

In silico prediction of linear B-cell epitopes for S1 protein of two Iranian 793/B isolates and their changes after 90 serial passaging

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Abstract

Neutralizing, serotype-specific, and hemagglutination-inhibiting antibodies against infectious bronchitis virus (IBV) are induced by epitopes in the S1 protein. Most changes in the virus genome due to mutation and recombination during serial passaging in embryonated chicken eggs occur in the S1 gene. In the current study, we tried to predict the potential linear B-cell epitopes of the S1 subunit of two Iranian 793/B isolates and then we analyzed their changes at passage level 90 due to mutations at this passage level. To predict linear B-cell epitopes of the S1 protein belonging to two Iranian 793/B isolates, we used two online epitope prediction programs called BepiPred and ABCpred. Some of the most important features of proteins including antigenicity, physicochemical properties, and secondary structure composition were analyzed. The predicted epitopes were studied between wild viruses and their passage level 90 viruses. We identified 15 potential linear B-cell epitopes among which six epitopes had the highest scores of physicochemical properties and antigenicity. Due to amino acid substitutions, seven predicted epitopes had different amino acid sequences at passage level 90. Among eight epitopes with no amino acid substitution at passage level 90, three epitopes had the highest scores. These three conserved epitopes including NH2-NQLGSCPLTGM1-COOH, NH2-GNFSDGFYPFTNSSLVKD-COOH, and NH2-GPIQGGC-COOH might be strategic and potential candidates for use in designing epitope-based vaccine researches. In conclusion, based on scores of physicochemical properties and antigenicity, it seemed that the sequence of most epitopes in wild viruses might be more antigenic and immunogenic compared to their sequence in viruses of passage 90.

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Introduction

Infectious bronchitis is a highly contagious respiratory disease caused by the infectious bronchitis virus (IBV).¹ The IBV was first described in 1930s and continues to be a major cause of disease in chickens of all ages worldwide.^{2,3} Spike glycoprotein is one of the four major structural proteins of the virus cleaving into S1 and S2 subunits.⁴⁻⁶ The S1 subunit forms the globular head of the virus spike.^{7,8} This subunit contains regions involving in neutralizing, serotype-specific, and hemagglutination-inhibiting antibodies and therefore, plays the key role in immunity against IBV.⁹ The S1 protein is very variable and the presence of different serotypes and genotypes is the

result of differences in its gene sequence.^{10,11} Live attenuated vaccine (LAV) is the primary method of controlling IBV infection in commercial chickens. To develop a LAV, IBV strains are passaged in specific pathogen-free (SPF) embryonated chicken eggs to achieve a reduction in virulence for the respiratory and urinary tracts. It has been demonstrated that most changes in the virus genome due to mutation and recombination during serial passaging in embryonated chicken eggs occur in the S1 gene. Some of these changes in S1 could relate to virus-host adaptation, antigenicity, and immunogenicity.^{12,13} Studies on mutations and their positions during serial passaging especially in the S1 gene as a gene with many key functions could be helpful to predict their effects on

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the changes of characteristics and structure of potential epitopes. Identification of potential B-cell epitopes is one of the important steps in designing epitope-driven subunit vaccines, antibody production, and immunodiagnostic tests. Efforts to design a subunit vaccine for IBV have been failed. These kinds of studies on changes of characteristics of potential epitopes due to mutations have the potential to help in designing subunit vaccines in the future by enhancing our knowledge over finding immunogenic epitopes remaining unchanged after serial passaging. In the current study, we predicted potential linear B-cell epitopes in the S1 protein of two Iranian 793/B isolates as one of the most dominant serotypes in Iran and we studied then amino acid changes of the S1 protein after 90 passages of two isolates and the effects of mutations on the predicted epitopes characteristics. There are a few studies on genetic changes of the S1 gene in 793/B serotype during virus passaging and their effects on the antigenic determinant of the S1 gene.^{11,14}

Materials and Methods

Viruses. We had two Iranian isolates of 793/B serotype (IR/794/2002 and IR/773/2001) isolated from broiler chicken flocks in our department.¹⁵ We had previously passaged both of these viruses to level 90 in SPF chicken eggs via the chorioallantoic sac route in our laboratory. The characteristics of viruses are shown in Table 1.

Table 1. Characteristics of viruses used in this study.

Virus	Accession number	Serotype
IR/794/2002	MH236618	739/B
IR/773/2001	MH252985	739/B

S1 Sequencing. Viral RNA of the parent viruses and their passage 90 viruses was extracted using a viral RNA extraction kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. To amplify the complete S1 gene of our five infectious bronchitis (IB) isolates, RT-PCR was carried out using an expanded high fidelity PCR system (Roche, Mannheim, Germany) according to the manufacturer's instructions. The RT-PCR was carried out as follows: 42.00 °C for 1 hr and then at 95.00 °C for 3 min. The PCR was carried out with 35 cycles of 94.00 °C, 45.00 °C, and 72.00 °C for 1, 1.50, and 3 min, respectively, followed by a final extension at 72.00 °C for 10 min. Published primers of Callison *et al.* (5'-TGAACTGAACAAA AGAC-3' as forward and 5'-CATAACTAACATAAGGGCAA-3' as reverse primers) were used in RT-PCR reaction.¹⁴ The PCR products (1720 bp) were confirmed by their size in 1.00% agarose gel electrophoresis with a 100 bp DNA marker. Purification of PCR products from agarose gel was performed using PCR purification kit (Roche) based on the manufacturer's instruction. Nucleotide sequencing of wild and passage 90 viruses was carried out using specific and internal primers by MWG-Biotech Co. (Ebersberg,

Germany). Nucleotide and amino acid sequences of the S1 were analyzed using CLC main workbench (version 5.5.0; Qiagen, Hilden, Germany).

Prediction of potential linear B-cell epitopes of the S1 protein. Linear B-cell epitopes of the S1 sequences were predicted by two servers including BepiPred and ABCpred.^{16,17} The length of amino acids was not set to any specific length to find all potential linear epitopes. Predicted peptides with scores above the threshold of 0.50 were selected. Two servers were used to find more potential epitopes. The most important characteristics of epitopes including antigenicity, physicochemical properties scores, and secondary structures were analyzed and finally, the most suitable epitopes with the highest scores were found. Location of mutations in the virus of passage 90 of each isolate was found and changes in the mentioned characteristics of epitopes were studied.

Analysis of physicochemical properties and antigenicity of the S1 protein. Physicochemical properties and antigenicity were studied for predicted epitopes of the S1 proteins of wild and passage 90 viruses. The physicochemical properties of the S1 proteins were analyzed using expasy-ProtScale (<http://www.expasy.org>) and IEBD servers. Physicochemical properties included hydropathy (Keyte and Doolittle algorithm), flexibility (Karplus and Schulz algorithm), and accessibility (Emini algorithm).¹⁸⁻²⁰ Antigenicity diagram was drawn for the S1 protein sequences using The kolaskar-Tongaonkar algorithm at database IEDB.²¹

Secondary structure composition. The secondary structure of the S1 proteins was analyzed using CFSSP (Chou and Fasman secondary structure prediction server) online server. All possible secondary structures including α -helix, β -sheets, turns, and loops were considered.

Results

Amino acid differences between wild viruses and their passage 90 viruses. Isolates 773 and 794 had seven and 35 nucleotide mutations at passage level 90, respectively. Most nucleotide mutations resulted in amino acid substitutions. Amino acid substitutions were seen at positions 23, 38, 87, 93 and 475 in 773 passage 90 and positions 24, 37, 51, 54, 55, 74, 76, 87, 130, 140, 142, 160, 164, 286, 298, 304, 382, 393 and 521 in 794 passage 90. Amino acid substitutions in each isolate are listed in Table 2.

Potential linear B-cell epitopes. Using BepiPred and ABCpred, linear B-cell epitopes of the S1 protein were predicted in both isolates. Predicted epitopes were identical in two isolates. Potential epitopes are shown in Table 3.

Physicochemical properties and antigenicity of the S1 protein. Considering antigenicity, physicochemical properties, and secondary structures, potential B cell

Table 2. Amino acid substitutions in isolates 773 and 794 after 90 serial passaging.

Amino acid position	773 wild	773P15	773P90
23	S	S	N
38	S	S	L
87	S	S	P
93	T	T	S
475	D	D	G
Amino acid position	794 wild	794P15	794P90
24	S	S	D
37	G	G	A
51	R	R	K
54	N	N	D
55	T	T	A
74	Y	Y	H
76	F	F	I
87	P	P	S
130	K	K	Q
140	G	G	D
142	A	A	T
160	L	L	F
164	G	G	S
286	L	L	S
298	H	H	R
304	Y	Y	C
382	R	R	T
393	S	S	K
521	P	P	S

linear epitopes of the S1 protein of the isolates with the highest scores were identified as follows: NH₂-NTTNNAGSAS-COOH, NH₂-NQLGSCPLTGMI-COOH, NH₂-GNFSDGFYFPFTNSSLVKD-COOH, NH₂-GPIQGGC-COOH, NH₂-QGPTRCKGV-COOH, and NH₂-ETGSEPIE-COOH.

Physicochemical properties and antigenicity scores of the predicted epitopes of the isolates and their passage 90 were compared and the scores are presented in Table 4. In some cases, characteristics of epitopes increased at passage level 90, while in some cases decreased.

Table 3. Predicted linear B-cell epitopes based on the S1 protein sequences of IR/794/2002 and IR/773/2001 using BepiPred and ABCpred servers.

Number	Server	Potential linear B-cell epitope	Start position
1	ABCpred	FQLYQHTAQDGYNF	292
2	ABCpred	KPSDFMYGSYHPKCNF	318
3	ABCpred	YAYSQGPTRCKGVYT	373
4	ABCpred	FVVQGAYGLNYYKVP	477
5	BepiPred	FRPGSGWHL	34
6	BepiPred	NTTNNAGSAS	54
7	BepiPred	NQLGSCPLTGMI	118
8	BepiPred	GNFSDGFYFPFTNSSLVKD	238
9	BepiPred	NAAPNLGGI	281
10	BepiPred	DFMYGSYHPKCNFRPE	321
11	BepiPred	GPIQGGC	354
12	BepiPred	QGPTRCKGV	378
13	BepiPred	RIQTRSEPLVLTQH	409
14	BepiPred	VTEATANYSYLAD	463
15	BepiPred	ETGSEPIE	516

Discussion

The S1 protein plays a key role in inducing virus-neutralizing antibodies, cell attachment, and serotype specificity.⁷ The S1 protein has three hypervariable regions (HVR) which their locations in European strains are as follows: HVR1 (amino acids 56-69), HVR2 (amino acids 117-131), and HVR3 (274-387).^{22,23} Using monoclonal antibodies, five antigenic sites with the ability to induce neutralizing antibodies have been mapped which were located at amino acid positions 24-61, 132-149, and 291-398. This finding suggests that HVRs are involved in antigenicity and serotype differences.²³⁻²⁵ It has been demonstrated that most changes in the virus genome due to mutation and recombination during serial passaging in embryonated chicken eggs occur in the S1 gene.^{12,13} It is worthwhile that we demonstrated Iranian 793/B isolates had some amino acid changes in the S1 gene compared to the foreign ones. Those changes, considered as amino acid markers for Iranian 793/B isolates, were located at variable regions of the S1 protein-containing epitopes playing role in inducing neutralizing antibodies.²⁶

In the current study, we predicted some linear epitopes for B cells based on the S1 protein sequences of two Iranian 793/B isolates and then we analyzed changes of these epitopes in passage level 90 of isolates to predict the potential effects of mutations after 90 passages on the topography of S1 epitopes.

Among epitopes predicted by ABCpred, two epitopes were overlapping with two epitopes predicted by BepiPred including KPSDFMYGSYHPKCNF with DFMYGSYHPKCNFRPE and YAYSQGPTRCKGVYT with QGPTRCKGV with the start positions of amino acids 318, 321, 373, and 378, respectively. Nine epitopes with the start positions of amino acids 54, 118, 281, 292, 318, 321, 354, 373, and 378 were located in the regions introduced as three HVRs. This is interesting because as mentioned above it has been suggested that HVRs have a role in inducing neutralizing antibody production. Regarding the position of five antigenic sites with the ability to induce neutralizing antibodies at amino acid positions 24-61, 132-149, and 291-398, it was found that epitopes with start positions of 34, 54, 118, 238, 281, 292, 318, 321, 373 and 378 were located at these antigenic sites.

Nine amino acid substitutions at positions 37, 38, 54, 55, 286, 298, 304, 382, and 475 were located inside the epitopes with the start positions of 34, 54, 281, 292, 373, 378, and 516. These are important data because some of these epitopes were located at HVRs including those with the start positions of 54, 281, 292, 373, and 378 and as mentioned above all of these epitopes are of those epitopes located at antigenic sites. These data show that some parts of changes of virus antigenicity or immunogenicity during serial passaging are results of

Table 4. Physicochemical properties (accessibility, flexibility, and hydrophobicity) and antigenicity scores of all of the predicted epitopes of isolates IR/794/2002 and IR/773/2001 and their passage 90.

Epitopes	Accessibility		Flexibility		Hydrophobicity		Antigenicity		
	Wild	Passage 90	Wild	Passage 90	Wild	Passage 90	Wild	Passage 90	
IR/794/2002	FQLYQHTHAQDGYYNF	0.540	0.545	0.969	0.960	-0.239	-0.022	1.018	1.039
	KPSDFMYGSYHPKCNF	1.211	1.012	1.040	1.031	-0.741	-0.561	0.945	0.962
	YAYSYQGPTRCKGVYT	0.630	0.592	0.996	0.998	0.144	0.159	1.034	1.037
	FVVQGAYGLNYYKVNPF	1.048	1.042	1.012	1.012	-0.430	-0.430	1.004	1.004
	FRPGSGWHL	0.656	0.88	1.008	1.053	-0.027	-0.436	0.995	0.971
	NTTNAGSAS	1.787	1.652	0.977	0.985	-1.018	-0.888	1.018	1.020
	NQLGSCPLTGM	1.441	1.432	0.997	0.997	-0.969	-0.969	1.030	1.030
	GNFSDGFYPFTNSSLVKD	1.493	1.484	0.993	0.993	-1.178	-1.050	1.012	1.012
	NAAPNLGGI	0.662	0.658	1.043	1.043	-0.337	-0.337	1.030	1.030
	DFMYGSYHPKCNFRPE	1.301	1.159	0.996	0.998	-0.743	-0.505	1.063	1.065
	GPIQGGC	1.315	1.0697	1.029	1.032	-1.055	-0.675	1.040	1.044
	QGPTCKGV	1.659	1.649	1.040	1.040	-0.807	-0.807	1.033	1.033
	RIQTRSEPLVLTQH	0.384	0.3818	0.997	0.997	0.968	0.968	1.031	1.031
	VTEATANYSYLAD	0.996	0.991	0.966	0.966	0.009	0.009	1.080	1.080
ETGSEPIE	1.864	1.682	1.082	1.078	-1.481	-1.404	0.942	0.937	
IR/773/2001	FQLYQHTHAQDGYYNF	0.750	0.540	1.015	0.969	-0.668	-0.239	0.991	1.018
	KPSDFMYGSYHPKCNF	1.212	1.213	1.040	1.040	-0.741	-0.741	0.945	0.945
	YAYSYQGPTRCKGVYT	0.631	0.631	0.996	0.996	0.144	0.144	1.034	1.034
	FVVQGAYGLNYYKVNPF	1.049	1.049	1.012	1.012	-0.430	-0.430	1.004	1.004
	FRPGSGWHL	0.664	0.664	1.007	1.007	-0.027	-0.027	0.995	0.995
	NTTNAGSAS	1.788	1.789	0.977	0.977	-1.018	-1.018	1.018	1.018
	NQLGSCPLTGM	1.442	1.442	0.997	0.997	-0.969	-0.969	1.030	1.030
	GNFSDGFYPFTNSSLVKD	1.494	1.495	0.993	0.993	-1.193	-1.193	1.012	1.012
	NAAPNLGGI	0.662	0.663	1.043	1.043	-0.337	-0.337	1.030	1.030
	DFMYGSYHPKCNFRPE	1.302	1.302	0.996	0.996	-0.743	-0.743	1.063	1.063
	GPIQGGC	1.427	1.4278	1.025	1.025	-1.055	-1.055	1.040	1.040
	QGPTCKGV	1.660	1.661	1.040	1.040	-0.807	-0.807	1.033	1.033
	RIQTRSEPLVLTQH	0.384	0.344	0.997	0.996	1.041	1.174	1.031	1.031
	VTEATANYSYLAD	0.990	0.991	0.966	0.966	0.009	0.074	1.080	1.080
ETGSEPIE	1.865	1.866	1.082	1.082	-1.481	-0.981	0.942	0.942	

at HVRs via structural changes of epitopes located at HVRs. Eight epitopes did not show amino acid substitutions in their sequence at passage level 90. Three epitopes (with start positions of amino acids 118, 238, and 354) having the highest scores of physicochemical properties and antigenicity were among eight epitopes with no amino acid substitution.

Amino acid substitutions in proteins could influence the characteristics of some parts of proteins. Amino acid substitutions at passage level 90 located near or inside predicted epitopes could change the physicochemical and antigenic properties of epitopes. Analyzing average scores of physicochemical properties and antigenicity of predicted epitopes, we found that some scores have been changed (increased and decreased) or remained without change. Interestingly, most changes of scores were seen in epitopes located in HVRs, and neutralizing antibody-inducing regions and other epitopes located outside these regions remained unchanged in most cases. This might be another sign that changes of antigenicity and immunogenicity of the viruses during serial passaging have a relationship with changes of HVRs and neutralizing antibody-inducing regions. In isolate 794, accessibility

scores decreased in all epitopes except one epitope (with the start position of amino acid 34), while in isolate 773, accessibility scores increased or remained without a change in most epitopes. It is expected that decreasing accessibility is accompanied by increasing in hydrophobicity and vice versa. In other words, hydrophobic residues tend to locate in the internal parts of proteins resulting in lower accessibility. Interestingly, our analyses showed this relationship between accessibility and hydrophilicity scores in epitopes with higher changes in accessibility score. In epitopes with very mild changes in accessibility score, the mentioned relationship was not seen and hydrophobicity did not change. Decreasing accessibility and increasing hydrophobicity are factors that have the potential to cover or weaken some of the predicted epitopes. We found no specific relationship between changes in accessibility and hydrophobicity scores with flexibility scores. In isolate 794, antigenicity scores showed a decrease in just two epitopes, and in other cases, it increased or remained unchanged at passage level 90. In isolate 773, we found just one change in antigenicity score and it was an increase in this score in the epitope with the start position of 292.

Secondary structures of linear epitopes are mostly composed of loops and to lesser degrees strands and then helices.²⁷ In our predicted epitopes, most secondary structures were loops and strands. Interestingly, some amino acid substitutions resulted in the changing of secondary structures predicted for every residue. Those substitutions included amino acids 51 (strand to helix), 55 (strand to helix), 142 (turn to strand), and 382 (turn to strand) in isolate 794 and amino acid 38 (loop to helix) in isolate 773. Among the mentioned substitutions, amino acids 38, 55, and 382 were located at HVRs. These changes in secondary structures could remarkably change the structure of epitopes in HVRs.

In conclusion, conservation of eight epitopes after 90 passages shows that these epitopes, especially three of them with the highest scores (with start positions of amino acids 118, 238, and 354), might be strategic for the virus and as potential candidates for use in designing epitope-based vaccine researches. Overall, it seems that in most cases, amino acid substitutions have weakened most epitopes and therefore, the sequence of epitopes in the wild viruses might be more antigenic and immunogenic compared to their sequence in viruses of passage 90. Our suggestions are evaluating the immunogenicity of predicted epitopes using the PepScan method and assessing introduced epitopes in other 793/B viruses to find suitable consensus epitopes in this serotype.

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Conflict of interest

The authors report no conflict of interest in this work.

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