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Original Article

Designing Polytopic Complex Vaccine Candidate against *Gallibacterium anatis*: An In-silico Study

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ABSTRACT

The haemolytic biovar of *Gallibacterium anatis* (*G. anatis*) is responsible for urogenital, gastrointestinal, and respiratory diseases in chickens. There are numerous reports on the resistance of *G. anatis* to antibiotics and recurrence of the disease, which raise concerns about antimicrobial treatment efficiency. Vaccination has been considered as the most feasible procedure of prevention in high risk farms. Subunit vaccines containing immunogenic components can have practical protective value in preventive measures regarding the infection. The present study aimed to introduce a polytopic vaccine candidate based on epitope detection. All registered sequences of four immunogenic proteins, includig Flfa, GTxA, Gab_1309, and Gab_2348 were retrieved and directed for variational analysis. A vaccine isolate was selected for each protein and tested for B-cell epitope mapping using different tools. Furthermore, consensus selected immunogenic regions with special patterns fused together by flexible linkers were integrated into two constructs and checked for the best status of proteasomal cleavage sites, as well as hydropathy plot. Moreover, back translations, along with codon optimization were performed, and then some tags were added to the constructs. The selected consensus B-cell immunogenic epitopes were for 12656: AA114-181, 7990: AA114-181, Avicor: AA42-77, 134-197, and IPDH: 61-155 for Flfa protein, AA185-235, AA372-457, and AA807-941 for GtxA-N, AA260-305, AA340-400, and AA110-146 for Gab-1309, and AA125-AA175 for Gab-2348. Two suitable patterns of attachment were selected from the different fusion patterns of epitopes in B-cell polytopic vaccinal constructs. Finally, the examination of these constructs showed their effect and efficacy for immune system stimulation. Based on bioinformatics results, these immunogens could be utilized as potential candidates to develop polytopic protective vaccines and design diagnostic kits.

Keywords: *Gallibacterium anatis*, Vaccine, Polytopic, In-silico

Conception du Candidat Vaccin Polytopique Complexe contre *Gallibacterium Anatis*: Une Étude in silico"

Résumé: Le biovar hémolytique de *Gallibacterium anatis* (*G. anatis*) est responsable de maladies urogénitales, gastro-intestinales et respiratoires chez les poulets. De nombreux rapports sur la résistance de *G. anatis* aux antibiotiques et sur la récurrence de la maladie soulèvent des préoccupations quant à l'efficacité du traitement antimicrobien. La vaccination a été considérée comme la procédure de prévention la plus réalisable dans les exploitations à haut risque. Les vaccins sous-unitaires contenant des composants immunogènes peuvent avoir une valeur protectrice pratique dans les mesures préventives relatives à l'infection. Cetteétude visait à présenter

un nouveau candidat vaccin polytopique basé sur la détection d'épitopes. Toutes les séquences enregistrées pour lesquatre protéines immunogènes Flfa, GTxA, Gab_1309 et Gab_2348 ont été extraites et utilisées pour une analyse variationnelle. Des souches vaccinales ont été sélectionnées pour chaque protéine et analysées pour la cartographie des épitopes de la céllule B en utilisant différents outils. En outre, des régions immunogènes sélectionnées par consensus avec des motifs spéciaux fusionnés par des lieurs flexibles ont été intégrées dans deux constructions et vérifiées pour les meilleurs statuts des sites de clivage protéasomal, ainsi que du tracé d'hydropathie. De plus, descontres traductions, ainsi que l'optimisation des codons, ont été effectuées, puis des tags ont été ajoutés aux constructions. Les épitopes immunogènes consensus à cellules B sélectionnés étaient pour 12656: AA114-181, 7990: AA114-181, Avicor: AA42-77, 134-197 et IPDH: 61-155 ; pour la protéine Flfa, AA185-235, AA372-457. et AA807-941 ; pour GtxA-N, AA260-305, AA340-400 et AA110-146 ; enfinpour Gab-1309 et AA125-AA175 pour Gab-2348. Deux modèles d'attachement appropriés ont été sélectionnés parmi les différents modèles de fusion d'épitopes dans des constructions vaccinales polytopiques à cellules B. Enfin, l'examen de ces constructions a démontré leur effet et leur efficacité pour la stimulation du système immunitaire. Sur la base des résultats bioinformatiques, ces immunogènes pourraient être utilisés comme candidats potentiels pour développer des vaccins protecteurs polytopiques et concevoir des kits de diagnostic.
Mots-clés: *Gallibacterium anatis*, Vaccin, Polytopique, In-silico

INTRODUCTION

Gallibacterium anatis (*G. anatis*) is a non-motile, Gram-negative, encapsulated, rod-shaped coccobacillus and classified in Pasteurellaceae family. Up to now seven species are diagnosed, from which *Gallibacterium anatis* haemolytic biovar is a virulent species causing infections in genital tract. The biovar triggers peritonitis and salpingitis problems in egg-laying chickens leading to decreased egg production; however, their symptoms are not pathognomonic (Bojesen et al., 2003; Christensen et al., 2003; Singh et al., 2016). Furthermore, the hemolytic biovar of *G. anatis* is responsible for the clinical complexities, such as egg dropping and oophoritis, hepatitis, salpingitis, peritonitis, follicle degeneration, septicemia enteritis, as well as respiratory disease, in both layer and broiler chickens (Bojesen et al., 2004; Proctor et al., 2006; Kristensen et al., 2011; Wang et al., 2016). Three different isolates of *G. anatis* haemolytic biovar were utilized to evaluate cross-protective potentials, namely the 12656-12 (biovar 4) homologous strain isolated from Danish chicken flock, strain IPDH isolated from infected chickens in Germany, and strain 7990 (biovar 3) isolated from a chicken with lesions in Mexico (Bager et al., 2013; Bager et al., 2014; Pedersen et al., 2015). Some of the virulent factors of bacterial invasions in *G. anatis* are GtxA part of RTX-toxins (Kristensen et al., 2010), secreted metalloproteases (e.g., Gab-1309, a lipoprotein) capable of denaturation of IgG avian immunoglobulin (Garcia-Gomez et al., 2005), and fimbriae (e.g., FlfA protein, subunit of the F17-like fimbriae helping tissue tropism) (Bager et al., 2013). In addition, some strains have the ability of hemagglutination of avian erythrocytes (Zepeda et al., 2009). The GtxA toxin is comprised of C-terminal domain (similar to other RTX-toxins with haemolytic function) and N-terminal domain (unknown function and no homology) (Kristensen et al., 2010). These proteins could be promising vaccine candidates for the stimulation of immune system (Pors et al., 2016).There are numerous reports on the resistance of *G. anatis* to antibiotics and recurrence of the disease, which raise concerns around antimicrobial agents, such as sulfa-based drugs, novobiocin, tylosin, clindamycin, tetracycline, and penicillin (Kristensen et al., 2011;

Singh et al., 2016; Jones et al., 2013). On the other hand, *G. anatis* field isolate has high antigenic variation (Bojesen et al., 2003; Jones et al., 2013). These facts cause concerns about financial issues and drug residues of antibacterials. Therefore, novel prevention strategies, such as classic and novel vaccines, are urgently needed. Recently, full genome sequencing of different *G. anatis* strains opened doors for conquering genetic variability and developing new vaccines using immunoinformatics approaches, such as reverse vaccinology approach (Bager et al., 2014) and polytopic (i.e., fusion epitopes) vaccines, followed in the present study. The absence of cross-protection between different strains originates the protection failure due to vaccination. This diversity in *G. anatis* immunogenic components has raised the need for a multivalent vaccine effective against a wide range of different strains. A subunit vaccine containing different immunogenic components shared between disease causative agents can be assumed as a solution to this problem. To the best of our knowledge, there was no report on epitope detection and polytopic vaccine candidates for *Gallibacterium* species, especially *G. anatis* haemolytic biovar. The present study aimed to apply novel immunoinformatics approaches for consensus epitope prediction and vigorous vaccine development for *G. anatis*.

MATERIAL AND METHODS

Retrieving sequences and structures. Complete protein sequences of Flfa, GTxA, Gab_1309 (Figure 4) and Gab_2348 of *Gallibacterium anatis*, along with other sequences were retrieved from UniProt, KB, and NCBI databases. The obtained sequences were aligned using ClustalW2 software (T-coffee server), trimmed, and analyzed by Bioedit software (version 7.7.9).

Entropy plot, signal peptides prediction, and transmembrane topology. Shannon entropy value was measured to find the regions of conservative mutations after the alignments of datasets. This plot was to calculate the variations of each amino acid position in a set of aligned sequences. SignalP 4.1 was used for the prediction of signal peptide of the selected proteins. The BLASTP, NCBI, and Pfam were utilized for the prediction of protein domains based on homologous known proteins. Transmembrane topology was predicted using TMHMM and TOPCONS servers.

Selection of vaccinal Flfa proteins and modeling of proteins structures. As Flfa protein sequences showed the high degree of variations in comparison with other selected proteins in entropy plot, it was tried to choose vaccine isolates in this protein and also visualize the extent of relatedness among Flfa protein isolates. In order to build phylogenetic trees, MEGA software (version 7) was used. The Robustness of phylogenetic analysis was measured by 10,000 bootstrap replications. The Estimation of average evolutionary divergence over all sequence pairs and similarity between isolates were also calculated by MEGA software (version 7). Although the homologus proteins in Protein Data Bank were detected by NCBI and BLASTP; however, similarity around 30% showed the modeled structures were not reliable; the 3D structure of protein in the prediction of B-cell epitopes was neglected.

B-cell epitope prediction. The majority of B-cell epitope prediction servers detected are linear (also called continuous) because they are convenient and easy to use. These prediction approaches are based on known amino acids specifications, such as solvent accessibility, hydrophilicity, flexibility, and antigenic property. Furthermore, from known linear epitopes databases, namely IEDB, BciPep, and FIMM learning machine approaches, such as artificial neural network, Hidden Markov Model, and support vector machine (SVM) were developed. In the present study, linear epitopes were predicted using following servers: LBtope (Support Vector Machine and Weka based on IEDB known linear epitope database), SVMTRiP (SVM applied by combined propensity score and tri-peptide similarity based on IEDB), ABCpred (by recurrent artificial neural network), BepiPred (by hidden Markov model propensity scale), Kolaskar and

Tongaonkar scale, parker hydrophilicity prediction, and Emini surface accessibility prediction (by physico-chemical properties). In addition, Conformational epitopes prediction (Also named discontinues) was carried out using the advantages of CBTOPE server (showing discontinuous antigenic amino acids by SVM score).

Selection of consensus epitopes and epitope engineering. With respect to the obtained results of the servers, consensus epitopes were selected for each protein. In addition, to achieve more broad spectrum and immunogenic epitopes some modifications and replacements were conducted regarding the epitopes.

Modelling and developing vaccinal constructs

B-cell epitope fusion by special pattern and concerns of conformational epitopes, as well as immunogenicity improvement to obtain maximum immunization responses are important. Moreover, it is essential to consider placing epitopes pattern in the correct positions near each other, composition method, and their antigenicity scores, each epitope number in a construct, immunodominancy, and spatial limitations of each epitope. In order to solve these problems, some arrangements were regarded in Flfa (A) and GTxA, Gab_1309 and Gab_2348 (B) constructs. For immunodominancy concerns or chance of new epitopes formation, NH2-GGSSGG-COOH flexible linker was used between the epitopes. The PADRE (Universal T-helper Pan-DR epitopes [AKFVAAWTLKAAA]) and the synthetic and immunogenic T-helper epitope, which can bind to a variety of DR molecules, were added to N-terminal of construct A design after NH2-KFERQ-COOH linker. To increase adjuvanticity, the activity of T-cell construct, and the best immunogenicity of the vaccine, polytope were fused by Small heat shock protein (HSP) of *Gallibacterium*. By the use of NH2-KFERQ-COOH linker C-terminal of construct A was added and separated from PADRE sequence. With the purpose of releasing the construct from host cells, metabolism control and rapid remove of B-cell constructs, constant heavy chain area of chicken´s immunoglobulin was fused to both NH2 end of B-cell RE constructs to form RE scIgG.

Prediction of construct primary structure. For A and B constructs, proteins sequence statistics, including molecular weight, length, isoelectric point, the total number of negative and positive residues, instability-aliphatic index, amino acid distributions, and Grand average of hydropathicity, were computed using the Expasy ProtParam server (http://expasy.org/cgi-bin/protpraram).

Hydrophobic region calculation. For the evaluation of A and B constructs of hydrophilic and hydrophobic regions, the algorithm of Doolittle and Kyte (1982) was utilized in the present study.

Codon optimization and reverse translation. Synthetic A and B constructs were then translated into nucleotide sequence using EMBOSS Transeq (version 6.0.1)(http://www.ebi.ac.uk/Tools/st/emboss_backtranseq/). The EMBOSS Transeq was designed to use codons that chickens can use. For the optimum expression of foreign genes in host cells, codon optimization was utilized, which causes higher gene expression in vectors. Vast C-G nucleotide numbers in an mRNA may increase the secondary formation of structures and as a result, inhibit protein translation. Small number of C-G nucleotides affects corresponding mRNA stability. The increase of mRNA stability significantly improves immune response. Codon optimization enhances the production of construct and results in higher yield antibodies. Constructs optimization was performed using J-Cat (Java Codon Adaptation Tool) server (http://www.jcat.de/Start.jsp). In addition, this server avoids restriction enzymes and useless cuttings in bacterial hosts.

Initiation/termination codon insertion and Kozak sequences. At the –COOH and $–NH_2$ terminals of constructs, the initiation codon ATG, termination codon TAA, and Kozak consensus sequence (to increase mRNA stability and *in vitro* gene expression) were added.

Open reading frame prediction. Gorf software was used at NCBI (http://www.ncbi.nlm.nih.gov/gorf/) to avoid undesirable open reading frame.

RESULTS

Variation analysis, signal peptides, and transmembrane topology of selected immunogenic proteins. The obtained results of this study were focused on three proteins, including FlfA, GtxA, and Gab-2348, introduced as protective components in a recent study carried out by Bojesen, using reverse vaccinology and one more protein identified as strong immunogen that is Gab-1309. The Flfa, GTxA, Gab_1309, and Gab_2348 protein datasets regarding the variation analysis of *G. anatis* showed that Flfa was a more variable protein than other selected proteins (Figure 1) and there were concerns on selecting only one strain for vaccine development, since the protein of one isolate does not cause protection against other isolates. Moreover, IPDH strain was the most divergent amino acid sequence, compared to other Flfa strains. Phylogenic basis, similarity matrix, and the knowledge of *G. anatis* haemolytic biovar were employed to select strains with broad spectrum of pathological and immunogenic Flfa. The obtained results of signal peptide region, transmembrane topology, variability evaluations, and putative conserve domains among bacterial species in mentioned protein of *G. anatis* are arranged in Table 1. As it can be observed in Table 1, GtxA does not have signal peptide and transmembrane topology, since it is a secretary toxin. The Flfa showed high variability along different places of its sequence. The GtxA displayed semi-variability, while Gab_1309 and Gab_2348 were highly conserved proteins. In all four studied proteins, there were conserved sequences, which demonstrated high similarity with other bacterial species with the same function and structure.

Phylogenetic tree for selection of Flfa vaccinal strains. As Flfa protein was a highly variable protein in alignment and in Shannon entropy plot analysis among other proteins, it was specially directed for the selection of vaccinal strains by phylogenetic and similarity matrix analysis. Previously reported Flfa *G. anatis* biovar haemolytic sequences were used for the construction of phylogenetic tree (Figure 2). Moreover, the overall mean distance between the sequences was reported as 0.304. The obtained results revealed that studied isolates were categorized into two major groups and two more strains showing divergence sequences of Flfa protein. Two strains from group 1 (7990 [from Mexico] and 12656/12 [from Denmark]), one strain (Avicor [from Mexico]) from group 2, one more divergence sequences from IPDH, and an isolate from Germany were selected to be used for further analysis.

Consensus epitope prediction of Flfa, GTxA, Gab_1309, and Gab_348 proteins. Linear and conformational B-cells epitopes were predicted for each protein based on their amino acid sequences. The obtained results of predicted epitopes using LBtope, SVMTriP, ABCpred, BepiPred, and CBTOPE servers, as well as selected consensus immunogenic regions among servers for each protein, are arranged in Table 2, 3, 4 and 5. Furthermore, five B-cell epitopes were identified in Flfa protein.

Development of Flfa, GTxA, Gab_1309, and Gab_2348 vaccinal constructs. In the next step, selected consensus immunogenic regions for designing two constructs named A and B were fused using special patterns to improve immunogenicity, minimize immunodominacy and conformations concerns. The construct A was designed as a vaccine candidate aimed to cause protection against the flagella of *G. anatis*. This construct contained eight segments, including E1, E2, E3, E4, E5, PDRE, HSP, and IgGk. The construct B was designed as a vaccine candidate with the purpose of causing immunity against three proteins, namely GTxA, Gab_1309, and Gab_2348. This construct contained seven segments including, E1, E2, E3, E4, E5, E6, and signal peptide. In this construct, the linker between E5 and E6 in design were duplicated to make it longer in order to give the construct more flexibility between the two segments. To enhance the host immune response and metabolism of the designed construct, the tags, including Pan-Dr epitope (PADRE) as a non-specific T-helper stimulator, Heat-shock protein (HSP) as an adjuvant, and IgG-k, acted as the

sequence leader (signal peptide) and better metabolism conditions were added to Construct A (Flfa)(Figure 3).

Determination of physico-chemical parameters. The primary structure of A and B constructs were predicted using Expasy ProtParam server (http://expasy.org/cgi-bin/protparam), the results of which are shown in Table 6. The Flfa construct is neutral and GtxA and Gab construct show the acidic nature. Both constructs are stable and soluble with a wide range of thermostability. Flfa construct needs some modification to change its neutral characteristics (Pi 7.7) for better immune response and metabolism. As it was described, this concern will be corrected by adding tags.

Reverse translation. Condon optimization and suitable GC content in the process of revers translation caused higher expression in hosts cell system. Graphic views regarding the construct after and before optimization are shown in Figure 5.

Final constructs, open reading frame, and hydropathy. Figure 6 illustrates the schematic views of final designed constructs for expression in vectors. The examination of open reading frame revealed no errors, and consequently the optimal expression of the construct was possible. Furthermore, hydrophilic plot by Parker algorithm revealed that all the epitopes (except the linkers between them) in the construct had hydrophilic characteristics. In detail, the average, minimum, and maximum of hydrophilicity by windows 7 size in Flfa constructs were reported as 2.824, -3.057, and 6.986, respectively (the plot was not shown). In case of GTxA/Gab construct, the average, minimum, and maximum hydrophilic characteristics were 2.256, -3.800, and 6.914, respectively.

DISCUSSION

Although some commercial local vaccines are introduced and used for three *G. anatis* prevalent biovars, antigenic diversity of strains affects the broad coverage and global usage of these vaccines for the prevention of *G. anatis* infections (Mendoza et al., 2014). An in-silico investigation for the identification of immunogenic regions of bacterial proteins had a major contribution in increasing the potency of subunit vaccines (Ranjbar et al., 2013; Ranjbar et al., 2015). In a recent study carried out by Bojesen et al. on using reverse vaccinology for the identification of protective protein components of *G. anatis*, five strong immunogenic proteins were introduced, and according to the results of in-vivo testing three of them showed the potential of being protective elements (Bager et al., 2014). In the present study, four out of five immunodominant proteins presented by Bager, et al. (2014), namely GtxA-N, FlfA, Gab_1309, and Gab_2348, were designated for B-cell epitopes prediction in those proteins and their incorporation in a polyepitope vaccine construct. *G. anatis* isolates represented difference in their lesions, pathogenicity, and virulence (Paudel et al., 2013). Previously, FlfA, GtxA-N, and Gab_1309 have been reported to induce a protective immunity and protein-specific antibodies against the homologous strains of 12656-12, 7990, and IPDH (Bager et al., 2013; Bager et al., 2014; Pedersen et al., 2015). However, the obtained results of cross-protection studies did not show full protection regarding other strains of bacteria (Pors et al., 2016). Pors et al. in 2016 investigated the cross-protective effects of GtxA-N (N is the terminal of GtxA), GtxAC, and FlfA recombinant proteins as promising in-vivo vaccines. In addition, the chickens challenged 12656-12, 7990, or IPDH 697-78 strains of three *G. anatis*. Immunization by all proteins showed protection against homologous strain12656-12 (Pors et al., 2016). Only partial protection against strain 7990 was induced by proteins, although FlfA decreased the rate of bacterial isolation. Furthermore, FlfA and GtxA-N caused protection against strain IPDH 697-78. Finally, it was concluded that they partially induced heterologous protection by the challenging egg-laying chickens with recombinant protein immunization. To solve these challenges, the present study attempted to apply variation analysis as well as phylogenetic tree, and similarity matrix selected four vaccinal Flfa strains to obtain protection against these strains, along with

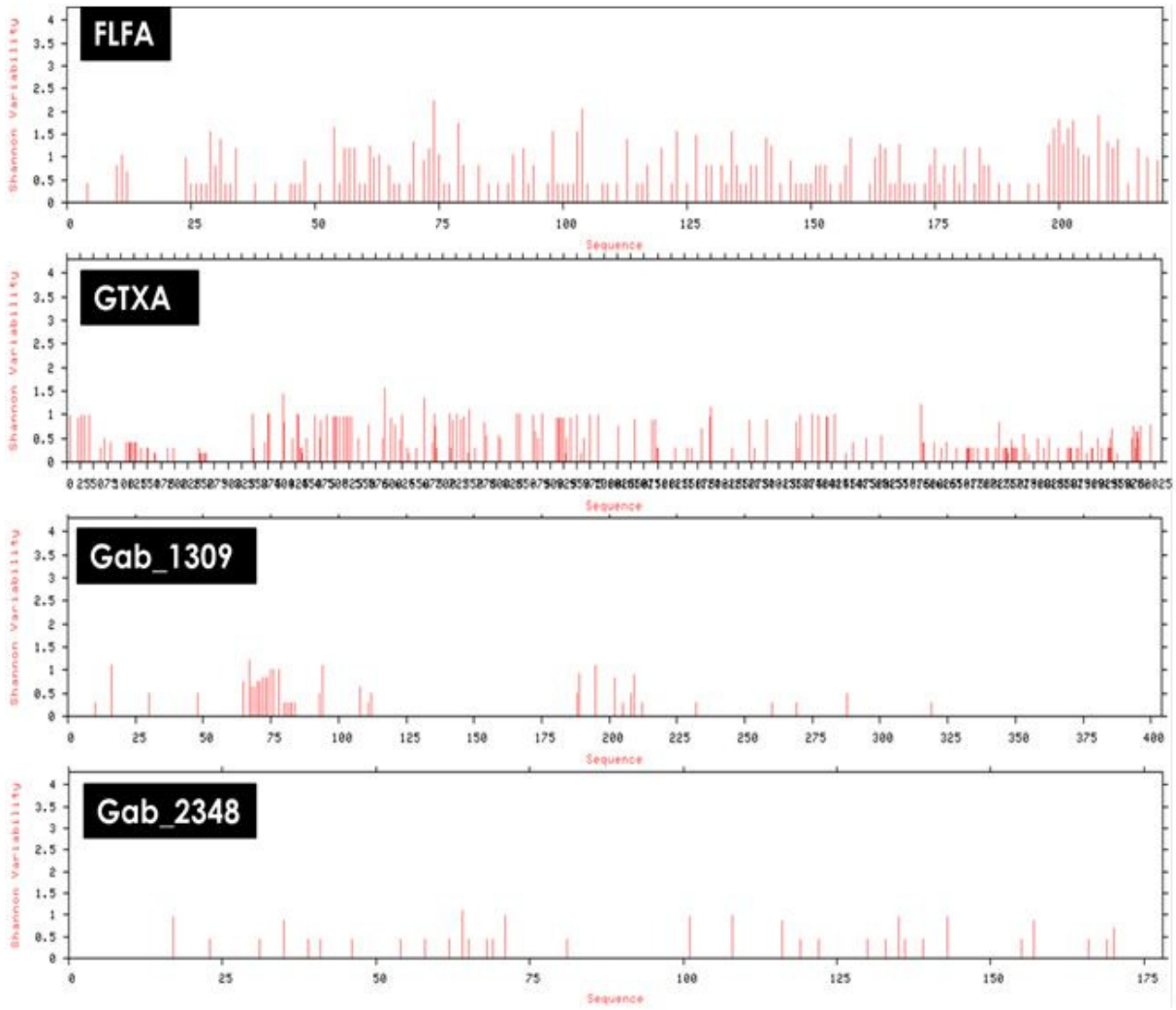

Figure 1. Protein variability analysis of Flfa, GTxA, Gab_1309, and Gab_2348 by entropy plot; threshold equal to protein variability server for depiction of plots (http://imed.med.ucm.es/PVS/)

Table 1. Protein sequence primary analysis results of Flfa, GTxA, Gab_1309, and Gab_2348

Protein name	Signal peptide (aa)	Transmembrane topology	High variations	Putative conserved domains
Flfa	1-24	1-22, and 172-200 (Transmembrane and inside)	+	Fimbrial super family (7-201)
GtxA	-	-	-/+	YhgE (252-628), RTX super family (1095-1577), Peptidase m10 (1763-1852), Peptidase M10 (1659-1743), COG2931 (1535-1839)
Gab_1309	1-25	Fully outside	-	LysM (142-184), N1Pd (142-402), PeptidaseM23 (303-396)
Gab_2348	1-27	1-32 (Transmembrane)	-	Omph (17-162)

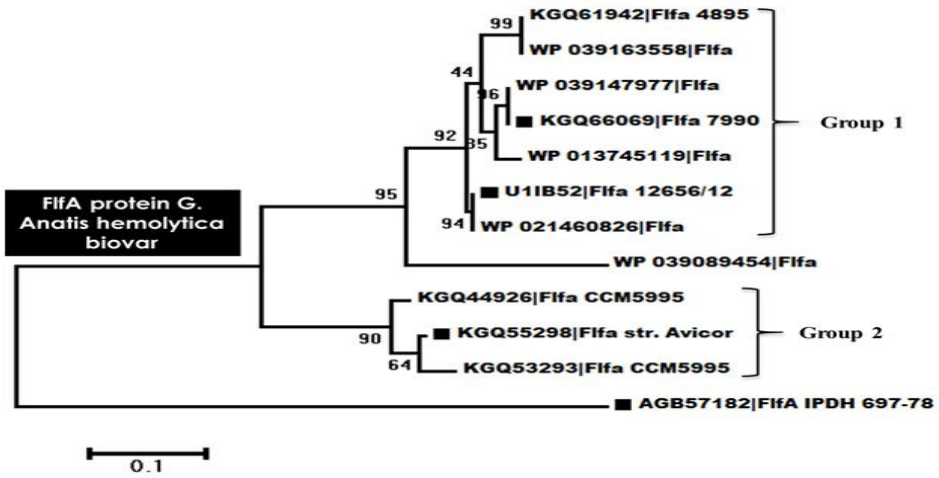

Figure 2. Phylogenetic tree of selected haemolytic biovar of *Gallibacterium anatis*; phylogeny test based on Bootstrap method by replication of 1000; maximum likelihood estimation

Table 2. Flfa epitope prediction of 2656, 7990, Avicor, and IDH isolates, along with consensus candidate epitopes

Epitope prediction	Server/Software name	Epitope sequence
Linear epitopes	**LBTope server**	**12656:** 43-50, 71-84, 114-116, 136-147, 155-156, **165-181** **7990:** 46-47, 69-84, 123-126, 136-147, 155-156, **165-181** **Avicor:** 43-51, **63-74**, 80-90, 106-108, 138-143, 176-192 **IPDH: 61-80,** 88-92, 119-121, 139-143, **154-185**
	SVMTriP server	**12656: 117-136** **7990: 115-134** **Avicor: 134-153,** 177-196 **IPDH: 96-115,** 130-149, 37-56
	ABCpred (Threshold 0.90)	**12656:** 95-106, 43-59 **7990:**95-111, **Avicor:** 42-58, 57-73 **IPDH: 139-155,** 173-189 (0.89)
	BepiPred	**12656:** 47-55, **63-77**, 84-94, 103-111, 117-126, 132-142, **147-170**, 176-192 **7990:** 47-54, **63-77**, 84-94, **103-111,114-126, 132-142, 147-170, 176-192** **Avicor:** 44-50, **63-75,** 84-94, 104-111, **118-127,** 132-141, **146-169,** 176-189 **IPDH:** 42-44, 53-50, 63-81, 89-97, **108-129, 138-168, 177-190**
Conformational	**CBTOPE (Antigenic regions were selected)**	**12656:** 79-81, 102-134, 142-163, 185-200 **7990:** 77-110, 130-163, 185-200 **Avicor:** 34-50, 67-72, 86-103, 102-131, **143-198** **IPDH:** 36-84, 108-143, 182-198

Selection of consensus immunogenic regions for Flfa isolates

12656: AA114-181
7990: AA114-181
Avicor: AA42-77, 134-197

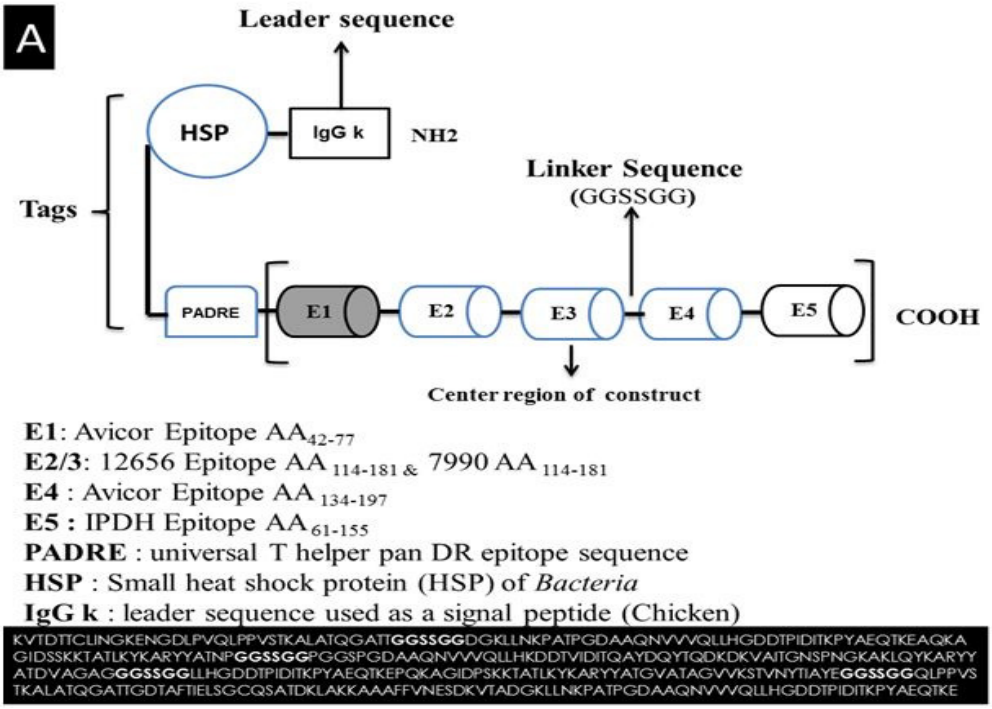

Figure 3. Flfa protein fusing epitopic regions and designing construct

Table 3. GTxA (N- and C-terminal) epitope prediction and consensus candidate epitopes

Epitope prediction	Server/Software name	Epitope sequence (aa)
Linear epitopes	**LBTope server**	121-130, 161-195, 225-226, 289-303, 381-389, 461-468, 490-502, 571-572, 630-631, 659-660, 680-690, 716-717, 826-837, 877-887, 932-939, 990-995, 1034-1036, 1152-1183, 1267-1272, **1361-1374,** 1443-1455, 1544-1552, 1656-1661, 1683-1693, 1714-1728, 1753-1797, 1865-1870, 1883-1893, **1929-1973,** 1988-1990
	SVMTriP server	**96 – 113, 203 – 220, 1067 – 1084**, 573 – 590,1178 – 1195, 50 – 67, 1730 – 1747,807 – 824, 1250 – 1267, 1203 - 1220
	ABCpred (Threshold above 0.90)	1816-1832, 1323-1339, 2020-2036, 1367-1383, 1239-1256, 989-1005, 78-94, 585-601, 1708-1724
	BepiPred	**N-terminal:** 125-137, 155-159, 183-192, 212-218, 249-256, 310-316, 342-349, 372-382, 403-410, 434-443, 497-504, 562-570, 591-599, 621-630, 679-688, 744-752, 775-811, 865-879, 898-908, 930-940, 992-1003
Conformational	**CBTOPE**	**N-terminal:** 56-77, 108-135, 180-189, 209-237, 266-268, 337-349, 386-418, 437-464, 480-482, 550-551, 587-604, 619-620, 631-633, 657-683, 737-739, 782-808, 837-849, 862-882, 902-956, 968-972

Selection of consensus immunogenic regions for GTxA

GTxA-N terminal:

1) AA185-235 (very conserved)
2) AA372-457
3) AA807-941

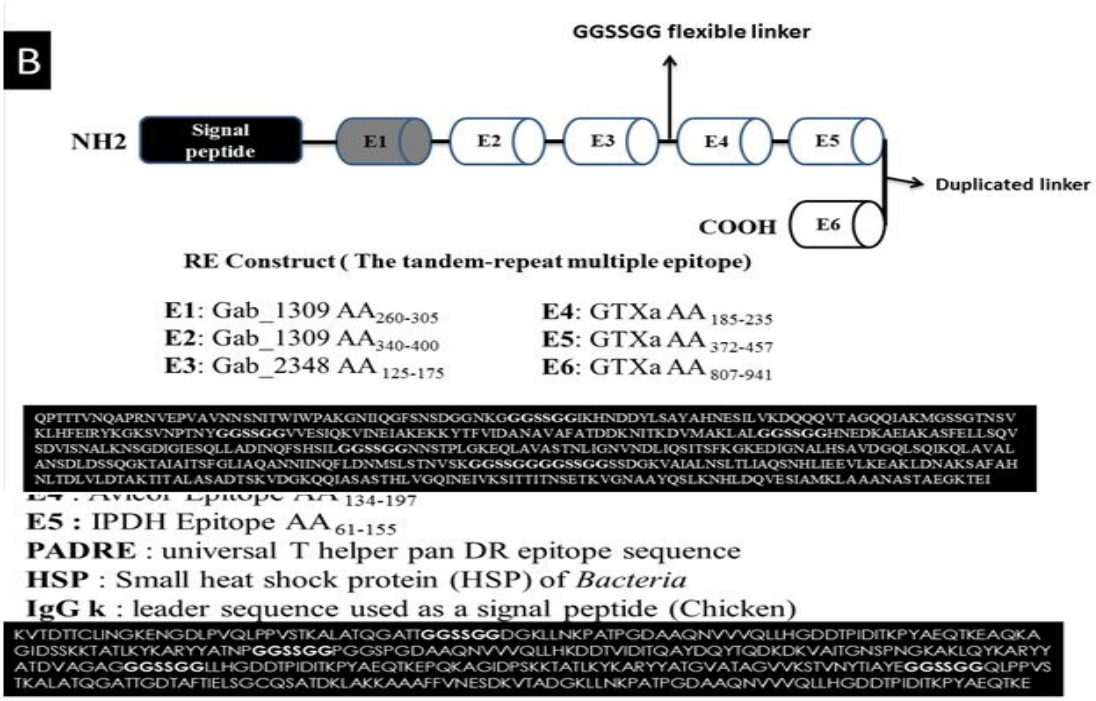

Figure 4. Schematic illustrations of GTxA, Gab_1309, and Gab_2348 epitopic fused regions and obtained vaccinal construct; adding signal peptide (chicken subcutaneous immunoglobulin) to N-terminal in construct

Table 4. Gab_1309 epitope prediction and consensus candidate epitopes

Epitope prediction	Server/Software name	Epitope sequence (aa)
Linear epitopes	**LBTope server**	**72-104,** 111-131, 221-233, **262-294,** 364-397
	SVMTriP server	**174 – 193,** 369 – 388, 269 – 288, 346 - 365
	ABCpred (Threshold 0.90)	**239-255,230-246, 384-400**, 51-66, 90-106, 81-97, 205-221
	BepiPred	**38-133, 117-132,** 135-146, 170-177, **187-278, 296-325,**362-380
Conformational	**CBTOPE**	29-46, 118-128, 166-182, 286-308, 326-361,370-403

Selection of consensus immunogenic regions for Gab_1309

AA260-305: QPTTTVNQAPRNVEPVAVNNSNITWIWPAKGNIIQGFSNSDGGNKG
AA340-400: Located in peptidase family (Cross-reactivity), fully conserved
IKHNDDYLSAYAHNESILVKDQQQVTAGQQIAKMGSSGTNSVKLHFEIRYKGKSVNPTNY
AA110-146: Conserved :KAISQDFTIPRNPQTNAPDYSKIDKGFYKGDSYTVRK

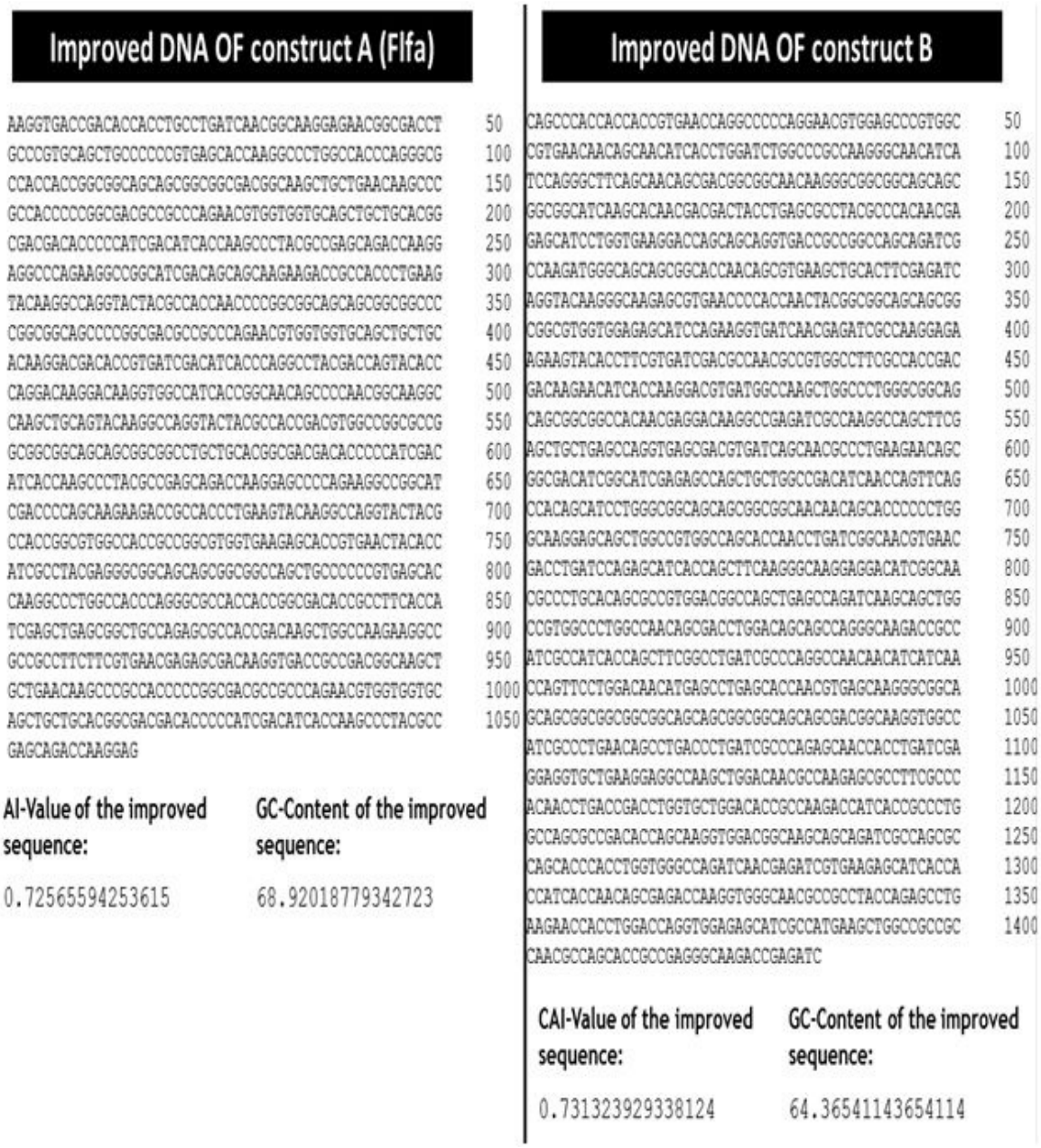

Figure 5. Codon optimization and reverse translation for constructs in a schematic image; depiction of GC contents of constructs following adaption

Table 5. Gab_2348 epitope prediction and consensus epitope candidates

Epitope prediction	Server/Software name	Epitope sequence
Linear epitopes	**LBTope server**	52-61, 79-91, 116-122, **134-152,** 162-173
	SVMTriP server	**148-167,** 111-130
	ABCpred (Threshold 0.87)	150-166, 136-151, 78-93
	BepiPred	42-47, 55-65, 82-84, 89-96, 103-111, 116-125,138-139, 153-161
Conformational	**CBTOPE (Antigenic regions were selected)**	44-56, 126-177
	Selection of consensus immunogenic regions for Gab_2348	
	Gab_2348: AA125 - AA175 VVESIQKVINEIAKEKKYTFVIDANAVAFATDDKNITKDVMAKLAL	

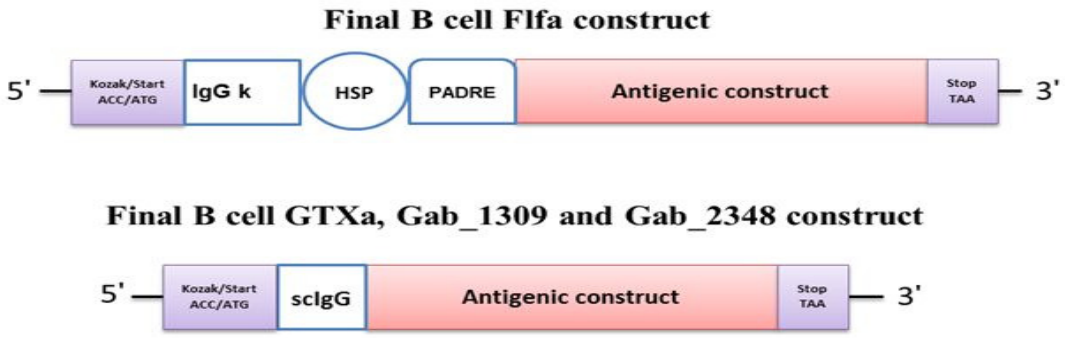

Figure 6. Final A and B constructs

Table 6. Calculated parameters for constructs using the Expasy ProtParam

Parameters	Construct A (Flfa construct)	GtxA and Gab construct (Flfa B construct)
Number of amino acids	355	478
Molecular weight	36669.77	49506.05
Isoelectric point	7.07	5.84
Overall −R and +R	38,38	45,38
Instability in stage II	24.27(stable)	31.20 (stable)
Grand average of hydropathicity, Aliphatic index	-0.529, 70.73	-0.225, 91.09
Half-life estimation	1.3 h (Mammalian reticulocytes, in vitro) 3 min (Yeast, in vivo) 3 min (Escherichia coli, in vivo)	0.8 h (Mammalian reticulocytes, in vitro) 10 min (Yeast, in vivo) 10 h (Escherichia coli, in vivo)

−R: number of negative-charged residues (Arg+Lys)
+R: number of positive-charged residues (Asp+Glu)
EC: extinction coefficient at 280 nm

cross-protection against other bacterial strains. In case of Gtxa-N, Gab_1309, and Gab_2348, peptide engineering was followed to cover sequential polymorphism between strains and more immune responses. As Gab_1309 and Gan_2348 are more conserved than Flfa and Gtxa-N, they may assist better protection than immunization regimes, which exclusively use Flfa and GtxA-N proteins. Moreover, with respect to sequence similarity and conserved domain analysis, vaccinal construct in the present study may cross-react to some extent with some other bacterial species, such as Pasteurellaceae family and *Yersinia*, which was also previously mentioned by Bager et al. (2014). Using a vaccinal construct based on a polytopic selection technique has the advantage of focusing the immune response of the vaccinated host in the most powerful immunogenic parts of the protein molecules from pathogen. This technique also decreases the side effects of the vaccination by the reduction of different foreign protein range introduced to the host. In addition, this procedure reduces financial issues considering vaccine development by minimizing the costs on the production of unwanted pathogen protein. Furthermore, this approach provides the possibility of incorporating different immunogenic components in a single construct of a subunit vaccine. This feasibility also is available for the inclusion of an immunogen from different strains of a pathogen to cause immunity against a wide range of several strains. Epitope mapping has broad applications in vaccine development, diagnosis, and therapy. Experimental challenges require time, cost, and resources (Ranjbar et al., 2013; Ranjbar et al., 2015); therefore, computer algorithms have been developed over decades regarding the prediction of T-cell and B-cell epitopes from structure and antigen sequences. It was shown that the prediction of linear B-cell epitopes was more difficult than other epitopes, such as T-cell epitope. The reason might be that those epitopes posse diversity length from 2 to 85 amino acids, compared with almost fixed length core of the epitopes in T-cells. Currently, *G. anatis* haemolytic biovar is known as an important opportunistic pathogen for layers and broilers in several countries, especially in Iran. However, there is limited scientific knowledge about pathogen and its protective vaccine(s); therefore, directed attention to control the outbreak and prevention against the disease is essential for poultry scientists and microbiologists.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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