selected as the following conditions: assembly selects "bioassembly 3" as viral pentamer, style select "surface", color select "by conservation", and select chain C, B, D to demonstrate that VP1 assembles with VP3, VP2 and VP4 capsid protein, respectively.

Prediction of ligand-interacting residues

EV-A71 protein structures (PDB code 4AED, 4CDQ, 3VBS, and 3ZFF) were applied to observe ligand-interacting residues. The display options of the NGL viewer were selected as the following conditions: interaction select ligand name: A, style selects "none", and ligand selects "Ball & Stick". The residues were shown when the ligand molecule interactions

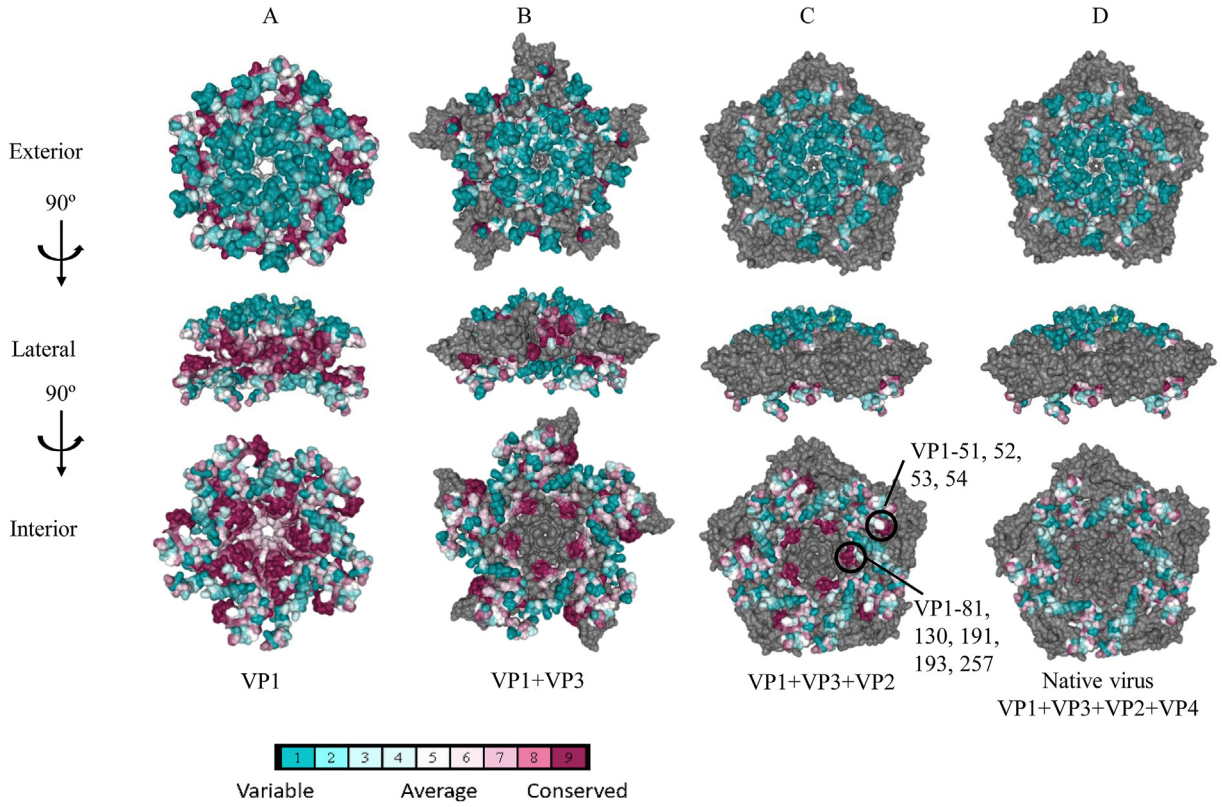

Figure 1. Secondary structure of VP1 of EV-A71 was shown by conservation score. (A) Diagram displaying VP1 of EV-A71 (PDB code 4AED) was applied to visualize the distribution of conserved residues by ConSurf with the NGL viewer. Blue to red color represents the conservation level. The blue-green color indicates more variable and dark red color indicates more conserved. The three-dimensional pentamers including exterior, lateral and interior views are indicated, respectively. The interaction among conserved residues of VP1 with other capsid proteins, pentamer formation during viral assembly was applied. (B) VP3, (C) VP2 and (D) VP4 was added into VP1 one by one sequentially through an NLG viewer. Gray color indicates VP2, VP3 and VP4.

by hydrogen bonds, halogen bonds, hydrophobic contacts, and *Pi* interactions within 5 Å. The interacted residues were filled into multiple sequence alignment tables.

Prediction of protein structure flexibility

CABS-flex 2.0 web server interface was used for assessing protein structure flexibility.[15] All analysis conditions were set as default. PDB code 6AKS was submitted for coxsackievirus A10, 5C4W for coxsackievirus A16, and 4AED, 3VBS and 4CDQ for EV-A71, respectively. Chain A(s) were selected for analysis. After the project was finished, the fluctuation plot was demonstrated from the website. The data download was as an excel file and filled into multiple sequence alignment tables.

Statistics

VP1 protein of EV-A71 (PDB code 4AED chain A) as the reference of secondary structure from previous study,[16] was added into the multiple sequence alignment tables for the comparison. Data comparison of conserved residues, conservation score, secondary structure, and fluctuation angle were calculated using excel. Figures were plotted using GraphPad Prism 5.0.

Results

Conserved residues of VP1 were found to be located at the interior

In order to identify the conserved residues of VP1 among enteroviruses, we applied the ConSurf software to investigate the diversity of conserved residues. The calculation was performed on a sample of 500 enterovirus sequences from the UniRef90 database. The deduced amino acid sequences contained fifteen enterovirus species including *Enterovirus A* to *L* and *Rhinovirus A* to *C*. Sequence homologs were shown in Table S1. Using EV-A71 (PDB code 4AED chain A) as reference, the default process was analyzed through a web-based server. From variable to conserved degrees, 9 categories of conservation score were classified by ConSurf. Using the 3D NLG viewer,[14] conserved residues of VP1 were found to be located at the interior area and variable residues were at the exterior area (Fig. 1A).

Conserved residues of VP1 interact with other capsid proteins

VP1 is the largest capsid protein and assembles with other capsid proteins as a pentamer unit. To investigate the

interaction among conserved residues of VP1 with other capsid proteins, pentamer formation during viral assembly was applied one by one through an NLG viewer. First, VP1 conserved residues were covered by VP3 in the exterior, lateral and central interior areas (Fig. 1B). Second, lateral conserved residues of VP1 were covered by VP2 (Fig. 1C). Lastly, two conserved regions were covered by VP4 (Fig. 1D). One of the covered regions is the lateral region, including VP1-51 to VP1-54, and the other is the central region, including VP1-81, VP1-130, VP1-191, VP1-193, and VP1-257. The result indicates that these regions' conserved residues of VP1 may interact with VP4. The structure of the mature viral pentamer formation showed no conserved residues were found on the surface, lateral and interior. Through the structural analysis of sequential enterovirus assembling, the observation indicates that highly conserved residues among enteroviruses may play a role in capsid protein interactions.

Conserved residues of VP1 were located at the β-barrel

To investigate the highly conserved residues distribution among enteroviral structure, we generate multiple sequence alignment in Table S2 by Consurf. The conservation of the secondary structure (loop and β-barrel) of VP1 were evaluated. The results indicate that highly conserved residues (conservation score 7, 8, 9) were found to be located at the β-barrels more than the loops (55.2% vs. 43.0%). A total of 81 variable residues (score 1, 2, 3) were located at the loop more than the β-barrels (35% vs. 10.4%). Similar results through filtering maximal amino acid percentage over 70% also showed that the β-barrel was more conserved than the loop structure (40.3% vs. 26.1%) (Table 1).

Conserved residues of VP1 were found at the hydrophobic pocket

The hydrophobic pocket is a switch for the infectious initiation process, leading to receptor interaction, particle destabilization, VP4 exposure, and followed by RNA release.[16] It is also a well-known potent target against enterovirus. To investigate whether the conserved residues are associated with the hydrophobic pockets, we used the protein structure of 4 EV-A71s (PDB code 4AED, 4CDQ, 3VBS and 3ZFF) to observe the interaction of residues. In order to investigate the residues of the hydrophobic pocket that interacted with ligand molecules within 5 Å by hydrogen bonds, halogen bonds, hydrophobic contacts, and *Pi* interactions, we used NGL viewer to observe the associated residues. A total of 28 residues were identified in the hydrophobic pocket. The conservation score of 22 residues was over 6 (mean 7.02) (Table S3). The result implies that highly conserved residues exist in hydrophobic pocket and could potentially act as a universal target for enterovirus drug development.

Proline and arginine are the most common conserved residues

To investigate the highly conserved amino acid properties, we focused on the 62 highly conserved residues where their conservation score was 9. Interestingly, we found arginine was the most common residue, and proline was the second most common residue in the VP1 of enteroviruses (Table 2 and Table S4). Conserved arginine residues were found at the CD loop VP1-(R120 and VP1-R121), at the β-barrel (VP1-R130, VP1-R189 and VP1-R236) and at the N- and C-terminus (VP1-R67, VP1-R86, VP1-R264, and VP1-R267). Conserved proline residues were found at the N-terminus (VP1-P60) and EF loop (VP1-P158 and VP1-P163), at the EF loop to β-barrel junction (VP1-P157 and VP1-P177), at the β-barrel G to GH loop junction (VP1-P193), and VP1-P263 was found at the β-barrel I to C-terminus junction (Fig. 2). This data suggests that the proline conserved residues were mostly located at the interior β-barrel to loop junction that may contribute to structure stabilization.

Table 2 Diversity of residues within the categorized conservation score 9.

Amino acid	No.	%	Amino acid	No.	%
Arg	9	14.52	Trp	3	4.84
Pro	7	11.29	Asn	3	4.84
Glu	6	9.68	Ile	2	3.23
Tyr	5	8.06	Phe	2	3.23
Thr	5	8.06	Asp	1	1.61
Gly	5	8.06	Leu	1	1.61
Lys	3	4.84	Met	1	1.61
Gln	3	4.84	His	1	1.61
Ala	3	4.84	Val	1	1.61
Ser	3	4.84			

Table 1 Comparison of the conservation and fluctuation angle of VP1 secondary structure among enteroviruses.

VP1 secondary structure	Conservation score (n = 297)									No. of maximal a.a. percentage >70%	Structure fluctuation angle (mean, Å)
	1	2	3	4	5	6	7	8	9		
	No. of variable a.a.			No. of undefined a.a.			No. of conserved a.a.				
Loop (n = 230)	81 (35.2%)			50 (21.7%)			99 (43.0%)			60 (26.1%)	2.22
β-barrel (n = 67)	7 (10.4%)			23 (34.3%)			37 (55.2%)			27 (40.3)	0.4
Structure fluctuation angle (mean, Å)	1.68			1.10			0.80			–	–

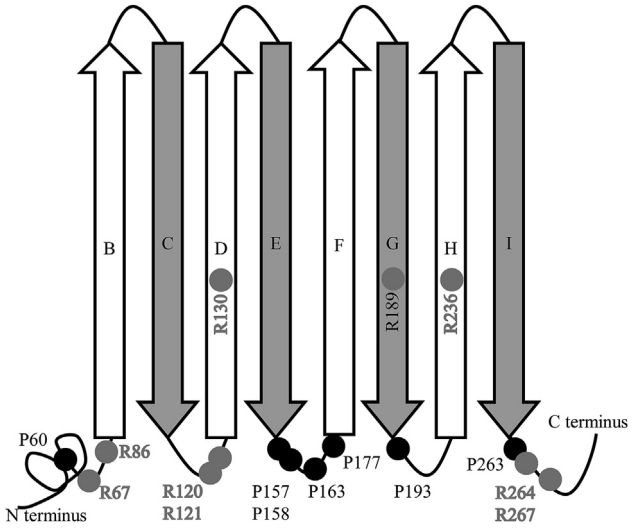

Figure 2. Diagram displaying location of most conserved arginine and proline residues in VP1 secondary cartoon structure. The arrow shape indicates β-barrel B to I and the link line demonstrates the loop structure. The black dots represent highly conserved proline. The gray dots represent highly conserved arginine.

Effects of the conserved residues on protein backbone fluctuation

The β-barrel is considered as rigid in order to maintain viral structure stability. Conserved residues were considered to stabilize the viral structure. To investigate how the conserved residues' flexibility and dynamics stability may affect their biological functions, enterovirus protein database was applied to CABS-flex 2.0.[15] The root mean square fluctuation (RMSF) could reflect the mobility of a certain residue around its position. We used the average of the fluctuation angle of VP1, which included 3 EV-A71 to investigate the flexibility between conservation and secondary structures. The result demonstrated that the fluctuation patterns among EV-A71s were similar. Proline and arginine showed a lower fluctuation angle. The β-barrel demonstrated low flexibility, and the interior or exterior loop demonstrated a fluctuation peak in our findings. When comparing the receptor binding associated residues (VP1-98, VP1-145, VP1-244, and VP1-245) and surface-exposed loop, higher fluctuation peaks were observed (Fig. 3A). Additionally, the conservation score was correlated with the fluctuation angle. Highly conserved residues demonstrated to have a lower fluctuation angle, and vice versa (Fig. 3B). The β-barrel also showed to have a lower fluctuation angle than loop (0.40 Å vs. 2.22 Å) (Table 1). There are some deviations among the loop of VP1, our data demonstrated conserved and β-barrel residues showed more significantly lower fluctuations, which may contribute to the stability of the EV-A71 structure (Table S5).

Discussion

Highly diverse enteroviruses have no effective universal antiviral drug. To our knowledge, this is the first report linked with protein structure and sequence information to characterize highly conserved residues of VP1 capsid protein in *enterovirus* genus. Conserved residues are important for viral structure stability and may interact with other capsid protein. In addition, most of these residues are adjacent to the interior hydrophobic pocket of the canyon area and loops to β-barrel intersection area. Through the analysis of conserved residues, we found that arginine is the most common conserved residue, while proline is the second common conserved residue. According to the information about the location and flexibility of conserved residues, this would thus provide more advantage on the development of a broad-spectrum anti-enteroviral drug.

We demonstrated that conserved residues interact with other capsid proteins. From the exterior view of viral particle, variable residues shown as blue color may be responsible for the facing of environmental challenges during the evolution among enteroviruses. Through the fluctuation pattern, the exterior receptor interaction region has a higher fluctuation angle, which may help viral particle search for host cells. From the interior view of the viral particle, variable residues may be involved in viral RNA interaction or initiate the infection process. However, our results demonstrate a highly conserved residues hidden inside which may preserve virus protein structure stability.

Unfortunately, currently no antiviral therapies are available for the treatment of pan-enterovirus infection. One barrier of development of efficient treatment would be the extremely high diversity of enteroviruses. Chen et al. reported ICA135 could be a broad-spectrum inhibitor for human enteroviruses, and they observed that this compound was targeted at the inside of the "pocket".[10] In our study, we also observed most of the conserved residues being located inside of the pocket, which may interact with other capsid proteins. Through cryo-EM, Flatt et al. also reported CP17 and CP48, that target VP1-VP3 interprotomer pocket, could inhibit virus assembly.[17] These recent reports support our protein simulation finding that the interior canyon pocket which contains highly conserved residues not only suggested to be a novel target region for the development of anti-enteroviral drugs, but is also important for viral life cycle.

Protein structure and fluctuation analysis were used to investigate the protein dynamic flexibility. In our study, we predicted low fluctuation means high level conservation of residues and vice versa. Receptor interaction switch residues of EV-A71 VP1-98, 145, 244, and 245 have high fluctuations. Similar fluctuation pattern was also discovered in echovirus and coxsackievirus. Using CABS-flex 2.0 server may rapidly simulate fluctuation patterns between different species. Furthermore, previous study showed that the Flaviviridae family, including *Flaviviruses* and a *Hepacivirus*, had a similar fluctuation pattern.[18] Echovirus 11 causes a severe neonatal outbreak in intensive care units during the spring-summer of 2018 in Taiwan.[19] Interestingly, coxsackievirus A10 (CA10) and coxsackievirus A16 (CA16) showed similar fluctuation patterns, and echovirus 11 after shift, 10 residues were also seen with the similar fluctuation patterns (Fig. S1). The results indicate the similarity among enterovirus species and the residues, in which fluctuation at peak may be potentially important neutralizing epitopes in the *enterovirus* genus. Further

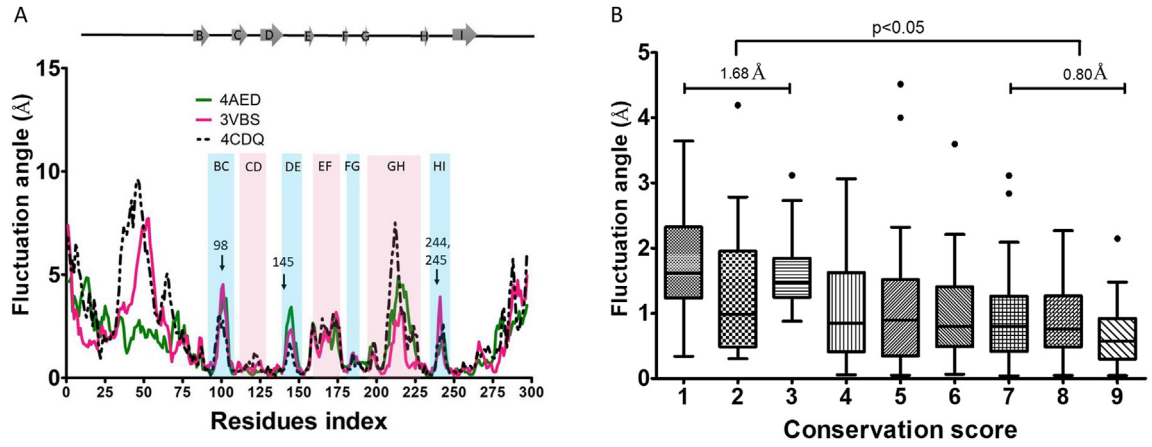

Figure 3. The fluctuation angle of VP1 of enteroviruses. (A) Diagram displaying PDB code 4AED, 3VBS and 4CDQ of VP1 of EV-A71were applied to CABS-flex 2.0. Blue block color indicated the exterior loops, and the pink block color indicated the interior loops. The gray arrow on the top figure referred to the residues position showing β-barrel B to I. (B) Comparison of protein fluctuation angle and conservation score of enteroviruses. The fluctuation angle of variable amino acids (conservation score 1 to 3) and conserved amino acids (conservation score 7 to 9) were shown. Statistical significance ($p < 0.05$) in fluctuation angle was indicated.

clarification of their roles in host—pathogen interaction during echovirus infection may be performed. Additionally, fluctuation patterns may accelerate the finding of viral receptor switches in other emerging species.

Since enterovirus species have been indicated with remarkable structural similarity, we tried to identify the most conserved residues for efficient treatment strategies which may cover a wider range of enteroviruses. Arginine depleting drugs have been used for the treatment of solid tumor and hematologic diseases in clinical trials. Arginine depletion may also be a potential therapeutic approach for SARS-CoV-2. Several studies had indicated that arginine is essential in the life cycle of Herpesviridae, adenovirus, influenza virus, and measles virus. Arginine is a semi-essential amino acid in humans, meaning that the amino acid needs to come from diet and synthesis in the body. Through enzyme metabolism, low arginine concentrations may decrease viral replication titer.[20] In our study, arginine is the most conserved residue in enterovirus genus. Through previous experience, arginine depletion may also work against pan-enteroviruses.

Proline was the second most common conserved residue of VP1 in *enterovirus* genus. It's also found in the loop to β-barrel intersection. In our unpublished data, VP1-P157 and VP1-P193 were shown to play a role in viral replication and thermal stability. We found alanine substitution of rgVP1-P157A demonstrated slower growth, smaller plaque size, unable to growth at 39.5 °C at low MOI, and loss viability after 1 h heating at 39.5 °C. The rgVP1-P193A found high RNA level after RNA transfection but no subsequent replication during passage, indicating this rgVP1-P193A affects initial step of infection or uncoating. A total of seven highly conserved prolines was shown in Table 3, the EF loop (position 157—177) contains four prolines. Three prolines were shown with low fluctuation angle except VP1-P158 in EF loop. The VP1-P157, VP1-P177, VP1-P193, and VP1-P263 located at the junction between β-barrel and loop were shown with lower fluctuation angle. The VP1-P60, VP1-P158, and VP1-P163 located at loop were shown with higher fluctuation angle. According to our supplementary table 5, we analyzed the fluctuation angle of VP1 residues of EV-A71 (PDB code 4AED, 3VBS, and 4CDQ) with the categorized conserved and variable residues. The overall mean of fluctuation angle was 1.83 Å. The conserved proline and arginine had fluctuation angle in 1.42 Å and 1.03 Å, respectively, which were lower than average (Table 4).

Like the arginine depletion strategy, proline depletion may also be a tool for universal drug against enterovirus. Qing et al. reported cyclosporin A, a known

Table 3 List of the fluctuation angle of conserved proline residues.

Position	Conservation score	Secondary structure	a.a.[a]	4AED (Å)	3VBS (Å)	4CDQ (Å)	Mean of RMSF (Å)	SD of RMSF (Å)
60	9	Loop	Pro	1.67	2.61	2.59	2.29	0.54
157	9	Loop	Pro	0.98	0.66	0.95	0.86	0.18
158	9	Loop	Pro	2.14	2.17	1.87	2.06	0.16
163	9	Loop	Pro	1.16	1.02	1.63	1.27	0.32
177	9	Loop	Pro	0.93	1.47	1.06	1.16	0.28
193	9	Loop	Pro	0.75	0.34	0.28	0.46	0.25
263	9	Loop	Pro	0.33	0.50	0.43	0.42	0.08

[a] a.a., amino acid.

Table 4 The mean of fluctuation angle in the EV-A71 VP1 residues.

Amino acids	Mean of RMSF (Å)		
	297 residues (n = 297)	Conserved[a] (n = 136)	Variable[a] (n = 88)
Cys	0.42	0.50	NA
Met	0.66	0.39	NA
Phe	0.75	0.88	NA
Trp	0.78	0.90	NA
Tyr	0.96	0.86	NA
Arg	1.33	1.03	3.32
Lys	1.34	0.53	1.83
Val	1.49	1.67	1.76
Leu	1.62	3.47	2.35
Ile	1.82	1.09	2.29
Pro	1.83	1.42	2.72
Gln	1.92	1.13	2.50
Asn	1.94	1.82	2.54
His	2.00	1.08	2.50
Ser	2.01	1.56	2.91
Glu	2.11	1.80	2.22
Thr	2.25	2.60	2.08
Ala	2.38	2.69	2.42
Asp	2.60	2.44	2.86
Gly	2.63	2.34	2.49
Mean	1.83	1.63	2.39

[a] The variable residues are defined as the residues within the categorized conservation score 1 to 3; The conserved residues are defined as the residues within the categorized conservation score 7 to 9.

immunosuppressor with peptidyl-prolyl cis—trans isomerase activity, plays an essential role in EV-A71 proliferation *in vitro*.[21] Proline residues were also applied in vaccine stabilization, which is a standard procedure during development. For example, the protein-based vaccines of SARS-CoV-2 were integrated in the dual-proline modification that could achieve vaccine stability.[22] The proline modification in HIV-1 Env trimer and respiratory syncytial virus prefusion protein showed that a conformational change could increase vaccine efficacy for several clinical trials.[23] Most of the conserved proline residues of enterovirus situated at the junction to β-barrel and loop surrounding the interior pocket of the canyon may act as important regulators in the viral life cycle.

Conclusion

In conclusion, a large-scale deduction of amino acid sequences in enterovirus genus were analyzed in detail. This study will help explore the importance of amino acid residues or areas in VP1 which may provide potential treatment in developing new antiviral therapy in the future.

Declaration of competing interest

No potential conflict of interest relevant to this article was reported.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2022.01.004.