Original Article

In silico experiment with an antigen-toll-like receptor-5 agonist fusion construct for immunogenic application to *Helicobacter pylori*

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BACKGROUNDS: *Helicobacter pylori* colonize the gastric mucosa of half of the world's population. Although it is classified as a definitive type I carcinogen by World Health Organization, there is no effective vaccine against this bacterium. *H. pylori* evade the host immune response by avoiding toll-like detection, such as detection via toll-like receptor-5 (TLR-5). Thus, a chimeric construct consisting of selected epitopes from virulence factors that is incorporated into a TLR-5 ligand (*Pseudomonas* flagellin) could result in more potent innate and adaptive immune responses.
MATERIALS AND METHODS: Based on the histocompatibility antigens of BALB/c mice, *in silico* techniques were used to select several fragments from *H. pylori* virulence factors with a high density of B- and T-cell epitopes.
RESULTS: These segments consist of cytotoxin-associated geneA (residue 162-283), neutrophil activating protein (residue 30-135) and outer inflammatory protein A (residue 155-268). The secondary and tertiary structure of the chimeric constructs and other bioinformatics analyses such as stability, solubility, and antigenicity were performed. The chimeric construct containing antigenic segments of *H. pylori* proteins was fused with the D3 domain of *Pseudomonas* flagellin. This recombinant chimeric gene was optimized for expression in *Escherichia coli*. The *in silico* results showed that the conserved C- and N-terminal domains of flagellin and the antigenicity of selected fragments were retained.
DISCUSSION: *In silico* analysis showed that *Pseudomonas* flagellin is a suitable platform for incorporation of an antigenic construct from *H. pylori*. This strategy may be an effective tool for the control of *H. pylori* and other persistent infections.

Key words: Cytotoxin-associated gene A, *Helicobacter pylori*, multi-epitope vaccine, neutrophil activating protein, outer inflammatory protein A

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Introduction

Approximately half of the world's population carry *Helicobacter pylori* in their upper gastrointestinal tract, with those in developing countries being the most affected.[1] Infection with this bacterium can lead to several clinical outcomes ranging from gastritis and duodenal ulcer to gastric cancer.[2,3] This infection usually affects the human stomach in childhood and persists for a lifetime, with the majority of infected persons (80-90%) remaining asymptomatic.[4] Furthermore, *H. pylori* was the first bacterium to be classified as a carcinogen by the World Health Organization.[5] Although the current multi-drug treatment is very effective at eradicating *H. pylori*, with cure rates higher than 90%, there remains a serious problem of increasing antibiotic resistance and reinfection with *H. pylori* after antimicrobial therapy, which

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occurs frequently in countries where infection rates are high.[6] Therefore, immunization against *H. pylori*, both to prevent and to treat infection, appears to be a better approach to control this widespread infection.[7] Great progress has been made in understanding the details of *H. pylori* pathogenesis and the role of its virulence factors in gastric disease and cancer. It has been shown that the main virulence factors, such as cytotoxin-associated gene A (CagA), neutrophil activating protein (Nap), and outer inflammatory protein A (OipA or HopH), in their native or recombinant forms can confer protection against *H. pylori* challenge in animal models.[2] CagA is the most important virulence factor associated with peptic ulcer or gastric adeno-carcinoma.[8] CagA-positive strains are associated with more severe clinical outcomes, especially in Western countries.[9] Nap is associated with severe gastro-duodenal mucosal injury and gastric cancer.[10] This protein (a toll-like receptor-2 [TLR-2] agonist) is able to elicit innate immunity and the production of interleukin IL-12 and IL-23, which promote a Th1 immune response,[11,12] and is therefore, a strong candidate as part of a multi-component recombinant vaccine against *H. pylori*.[13] OipA is another important protein that may be linked to gastro-duodenal diseases because of its association with other virulence factors, such as CagA[14] and associations with increased bacterial colonization.[15] Immunological studies have shown that *H. pylori* infection leads to a Th1 immune response but that this response is inadequate to eradicate the organism and as result, infection can persist. Insufficient adaptive immunity can be explained by incomplete activation of TLRs, suppressive effects of bacterial factors and, finally, the induction of regulatory T-cell responses.[16] Recent advances in the field of innate immunity have resulted in the identification of the TLR receptor family and their pathogen associated molecular pattern (PAMP) agonists.[17,18] Flagellin, as a PAMP (TLR-5 agonist), can polarize the immune response towards a Th1 response and lead to adjuvant properties and effects on dendritic cells.[19,20] A major role for TLR-5 in the innate immune response can be excluded by the fact that *H. pylori* flagellins have very low intrinsic activity to trigger TLR-5 receptors.[21,22] Because it has been proven that the Th1 immunity is necessary for protection and clearance of *H. pylori*,[23] vaccine research has been focused on induction of Th1 immunity. It has been demonstrated that although Th2 promoting vaccines induced strong systemic and local immune responses, only Th1 promoting vaccines are protective.[16] The intracellular location of *H. pylori* strengthens the argument that a Th1 immune response is necessary to eradicate infection.[24,25] Classical protocols have relied on a mixture of both antigen and adjuvant, but the disadvantage of this strategy was that the real-time loading and activation of antigen presenting cells (APCs) was not guaranteed. Due to the nature of the flagellin protein, it can be encoded into a protein fusion combination providing both constituents to the same APCs to generate a homogeneous antigen-presenting and activated cell population.[26] It was demonstrated that the immune response induced by *H. pylori* did not efficiently clear these bacteria.[16] Therefore, it is favorable to trigger the immune response in a way that differs from natural infection. For this purpose a newly designed antigen that differs from the naturally occurring antigen should be constructed.[27] Epitope-based vaccines represent a new strategy for generating a specific immune response and avoiding the side-effects of unfavorable epitopes in the complete antigen. Furthermore, an epitope-based immunogen could also include single antigenic molecules combined from different epitopes for increased potency.[28,29] Moreover, it has been shown that the immunogenicity of predefined epitopes is significantly increased by this strategy[30] for tuberculosis bacilli[31] and influenza virus.[32] Here, we designed a new chimeric construct containing the flagellin protein, replacing its D3 domain with a combination of selected antigenic segments of *H. pylori* virulence factors (CagA, Nap, and OipA) directly fused together. For efficient expression in an *Escherichia coli* host, this synthetic construct gene was analyzed by bioinformatics algorithms. Finally, a novel *in silico* approach was used to analyze the structural characteristics of this chimeric protein.

Materials and Methods

Sequence analysis

Related sequences of *H. pylori* virulence factors (CagA, Nap, and OipA) and *P. aeroginosa* flagellin type A (flaA) were obtained from GenBank. Using Clustal W software

http://www.ebi.ac.uk/Tools/clustalw2/, multiple sequence alignment was performed to identify the common and conserved antigenic fragments in putative *H. pylori* strain sequences.

Segment selection in virulence factors

Based on BALB/c histocompatibility molecules, all virulence factor sequences were analyzed separately using DNASTAR (DNAstar, Inc. www.dnastar.com) to identify the best segments containing both B and T-cell epitopes. Furthermore, the presence of appropriate epitopes was confirmed with other web-based B- and T-cell epitope prediction algorithms in single or assembled forms of selected segments.

Prediction of immunogenic epitopes

B-cell epitopes

The amino acid sequences were analyzed to predict continuous and discontinuous B cell epitopes using the Bcepred program (http://www. imtech.res.in/raghava/bcepred)[33] and the Disco Tope 1.2 server (http://www.cbs.dtu.dk/services/DiscoTope/), respectively.[34]

T-cell epitopes prediction based on BALB/c mice major histocompatibility molecules ($H2^d$)

Among different haplotypes of major histocompatibility molecules (MHC) in mice, those of the BALB/c mice harboring $H2^d$ were selected. These alleles contain I-A^d and I-E^d of class II and H2-K^d, H2-L^d, H2-D^d of class I MHC.[35]

Based on position specific scoring matrices or profiles from sets of aligned peptides to bind to given histocompatibility molecules, analyses of peptides related to H2 class II sequences were performed with the web-based program Rankpep (http://imed.med.ucm.es/Tools/rankpep.html). Similarly, the cytotoxic-T lymphocyte epitopes (CTL) related to H2 class-I was predicted with the web-based CTL-Pred program (http://www.imtech.res.in/raghava/ctlpred).[36]

CagA-Nap-OipA constructs design

The *H. pylori* antigenic construct (HAC) was assembled by fusing three selected segments into a single engineered molecule without any linker. The N-terminal of cagA (NP_207343.1), middle fragment of Nap (NP_207041.1) and C-terminal of OipA (AAQ57665.1) segments were linked together. The synthetic construct was deposited into GenBank (Accession No.: 1343925).

HAC design, replacement with the D3 domain and codon optimization

Upon three-dimensional (3D) study of *Pseudomonas* flagellin and homology comparison with *Salmonella* flagellin by protein visualizer, the D3 domain of this molecule was predicted and replaced with the antigenic construct (HAC) to develop complete flaA-HAC.

The designed construct was optimized with the Genscript Optimum Gene™ algorithm (www.genescript.com, Piscataway, New Jersy, USA). The chimeric gene was prepared for cloning and expression in the *E. coli* prokaryotic system.

Bioinformatics analysis of chimeric protein

The messenger RNA secondary structure of the designed construct was predicted by the centroidfold program[37] and was compared before and after gene optimization. The prediction of the secondary structure of the recombinant protein was performed using the Advanced Protein Secondary Structure Prediction Server 2 (http://www.imtech.res.in/raghava/apssp2/) and GOR-IV.[38] For prediction of 3D structure of each selected segment and the complete designed construct, several *ab-initio* online programs such as 3Dpro http://scratch.proteomics.ics.uci.edu/and Robetta http://robetta.bakerlab.org/submit.jsp were used. The 3D protein models calculated by comparative modeling such as Mod-Base http://modbase.compbio.ucsf.edu/modbase-cgi/index.cgi and LOOPP server (cbsuapps.tc.cornell.edu/loopp) were used. The stability of the 3D structure of the synthetic protein was further analyzed by swiss-PDB viewer for energy minimization. Prediction of solubility upon overexpression[39] and antigenicity of recombinant proteins were performed with online programs such as SOLpro and ANTIGENpro http://scratch.proteomics.ics.uci.edu/.

Results

Antigenic segment selection

The alignment of CagA, Nap and OipA amino acid sequences of different *H. pylori* strains resulted in the

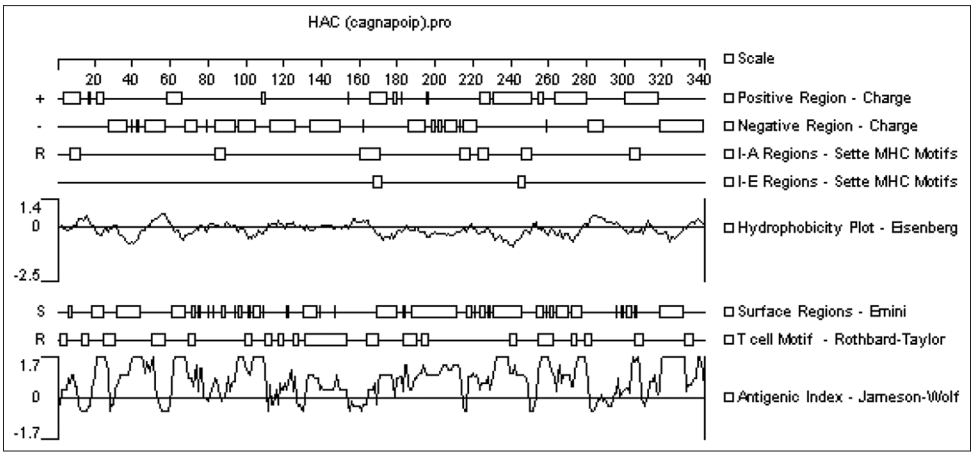

Figure 1: Analysis of the *H. pylori* antigenic construct by DNAstar software (see the text for details)

identification of three conserved segments. DNAstar software analysis primarily showed they were contained within both the B- and T-cell epitopes [Figure 1]. These segments, covering 342 residues, consist of 122 residues of the N-terminal portion of CagA (162-283), 106 residues of the middle portion of Nap (30-135) and 114 residues of the C-terminal portion of OipA (155-268). Without using any extra amino acids, these fragments were directly linked together to produce the synthetic HAC.

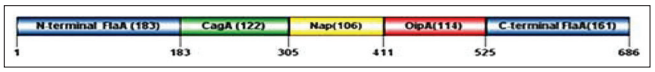

Figure 2: Schematic representation of the *H. pylori* antigenic construct consisting of flagellin type A and the *H. pylori* antigenic construct fragment. The lengths of the amino acid residues are listed in parentheses

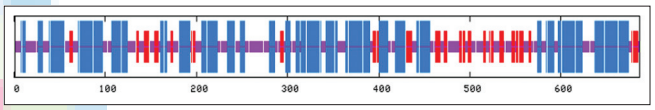

Figure 3: Secondary structure analysis of the flagellin type A/*H. pylori* antigenic construct protein helix: Blue, sheet: Red, coil: Violet

HAC replacement with D3 domain of flaA

There are four conserved domains consisting of D0, D1, D2, and D3 in the *P. aeroginosa* flaA structure. The conserved D0 domain consists of the C- and N-terminal of the flagellin hairpin molecule and has been reported as the agonist of TLR-5, and the D3 domain is a variable region. Upon 3D structural prediction of *Pseudomonas* flaA (Accession No.: Gu060499) and homology comparison with *Salmonella* flagellin (Accession No.: P06179), it was clear that the D3 domain in flaA molecule consists of 50 amino acid residues, which were replaced with the HAC. The schematic arrangement of the chimeric flaA-HAC molecule is shown in Figure 2.

Secondary and tertiary structure prediction

Secondary structure was predicted by several on line programs, and the best result was achieved by GOR-IV, as shown in Figure 3. The accuracy and quality of the model depends on two factors: First, the sequence alignment of query and template proteins and, second, the structures of the loop regions.

Three hundred models were predicted by LOOPP (learning, Observing and Outputting Protein Pattern) online software. Based on the analysis of the structures, two models were selected. Further analysis of selected models by Discovery Studio viewer showed two essential C- and N-terminals of the alpha helix structures (D0) that are needed for TLR-5 interaction and were retained in these models. Moreover, the HAC replacement within the D3 domain has no effect on the conformation of the C- and N-terminal domains of the flaA molecule [Figure 4].

Model stability for flaA-HAC

The stability of the model was first assessed by the computation of a Ramachandran plot based on each residue and then by energy minimization based on the total energy of the model. The majority of residues are in the stable region in the Ramachandran plot, which is shown in Figure 5.

Prediction of B- and T- cells epitopes for HAC

The predicted epitopes were candidates for inducing humoral or cell-mediated immunity against *H. pylori* in a BALB/c mouse model. For continuous B-cell epitope prediction, several physico-chemical properties of the HAC sequence, including hydrophilicity, flexibility, accessibility, exposed surface, polarity, and antigen propensity were analyzed [Table 1]. The results showed there were no continuous epitope sites in the junction of different fragments. Furthermore, with the Disco Tope server and based on model A [Figure 4], the discontinuous B-cell epitopes were predicted [Table 2]. In comparison with model B [Figure 4], the Nap segment in the model A shows more epitope pattern similarity with the standard NapA protein (PDB code: 1JI4). Based on SOLpro and ANTIGENpro software, the solvent accessibility and antigenicity were estimated with probabilities of 0.51 and 0.89, respectively.

The HAC sequence was analyzed, and the T-cell epitopes sites related to H2 class II [Table 3] and class I [Table 4] of BALB/c mice histocompatibility molecules were predicted.

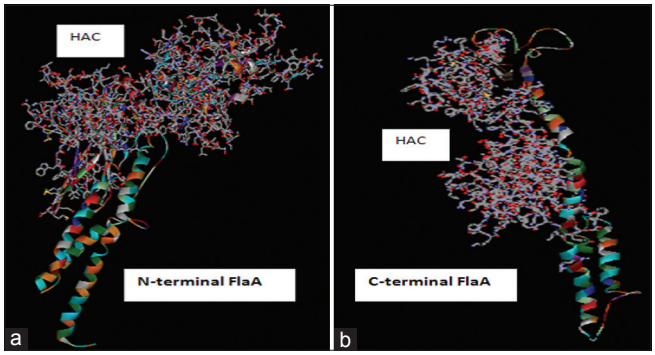

Figure 4: The position of the replaced *H. pylori* antigenic construct and flagellin segments in models predicted by LOOPP server and visualized by Discovery Studio viewer. (a) Intact N-terminus of flagellin type A (flaA) (183 residues) attached to the complete *H. pylori* antigenic construct construct and only a small fragment of C-terminal flaA (1-68 out of 163 residues). (b) The incomplete *H. pylori* antigenic construct consisting of a partial cytotoxin-associated gene A fragment (64-122) and intact C-terminus of flaA

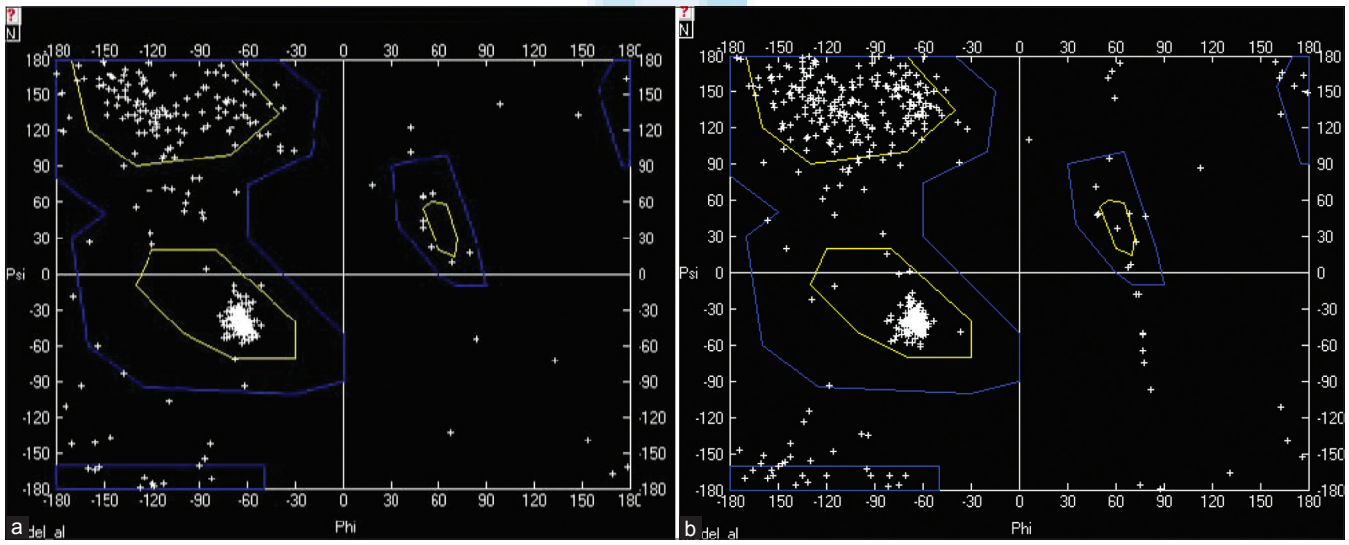

Figure 5: Ramachandran plot for two selected flagellin type A-*H. pylori* antigenic construct models. The a and b models are the same as those presented in Figure 4

Table 1: HAC continuous B- cell epitopes prediction in flaA-HAC chimeric protein based on physical and chemical properties by Bcepred

Prediction parameters	Epitope positions and segments		
	CagA	Nap	OipA
Hydrophilic	214-233, 244-254, 286-292	352-361, 386-397	415-423, 437-443, 503-511
Flexibility	185-191, 212-230, 240-251, 284-291	352-359, 383-394	412-421, 423-429, 447-461, 481-488, 516-522
Accessibility	187-194, 200-210, 213-230, 242-262, 277-293	311-323, 350-397, 404-411	412-432, 436-460, 481-493, 500-517
Exposed surface	203-208, 214-226, 243-252	350-361, 373-386, 389-396	412-430
Polarity	213-228, 252-258, 280-293	311-323, 327-334, 347-397	413-434, 448-461, 501-516
Antigen propensity	235-244, 258-267	333-347	429-435, 459-463

HAC: *Helicobacter pylori* antigenic construct, CagA: Cytotoxin-associated gene A, Nap: Neutrophil activating protein, Oip: Outer inflammatory protein

Optimization and synthesis of chimeric construct

The sequence encoding the flaA-HAC chimeric gene was optimized by changing a variety of factors that could regulate and influence gene expression.

The analysis of the wild-type gene and the optimized synthetic chimera is shown in Figure 6.

The codon usage bias in *E. coli* was increased by upgrading the codon adaptation index (CAI) to 0.87. The GC (guanine plus cytosine) content and unfavorable peaks have been optimized to prolong the half-life of the mRNA. The Stem-Loop structures, which impact ribosomal binding and stability of mRNA, were changed. In addition, the optimization process has screened and successfully modified any negative *cis*-acting sites.

In brief, within the synthetic construct, the splice sites, instability elements and all the *cis*-acting sites that may have a negative influence on the expression rate were removed. Furthermore, the necessary restriction enzyme

Table 2: HAC discontinuous B-cell epitopes prediction in flaA-HAC chimeric protein by Discotope server

Chimeric segments	Epitope positions (Starts and ends)
HAC	
CagA	200-21, 201-19, 202-10, 203-13, 204-8, 205-7, 206-10, 207-7, 208-7, 209-7, 210-8, 211-12, 212-10, 213-9, 214-7, 215-11, 216-13, 217-10, 218-8, 219-8, 220-8, 221-9, 222-9, 223-14, 224-12, 225-10, 226-13, 227-17, 228-22, 229-21, 246-12,247-12, 248-13, 249-8, 250-15, 264-14, 266-9, 267-8, 268-9, 298-6, 299-7
Nap	317-10, 338-11, 339-9, 354-10, 355-8, 356-11, 357-8, 358-14, 361-19, 363-17, 365-16, 383-10, 384-8, 385-7, 386-8, 387-8, 388-12, 389-12, 390-12, 397-10, 398-7, 399-7,400-8
OipA	413-18, 414-13, 415-11, 416-9, 417-16, 418-15, 419-19, 420-20, 422-15, 423-18, 426-14, 427-17, 432-14, 433-10, 434-7, 435-9, 436-14, 438-19, 454-12, 455-15, 484-12, 485-10, 486-9, 487-7, 488-7, 489-10, 490-13, 505-17, 506-20, 507-18, 508-11, 509-11, 510-22
Cag- nap	300-11
Nap-oip	410-16, 411-18
N-terminal FlaA-Cag	0
OipA-C-terminal FlaA	511-21, 512-18

Table 3: HAC T- cell epitopes prediction based on BALB/c histocompatibility molecules by rankpep

H2 Histocompatibility molecules	HAC segments/Ranking and epitope position prediction		
	CagA	Nap	OipA
BALB/C			
I-A d	272-280	310-318, 339-347, 347-355, 392-400, 394-402, 373-380, 333-341	485-493,419-427
I-E d	188-196,217-225, 198-206, 285-293	399-407, 306-314, 382-390, 383-391	442-450, 474-482, 423-431, 484-492, 437-445, 456, 464, 434-442, 511-519, 499-507, 451-459
Other			
I-A b	262-270, 194-202	327-335	441-449, 483-491, 398-406, 436-444, 474-484
I-E b	239,247	375-383, 351-359, 368-376,316-324	422-430, 432-440

Table 4: HAC cytotoxicity T-cell epitopes prediction based on different histocompatibility molecules by CTLpred and nHLApred

HAC segments	Ranking and epitope position prediction based on ANN and SVM*	Histocompatibility molecule restriction (H2) based on nHLApred					
		BALB/C			Other mice		
		Dd	Kd	Ld	Db	Kb	H-2QA
CagA	238-247, 234-243, 286-295, 204-213, 231-240, 186-195, 283-292, 189-198, 276-285, 288-297, 209-218, 294-303, 291-300, 191-200, 233-242, 195-204, 252-261, 237-246, 230-239, 280-289, 223-232	+	+	+	+	+	+
NAP	400-408, 306-315, 363-372, 346-355, 343-352, 329-338, 326-335, 361-370, 315-324, 319-328, 399-408, 337-346, 322-331, 377-386, 318-327, 360-369, 307-316,372-381, 374-383, 340-349, 334-342	+	+	+	+	+	+
OipA	510-518, 419-427, 428-436, 472-481, 489-498, 448-457, 478-487, 383-392, 421-430, 451-460, 431-440, 424-433, 427-436, 441-450, 459-468, 499-508, 485-494, 479-488, 517-526, 452-461, 443-452, 531-540,547-556, 486-495, 406-414, 467-476, 520-529, 370-379, 471-480, 429-438, 438-447, 490-499, 505-514, 548-557, 493-502, 475-484, 435-444, 463-472	+	+	+	+	+	+

* ANN: Artificial neural networks, SVM: Support vector machine

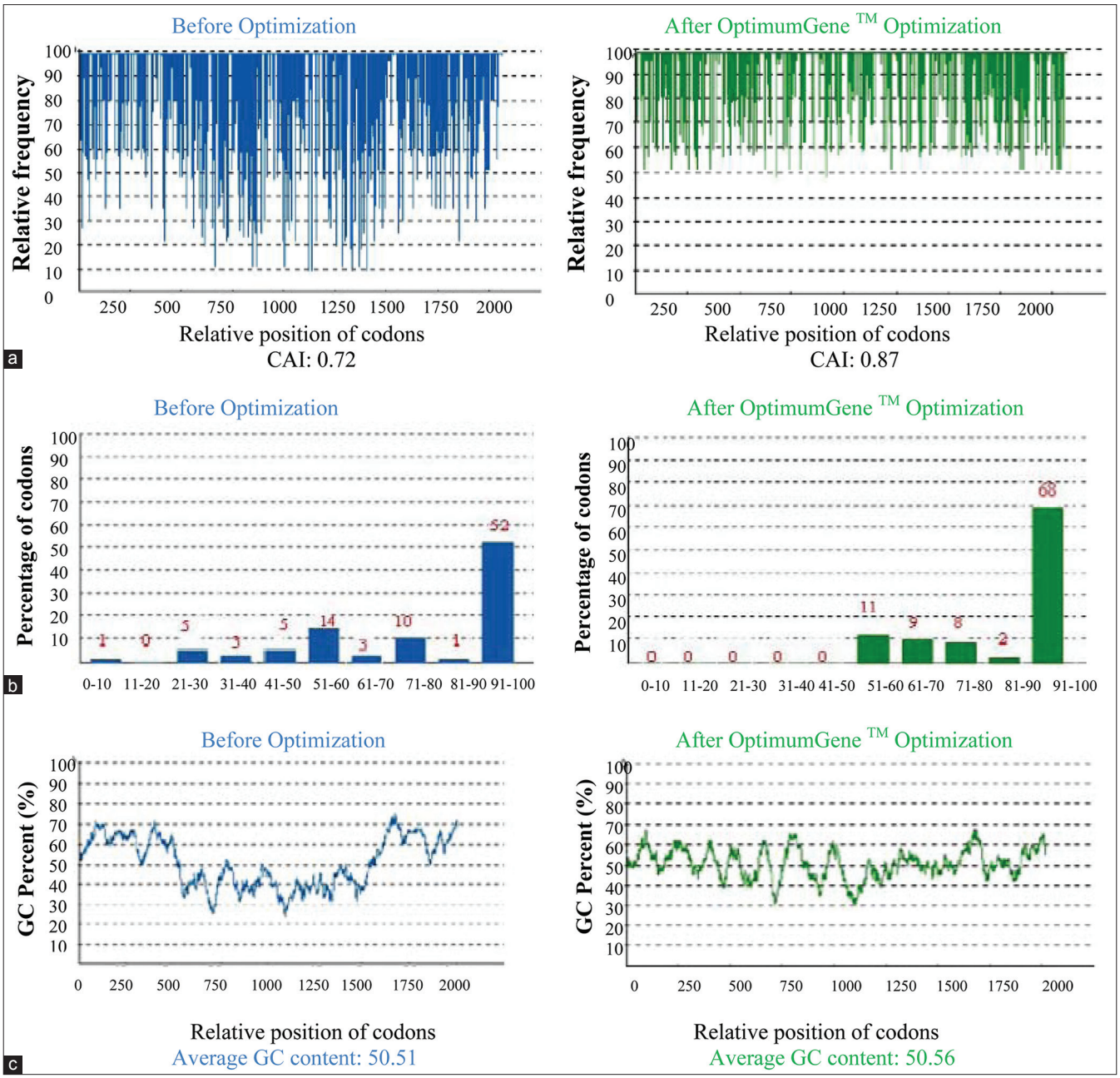

Figure 6: The sequence of the flagellin type A - *H. pylori* antigenic construct gene was optimized by changing some factors to increase gene expression. (a) Codon adaptation index, (b) frequency of optimal codons, and (c) G+C content adjustment

sites (*Bam*HI and *Hind*III) were introduced at the ends of the sequence for subsequent cloning [Figure 6].

Discussion

Most investigations in vaccine candidate development against *H. pylori* involve whole bacteria lysates,[40,41] recombinant purified subunit antigens,[41] and DNA vaccines, but no major breakthroughs have been achieved. Recently, the focus of vaccine development has shifted to multi-epitope vaccines as a new strategy to control certain severe and chronic infectious diseases, such as AIDS,[42] meningitis,[43] malaria,[44] tuberculosis,[31] and hemorrhagic diarrhea.[45] Potential advantages of epitope-based vaccines could result in this type of vaccine being an effective strategy to control *H. pylori.* Earlier studies revealed that many bacterial flagellins had adjuvant behavior for inducing humoral and cellular

immunity.[46] Biochemical analysis of flagellin showed that conserved domains, D1 and D2,[47] in the amino and carboxy, terminus were sufficient for TLR-5 receptor activation and concomitant nuclear factor kappa B signaling, which resulted in expression of cytokines, such as tumor necrosis factor-α, chemokine including IL-8 and free radicals such as nitric-oxide. Moreover, the hyper variable domain (D3) of flagellin was not involved in activation of this receptor.[48] It was shown that the leucine-rich region of TLR-5 containing amino acid residues 386-407 was a likely binding site for flagellin.[49]

In this study, special emphasis was placed on designing a new single construct that consisted of *Pseudomonas* flaA as an adjuvant and *H. pylori* antigens (HAC) to elicit an innate and adaptive response. Based on mechanisms of antigen selection in dendritic cells,[50,51] the fusion of adjuvant-antigen in this construct is a crucial advantage for simultaneous loading and activation of professional APCs, which could potentially create the optimal immune response. Moreover, many studies have shown that bacterial flagellin was a good platform[52] for B- and T-cell epitopes to elicit cross presentation of antigen.[26,53-56]

In this design, the HAC construct was replaced with the D3 domain of the *Pseudomonas* flaA. The 3D structure predictions confirmed that the HAC created separate domains in the flagellin structure. Moreover, the conserved N- and C-terminal segments of the molecule, which are necessary for TLR-5 activation, was retained [Figure 2]. Recent evidence in support of this design demonstrated that the D3 domain of flagellin was structurally independent and that its replacement with other antigen such as the hemagglutinin of swine influenza[57] virus did not interfere with TLR-5 activation and was more efficient than other domain fusions. In addition to deficient intrinsic TLR-5 activity of flagellin in *H. pylori*,[22] the secretory protein VacA (vacuolating cytotoxin A) suppresses host adaptive immunity, so this virulence factor was not incorporated into the HAC antigen.[23,58] This construct contains the main virulence factors that are involved in the pathogenesis of *H. pylori*. CagA, as an intracellular bacterial cytotoxin, is injected into host epithelial cells by a bacterial type IV secretory system. This antigen would be expressed by MHC class I molecules to induce a cell-mediated immune response. In this design, although we used recombinant cagA as an external antigen, which is mainly presented by the MHC class II molecule, integration of this protein into flagellin would increase cross-presentation and result in increase in MHC class I presentation. The highly conserved immunogenic amino terminal of CagA was the subject of segment selection in this study. Nap, as a ferritin like protein, has important roles in attachment to the carbohydrates of the host cell membrane matrix. The induction of an immune response against the immunogenic segment of this protein may result in effective protection by preventing bacterial host cell attachment[59] and induction of a Th1 immune response.

The last virulence factor was OipA. The evidence suggests that OipA participates in bacterial colonization[15] and adherence. This coupled with its association with other virulence factors suggested including the OipA segment as a component of the HAC.[15] For some limitations in gene expression, such as the size of synthetic gene and the capacity of a heterologous host to express a recombinant protein, only small segments of CagA, Nap, and OipA with the highest density of B- and T-cell epitopes were selected. In the flaA-HAC, the *Ab-initio* (data not shown) and homology structural prediction confirmed the presence of a random coil strand in the N- and C-terminus of each selected segment, which caused the effective separation of different domains. Because of the presenting N- and C-terminal coil strand in each segment, three selected fragments were linked directly in the synthetic HAC. The analysis also showed that three distinct parts were developed completely in this chimeric protein. The CagA-Nap-OipA combination presented in Figure 2 was more stable than any other possible arrangement of the three segments (Data not shown). Both the Nap segment in the HAC and the D2 domain of flaA revealed an α-helix structure. To avoid the linking between these two α-helices and to maintain the flagellin structure, the Nap segment was located between CagA and OipA as the central fragment of the HAC.

The α-helical structure of the Nap segment suggested it as the central fragment in the HAC. Using this strategy, the linking of the two α helix structures related to this segment and the D2 domain of flagellin was avoided, and the flagellin structure was maintained. The accuracy of the construct was also supported by the epitope prediction results. There were no undesired continuous

B- and T-cell epitopes in the different segment junctions and only a few undesired conformational epitopes, including one epitope in the CagA-Nap junction and two other epitopes in each of the Nap-OipA and OipA-amino terminal flaA junctions. It is possible that these undesired epitopes could raise a few nonspecific immune responses, but they have no effect on protection against *H. pylori* infection. The presence segments with differing G+C percentages and codon usage variations could reduce the overall expression level of the HAC in an *E. coli* host. Therefore, the chimeric flaA-HAC was codon optimized. To that end, the CAI, G+C percent and half-life of the mRNA were also analyzed. Additionally, the stem-loop structures of the mRNA that impact ribosomal binding and mRNA stability were modified, and negative cis-acting sites were avoided [Figure 6]. The prediction of the minimum free energy of the chimeric protein compared to its original structure showed that the protein had adequate stability. This prediction study also revealed that the chimeric construct had the potential to induce humoral and cellular ($CD4^+$ and $CD8^+$) immune responses against this pathogen in the BALB/c model.[60] These responses were demonstrated to be the crucial factors for protective immunity against this pathogen.[16]. In addition, some of the predicted HAC epitopes [Table 1] could be presented to other mouse and human immune systems. This potential may be valuable for a heterogeneity study of this construct.[60]

In conclusion, to overcome the persistent infection, induction of targeted innate and adaptive immunity using a novel strategy is essential. The synthetic chimeric polytope containing several B- and T-cell epitopes appears to be a convincing method for this purpose. Here, we have described multi-epitopes of several *H. pylori* virulence factors fused to *Pseudomonas* flagellin as a TLR-5 agonist for effective induction of innate and adaptive responses against this pathogen in an animal model. This strategy may be a useful tool to control persistent infection by this bacterium.

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