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ORIGINAL ARTICLE

The chimeric UreB, FliD and Omp18 proteins for a sensitive and specific diagnosis of *Helicobacter pylori* infections

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ABSTRACT

Background and Objectives: *Helicobacter pylori* is known as the main cause of gastrointestinal diseases including gastritis, gastric ulcer and stomach cancer. Serodiagnosis of *H. pylori* infection is a noninvasive and rapid method but the efficiency of this method is highly dependent to the antigens used. This study evaluated the efficacy of recombinant UreB-Omp18 and FliD for serodiagnosis of *H. pylori* infection.

Materials and Methods: The genes encoding for *fliD*, *ureB*, and *omp18* was amplified by PCR and cloned into pET-22b and pET-28a vectors. The constructs were expressed in *E. coli* BL21 and purified by affinity chromatography. The antigenic properties and diagnostic potential of the recombinant proteins were analysed by immunoblotting and ELISA, respectively.

Results: The recombinant UreB-Omp18 and FliD with molecular weights of 48 kDa and 25 kDa were observed on SDS-PAGE and purified by the Ni-NTA column. The ELISA results showed that the sensitivity and specificity of recombinant UreB-Omp18 protein in serodiagnosis of *H. pylori* infection were 89% and 83%, respectively. Also, the sensitivity and specificity of the recombinant FliD protein were calculated to be 91% and 76%, respectively.

Conclusion: The results indicated that the recombinant UreB-Omp18 and FliD could diagnose *H. pylori* infection with high sensitivity and specificity.

Keywords: *Helicobacter pylori*; UreB; Omp18; FliD protein; Chimeric antigen

INTRODUCTION

Helicobacter pylori (H. pylori) is a Gram-negative, spiral-curved, microaerophilic bacteria found in the mucosal layers and apical surface of human stomach epithelial cells (1, 2). It is estimated that more than 90% of the population in developing and eastern countries and half of the global population are infected with *H. pylori* (3). Several virulence factors, including the FlaB, FlaA, VacA, CagA, HspB, UreA and Omp18 proteins, are involved in the bacterium's pathogenicity (2, 4). A strong association has been established between *H. pylori* and gastritis, peptic ulcer, gastric cancer, atrophic gastritis, lymphoma, and

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non-Hodgkin's lymphoma. Every year, gastric cancer kills about 700,000 people globally. On the other hand, the prevalence of *H. pylori*'s antibiotic resistant strains is increasing (5).

Early detection of *H. pylori* infection is the primary approach for controlling the infection and its associated disorders. Several diagnostic techniques with different levels of accuracy and sensitivity have been developed to diagnose *H. pylori* infection. These approaches are considered invasive or non-invasive based on whether or not endoscopy is required. Serological methods are very useful for diagnosing *H. pylori* and are also commonly utilized for screening in epidemiological studies (6). Serological tests are inexpensive and quick, and their accuracy is unaffected by situations such as wound bleeding, stomach atrophy, or antibiotic use; and false-negative results are uncommon. The antigen(s) used in commercial kits determines the accuracy of serological tests (7). VacA, CagA, Omp18, UreA, UreB, and GroEL antigenic markers have been employed to identify anti-*H. pylori* IgG antibodies. The FliD protein is involved in flagellate production and has been considered a potential marker for *H. pylori*-associated illnesses (8). CagA, VacA, and GroEL have also been utilized to diagnose *H. pylori*-related gastric cancer. As a result, predictions can be made about the infection with highly pathogenic strains of *Helicobacter pylori* (9). The diagnostic kit outcomes have proven to be inadequate as a result of the genetic diversity within the strain, potential cross-reactivity with various gastrointestinal pathogens, or alterations in the immune system's response to bacterial antigens (10). Consequently, the utilization of antigens derived from indigenous strains has been suggested as a potential approach to enhance the sensitivity and specificity of diagnostic tests. The production of antigens through recombinant DNA technology is a crucial method in the development of more accurate and precise diagnostic tests. The utilization of this approach has facilitated the establishment of diagnostic tests using UreB, GroEL, CagA, Ggt, HcpC, ICD, VacA, and Omp18 (11).

In this study, the production of chimeric diagnostic antigens has been achieved through the expression and purification of a recombinant multi-antigen. Previous research has demonstrated the utility of UreB, FliD, and Omp18 antigens for *H. pylori* serological detection. It has been shown that the specificity and sensitivity of detection based on conserved FliD antigen is more than 90% (8). UreB, a subunit of urease, possesses a B cell stimulation epitope and constitutes approximately 10% of the proteins found in *H. pylori* (8). Roufi et al. conducted a study to investigate the antigenicity of the recombinant UreB protein and its ability to produce antibodies against *H. pylori* using ELISA on the serum of infected individuals. Analysis for antibodies in patients have been shown that UreB is highly immunogenic and could be used as a suitable antigen for the diagnosis and also in the preparation of a vaccine against *H. pylori* (12).

Omp18 from *H. pylori* is an outer membrane protein with little homology to similar proteins of other bacteria. It is capable to stimulates the immune system in *H. pylori* infected patients (13). In a study, Talebkhan et al. reported a sensitivity of 93.25% for rOmp18 in diagnosing infection. They showed that the Omp18 is a reliable serological marker for diagnosing the infection and can be used in screening studies (14). In the present study, the antigenic epitopes of FliD, UreB, and Omp18 proteins were used to create a chimeric protein. The purified fusion proteins exhibited a promising potential in detection of *H. pylori* infection.

MATERIALS AND METHODS

Bacterial strains and plasmids. *H. pylori* isolate used for amplification of target genes was isolated from an Iranian patient and verified by biochemical and molecuar methods. DNA extraction and plasmid isolation was carried out by commercial kit from GeneAll Co (GeneAll Co.Kr). Plasmids pET 22b and pET 28a were purchased from Novagen Co.

Gene amplification procedure. *H. pylori* genomic DNA was extracted using commercial DNA extraction kit following manufacturing instruction and analysed by ecectrophoresis on 1% agarose gel. PCR was used for amplification of genetic fragment encoding antigenic epitopes of UreB, FliD, and Omp18 proteins of *H. pylori* using specific primers (Table 1). The PCR program for amplification of the *fliD* included initial denaturation at 94°C for 30 s, followed by 31 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 30 s, extension at 72°C for 40 s, with a final extention at 72°C for 5 min. Amplification of *ureB* and *omp18* genes was performed at an initial denaturation temperature of 95°C for 5 minutes, followed by

Table 1. The sequence of primers used for PCR amplification of target genes.

Genes		Primers (5' to 3')	Restriction enzymes	Size (bp)
fliD	Forward	CATATGGCTCAAAGCATCACGGACG	NdeI and XhoI	624
	Reverse	CTCGAGGTCTGTGTTTTCTTCACTGGTG		
ureB	Forward	CATATGATGTATGGCCCTACTACAGGCGA	NdeI and BamHI	597
	Reverse	GGATCCTTGATCGGCTAAGCTTGCATC		
omp18	Forward-1	TGGAGGTGGtTCtATGGATAATAAGACTGTGGCC	BamHI	597
	Reverse-1	ATGGATCCGGTGGAGGTGGTTCCGGTGGAGGTGGtTCT		
	Forward-2	CCACCTCCACCCTTCATTAATTTGACATCCACTCTTCTG	HindIII	479
	Reverse-2	TAAAGCTTTCCACCGGATCCACCTCCACC		

30 cycles at 95°C for 20 s, 56°C for 20 s, and 72°C for 40 s using Eppendorf thermocycler (Germany). The PCR reaction contained PCR mastermix, forward and reverse primes(5pM each) and 100 ng of *H. pylori* genomic DNA. The PCR products were analysed using 1% agarose gel along with 1 Kb size marker. PCR products were then purified by PCR product purification kit (GeneAll) according to the manufacturere instructions.

Expression and preparation of recombinant proteins. PCR products of *ureB* and *omp18* were cloned in pET22b and *fliD* was cloned in pET28a vectors (Novgene, USA). pET28a vector double-digested with NdeI and XhoI enzymes (Fermentas USA) were used for *fliD* cloning. Also, The pET-22b vector was used for cloning of *ureB* and *omp18* by double digestion with NdeI-BamHI, and BamHI-HindIII(Fermentas, USA), respectively. Ligation of double digested PCR products into vectors were performed by T4 DNA ligase (Fermentas, USA).

Expression and purification. A single colony of *E. coli* BL21(obtained from Pasteur Institute of IRAN) containing 22b-UreaB-Omp18 or 28a-FliD were cultured in LB broth medium containing ampicilun and kanamycin, respectively. After reaching the logarithmic growth phase, 1 mM of IPTG was added at OD of 0.6 to the culture tube and incubated for another 16h hours at 37°C. The expression of recombinant proteins were analysed by 12% SDS-PAGE. For purification of recombinant antigens, the starter culture of expression clones were added into 500 ml of LB culture media contained appropriate antibiotic and incubated at 37°C for 2-3 h. After induction with 0.3 mM IPTG, the cultures were continued for another 8 h at 25°C. The bacteria were harvested and disrupted by sonication and the soluble fraction was used for purification. For purification, two ml of Nickel-NTA resin was packed into a 10 ml column, and after washing and equilibration, sonicated bacterial supernatant was passed through the column. The column was washed with 10 column volume of washing buffer containing 10 to 25 mM Imidazole. After washing the bound proteins were eluted with 250 mM Imidazole.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). 20 to 30 µl of each samples were mixed with the 4× sample buffer and boiled for 10 minutes before running on a SDS-PAGE gel containing 5% and 12% stacking and resolving gels, respectively. Electrophoresis was performed under a voltage of 20 to 30 mA for 90 minutes. After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue for 30 minutes and destained by washing with warmed water. A low molecular weight protein size marker (Fermentas) were used for comparison.

Sample collection and evaluation of the efficacy of recombinant chimeric antigen. Purified chimeric antigens were used to design an ELISA test. Blood samples were taken from 200 patients with gastric and duodenal symptoms referred to endoscopy unite of Shahid Madani Hospital in Khoy, Iran, in 2018-2019. The samples included 200 sera from infected and 200 healthy control individuals. Patients with consumption history of proton pump inhibitors or antibiotics in the last 3 months were excluded. 5 ml of blood was taken from each person and after centrifugation and serum separation, it was kept at -20°C until analysis.

Immunoblotting. Western blot was used to determine the integrity of the prepared antigens and their reactivity with patients' sera. For this purpose,

the antigens were separated using SDS-PAGE gel and transferred to the PVDF membrane. The sera of patients were analyzed for presence of anti-*H. pylori* FliD, Omp18-UreaB antibodies. Accordingly, serum containing anti-*H. pylori* antibody was diluted 1:200 and added on the membrane containing antigens. The membrane was washed three times with PBS buffer and incubated with an anti-human IgG antibody (Sigma, USA) at a dilution of 1:5000. Finally, the membrane was washed and stained with Diaminobenzidine (DAB) substrate (15). After the bands appeared, the membrane was washed with distilled water and dried.

ELISA test. Serum samples were analyzed using recombinant chimeric antigens. For this, the appropriate concentration of antigens was determined using the checkerboard method. 100 µl of antigens (5µg/ml in carbonat-bicarbonate buffer) were coated into wells of the ELISA plate overnight at 4°C. On the next day, the plate was washed with PBS containing 0.05% tween-20 (PBS-T), and blocked with 300 µl of PBS-T containing 3% bovine serum albumin (BSA). The plate was incubated for 1 hours at room temprature and washed three times. 100 µl of sera were added in 1:100, 1:200, and 1:400 dilutions. Two wells containing PBS-T were used as control. The plate was incubated at room temperature for 60 minutes. After 4 times washing, 100 µl of of horseradish peroxidase (HRP)-conjugated anti-human IgG antibody diluted with blocking buffer in a ratio of 1:2000 were added to the wells. After one hour incubation at room temperature and rinsing, 100 µl of tetramethylbenzidine (TMB) substrate was added to each well. The plate was incubated in a dark chamber at room temperature for 10 minutes. The reaction was stopped using 100 µl of 1N sulfuric acid, and the absorption was read at 450 nm with an ELISA reader.

Statistical analysis. The results of the experiments were statistically analyzed by ANOVA and Tukey tests using SPSS v22. A p-value <0.05% with 95% confidence level (95% CI) was considered as significant. The graph used in the study were drawn by MS-Excel 2019. The following formula was used to determine the ELISA sensitivity and specificity:

$$Sensitivity = \frac{TP}{TP + FN}$$

$$Specificity = \frac{TN}{TN + FP}$$

TP stands for true positive, TN stands for true negative, FP stands for false positive, and FN stands for false negative. Further positive predictive value (PPV) and negative predictive value (NPV) were calculated as follows:

$$PPV = \frac{TP}{TP + FP}$$

$$PV = \frac{TN}{TN + FN}$$

Ethical statement. The present study was approve by ethical committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1397.056). After explaining the details of the research, written consent was obtained from all the study participants.

RESULTS

Descritpion of the subjects features. The features of the patients are described in Table 2. *H. pylori* infection was higher in women than men. Furthermore, around 82% of the patients (164 persons) suffering from gastritis, of which approximately 111 (68%) were women. However, 58 patients (29%) have gastric ulcers, with women making up the majority of these cases. The patients' drug usage status is also included in Table 2. As a result, approximately106 people (53%) and 159 people (80%) of them take antiacid and antibiotic medications. Table 2 also includes information of patients' urease status. Urease was positive in 52 and 46 percent; and negative in 48 and 50 percent of men and women, respectively.

Gene amplification. Amplification of *H. pylori ureB*, *omp18*, and *fliD* genes resulted in PCR products of 597, 479, and 624 bp, respectively (Table 1). Subsequently, the PCR products were purified and used for cloning by restriction digestion method (Fig. 1).

Gene expression and purification of proteins. After amplifying, the genes *ureaB* and *omp18* were cloned into pET 22b and the *fliD* the pET28a vectors. The results are shown in Fig. 2. Vectors harboring *fliD* showed and insert of 624 bp. Screening of *omp18* clones by PCR and T7F-T7-R primers is shown in Fig. 2. The band length for this gene was obtained with a fragment of the 679 bp. The empty vectors formed a

Table 2. Frequency (percent) for features of the patients in the study adjusted to Gender.

Feature		Gender		Feature		Gender	
		Male	Female			Male	Female
H. pylori Infection				Antibiotic Usage			
	Yes	44 (61)	110 (86)		Yes	48 (66)	111 (88)
	No	28 (39)	18 (14)		No	25 (34)	16 (12)
Gastiritis				Endoscopy			
	Yes	53 (72)	111 (88)		Yes	25 (35)	41 (32)
	No	21 (28)	15 (12)		No	48 (65)	86 (68)
Gastric Ulcer				Urease			
	Yes	20 (28)	38 (30)		Positive	39 (52)	56 (46)
	No	53 (72)	89 (70)		Weak Positive	0 (0)	3 (2)
Antiacid Usage					Negative	37 (48)	62 (50)
	Yes	38 (52)	68 (54)		Weak Negative	0 (0)	3 (2)
	No	36 (48)	58 (46)				

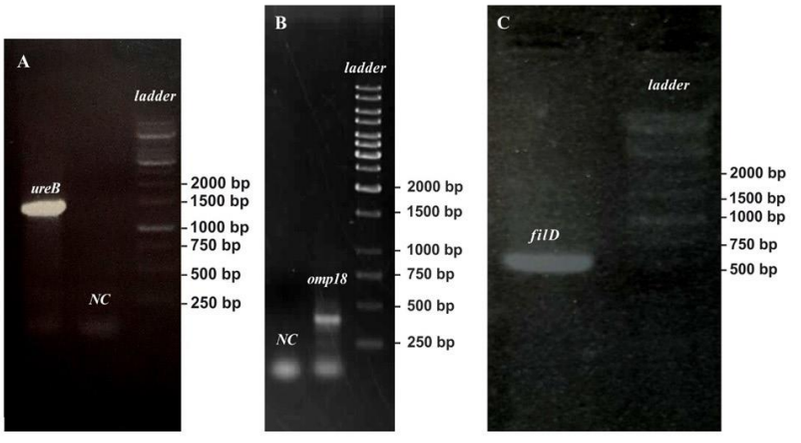

Fig. 1. Amplification of *H. pylori* genes. A) *ureB* amplicon with 597 bp size. B) *omp18* gene product with 679 bp size. C) *fliD* gene amplicon with the size of 624 bp. NC, negative control.

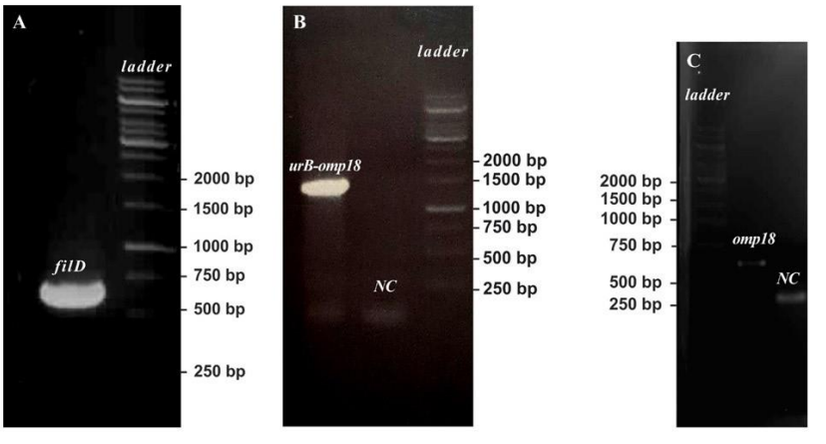

Fig. 2. Cloning confirmation by PCR. A) *fliD* product in vector pET28a with 624 bp product size. B) *omp18* in vector pET28a with size 679 bp. C) Electrophoresis of *uraB-omp18* product in pET 22b vector with about 1300 bp size. NC, negative control.

band in the range of 200 bp in the PCR product. The pET-22b vector was used for cloning *ureB* and *omp18*. The size of the *ureB-omp18* was about 1300 bp. The amplification results of these two genes using PCR are shown in Fig. 2.

Analysis of expression by SDS-PAGE. The expression of recombinant proteins was assessed using SDS-PAGE. IPTG was used to induce expression of *fliD, omp18,* and *ureB-omp18,* and production of recombinant proteins was observed at approximately 25, 20, and 48 kDa positions for FliD, Omp18, and UreaB-Omp18 fusion protein, respectively. Gene constructs were transformed in *E. coli*, and the resulting proteins were purified after expression. Production of recombinant proteins at approximately 25, 20, and 60 kDa positions was observed for FliD, Omp18, and UreB-Omp18 fusion genes, respectively (Fig. 3). The molecular weight of the recombinant protein UreB-Omp18 in the chromatographic test was 48 kDa, which was obtained due to the addition of his-tag.

Immunoblotting results. At this stage, the reactivity of recombinant proteins was investigated using the immunoblotting method, and the results confirmed the accuracy of the reactivity of the proteins as antigens with the serum of patients infected with *H. pylori* (Fig. 4). The western blot was used to evaluate the accuracy of the recombinant antigen's reactivity with antibodies in the serum of infected patients. As shown in Fig. 4, the purified recombinant fusion protein reacted with high intensity to the antibodies in the patients' serum and became positive.

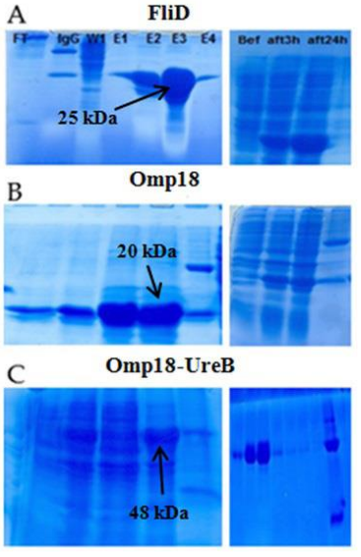

Fig. 3. Recombinant protein expression in *E. coli* and purification. A) *fliD* gene expression in *E. coli* (right) and purification of FliD protein with a molecular weight of 25 kDa (left). B) Expression of the omp18 gene in *E. coli* (right) and purification of recombinant Omp18 with a molecular weight of 20 kDa (left). C) Expression of *omp18-ureB* construct in *E. coli* (left), and purification of recombinant protein with molecular weight 48 kDa (right)

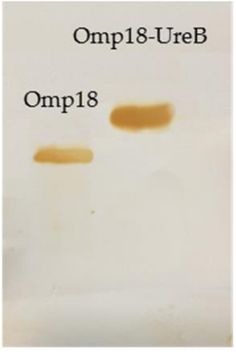

Fig. 4. Western blot results. Column 1, Omp18 antigen (molecular weight 20 kDa), and column 2, Omp18-Urea B antigen (molecular weight 48 kDa)

Evaluation of FliD and UreB-Omp18 reactivity by ELISA test. The efficiency of recombinant chimeric protein UreB-Omp18 and FliD in the serological diagnosis of *H. pylori* infection was assessed with an ELISA test. For this purpose, sera containing antibodies of *H. pylori* was prepared in dilutions of 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600. A constant dilution of the recombinant proteins (10 µg/ml) was used as the antigen.

In the ELISA test with UreB-Omp18, 89/100 (89%) of *H. pylori*-positive cases exhibited higher absorption than the cut-off, (2 × mean of negative samples) whereas 11/100 (11%) showed lower absorption and were negative. Out of 100 samples tested with negative sera, 83/100 (83%) had lower absorption than cut off, and 17/100 (17%) had higher absorption than cut off in the ELISA test with *omp18-ureB* antigen. Out of 100 positive samples tested for FliD antigen, 91/100 (91%) showed higher absorption than the cut-off, while 9/100 (9%) showed lower absorption than the cut-off. Out of 100 *H. pylori*-negative serum samples, in the FliD antigen test, 76/100 (76%) showed lower absorption than cut off, whereas 24/100 (24%) had intermediate or higher absorption than cut off.

The statistical comparison showed that the sensitivity and specificity of recombinant UreB-Omp18

protein in serological diagnosis of *H. pylori* infection were 89% and 83%, respectively. The sensitivity and specificity of the recombinant FliD protein were calculated to be 91% and 76%, respectively.

DISCUSSION

Most people in the world are infected with *H. pylori* in their lifetime. Chronic infection with this bacterium occurs in some patients as gastric ulcer or gastric cancer (16-18). *H. pylori* is classified as a human class I carcinogen (19). Therefore, preventing the disease with early diagnosis and treatment can reduce the mortality rate in infected patients.

There are several methods for diagnosing *H. pylori* infection, which are classified as invasive or non-invasive. The serological approach is one of the most important non-invasive diagnostic methods. Serological techniques are also appropriate for demographic surveys (20). Current routine tests, however, cannot differentiate between a newly developed infection and an infection already existing in the body (21). For many years, various purified antigens have employed in ELISA for serodiagnosis. This technique is costly, but it improves the test's sensitivity and specificity (11, 22-25). Furthermore, due to antigenic variations between prevalent strains in different groups, native strains must be used for antigen manufacturing. As a result, this study aimed to create a recombinant construct encoding multiantigen from native strain for the detection of *H. pylori* infection. Economically, this method is cost-effective and has significant sensitivity and specificity. Three *H. pylori* antigens *ureB, fliD,* and *omp18* were amplified and employed as antigens in the current investigation to prepare a diagnostic ELISA test. *H. pylori* largely expresses the *ureB* virulence factor, a particularly good antigen for diagnostic kits (26). This bacterium also produces significant amounts of UreB antigen, which is necessary to develop bacterial colonies (27). It has been reported that the use of UreB in combination with other antigens increases the sensitivity and specificity of *H. pylori* detection (22-24).

One of the most important pathogenic components of *H. pylori* is flagella protein (FliD), which leads to antibody responses in almost all infected people (8). Furthermore, the detection of FliD antibodies is associated with a 10.6-fold increase in the incidence of gastric cancer (28). As a result, evaluating the level of antibodies against FliD is one of the most reliable methods for detecting *H. pylori* infection (2).

The outer membrane protein HP1125 (Omp18) has been shown to specifically react with patient sera (29, 30). Omp18 is a peptidoglycan-dependent lipoprotein and structurally related to Omp18 from *Campylobacter jejuni (C. jejuni)* and Omp22 from *H. pylori*. Omp18, a key immunological antigen found in *C. jejuni,* is being used as a poultry vaccine (31, 32).

This investigation used immunoblotting and ELISA to measure the antigenicity of three recombinant antigens, FliD, UreB, and UreB-Omp18. The results showed that recombinant proteins are a suitable option for use in commercial diagnostic kits. Previous researches employed Omp18, UreB, and FliD antigens, separately. Because preparing recombinant proteins is time-demanding and expensive, preparing chimeric recombinant antigens is exceptionally rational. Furthermore, the virulence factors of *H. pylori* are typically quite large and challenging to combine and so do not produce the desired result. As a result, the technique used in this study to produce a recombinant chimeric antigen can be significantly more successful and convenient in diagnostic research as well as making vaccines against *H. pylori* (33-35).

In previous studies, when Omp18 and UreB antigens were combined, they demonstrated greater sensitivity and specificity for *H. pylori* detection compared to commercial kits (13, 14). Omp18 is a conserved bacterial antigen with limited homology to its equivalents in other bacteria. As a result, this protein is introduced as a suitable antigen for serological diagnostics (13, 14). Previous research has found that the Omp18 protein is particularly effective in identifying infection and can be employed in producing commercial kits (14). FliD antigen was previously described as an essential and sensitive factor for serological detection of *H. pylor*i infection, with more than 97% of serum samples responding positively to this antigen (8). Given that three antigens, FliD, UreB, and Omp18 of *H. pylori* are antigenic and induce high immune response in human, they might be utilized to make vaccines after testing their efficiency.

CONCLUSION

According to the findings of this study, chimeric

antigens generated from FliD, UreB, and Omp18 can be utilized successfully in serological tests to detect *H. pylori* infection. The simultaneous use of all three virulence factors can increase the sensitivity and specificity of serological diagnosis by more than 80%. This demonstrates that fusion proteins of *H. pylori* virulence antigens can be a cost-effective and rapid method for reliable detection of *H. pylori* infection in large populations.

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