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ORIGINAL ARTICLE

Immunological detection of 34 KDa outer membrane protein as a functional form of OipA in clinical isolates of *Helicobacter pylori*

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ABSTRACT

Background and Objective: An outer membrane protein (OMP) of *Helicobacter pylori* namely OipA, is an important virulence factor associated with peptic ulcer and gastric cancer risks. The purpose of this study was to isolate the 34 KDa OMP of *H. pylori* and evaluate its immunogenicity in experimental animals for rapid detection of more virulent *H. pylori* isolates.
Material and Methods: Sarcosine insoluble fraction of membrane proteins (OMPs) were prepared from 15 clinical isolates of *H. pylori* and their profiles were analyzed by SDS-PAGE. Two out of 15 isolates which demonstrated higher expression for apparent 34 KDa proteins were selected. Under optimal conditions, 34 KDa protein was recovered from 5% SDS-Agarose gel, purified and injected into the New Zealand white rabbits with Fruend′s adjuvant in multiple stages with two weeks intervals. Collected antiserum was purified through affinity chromatography with Sepharose column and its titer was determined by ELISA. Specific immune response was demonstrated by Dot blot and western blotting methods.
Results: The titer of antibody was determined about 1/3000 and western blotting demonstrated a 34 KD-protein .Screening of various strains by Dot blot method for its presence showed that its expression was more frequent in strains isolated from the patients with more severe pathology.
Conclusion: High titer obtained for pAbs antibody, suggested the high immunogenicity of this protein in experimental animals. Detection of 34 KDa OMP in strains isolated from the patients with more severe pathology proposes the possible application of this pAbs in detecting more virulent strains of *H. pylori*.

Keywords: *Helicobacter pylori*, Outer membrane proteins, 34 KD protein, OipA

INTRODUCTION

Helicobacter pylori colonizes the gastric tissue of one-half of the world population and causes gastritis, peptic ulcer, mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma (1). During the last decades, there has been many efforts to understand the role of putative bacterial virulence factors and probably the most studied factor is the *cag* pathogenicity island (PAI), which encodes a type IV secretory apparatus that injecting the CagA protein into eukaryotic cells (1-4). Other putative virulence factors are the surface-exposed components of *H. pylori* that mediate tight adherence to gastric-epithelium, most of which are members of the outer membrane protein (OMP) family (4-6). The Hop (Helicobacter outer membrane proteins) and Hor (Hop-related proteins) family consist of 33 members, of which BabA (HopS), SabA (HopP), AlpA, AlpB, homB, homA, and OipA (HopH) are the best studied (7-9). Association of BabA (blood group antigen binding adhesin) and SabA (sialic acid binding adhesin), with peptic ulcers and probably with gastric cancer has been reported (10-14). Among these OMPs, a 33-35 kDa outer inflammatory protein

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(OipA) is more important since its gene is located on the *H. pylori* chromosome approximately 100 kb from the *cag* PAI and its presence is associated with enhanced interleukin-8 secretion. Studies performed by several investigators have demonstrated that *oipA* mutagenesis results in reduced bacterial adherence to gastric epithelial cells but does not alter IL-8 secretion in vitro, probably due to the presence of *cag* Pathogenicity Island in the studied isolates (9, 14).

Expression of *oipA* is regulated by the slipped-strand repair mechanism based on the number of CT dinucleotide repeats in the 5' region of the *oipA* gene in a way such that its switch on would be functional and its switch off would be nonfunctional (15-16). This is consistent with the observation that an OipA-positive status has been significantly associated with the presence of duodenal ulceration and gastric cancer, high *H. pylori* density and severe neutrophil infiltration (15-18).

The purpose of this study was to isolate and evaluate the immunogenicity of this predicted 34 KD protein in experimental animals for immunological and operational purpose.

MATERIALS AND METHODS

Bacterial strains. *H. pylori* strains isolated from adults suffering from gastrointestinal disease were included in this study. For isolation of primary *H. pylori* strains, the antral biopsies were processed according to the previously described protocol (19). Briefly, the strains were cultivated on modified campy-blood agar plates containing brucella agar base (Merck), 10% Sheep Blood, and antibiotics (Merck). After the initial growth of *H. pylori* from biopsy specimen, pure cultures were produced from each isolate and identification was performed by Gram staining, positive urease, oxidase and catalase tests. Biochemical identification was confirmed by PCR amplification of *H. pylori 16S rRNA* and *UreC*. Strains identified as *H. pylori*, were stored in skim milk containing 15% glycerol (Merck), and 10% fetal calf serum (Gibson) at -70°C.

Isolation of outer membrane proteins. Three day cultures plates were harvested by centrifugation (Beckman, 12,000 × g, 20 min, 4°C) and washed twice with phosphate buffer saline (PBS, pH: 7.4). The suspension was sonicated on ice a few times (45 s at 120W each) until the cells were disrupted (as determined by microscopic examination). Cell debris was removed by centrifugation and the crude membranes were recovered from the supernatant by centrifugation (Beckman) at 50,000-× g for 1h at 4°C. The resulting pellet was suspended in PBS (pH 7.4) and mixed with 10% sodium N-lauryl-sarcosine (Sigma). Then the preparation was incubated at 37°C for 30 min before it was centrifuged at 50,000-× g for 60 min at 4°C. After two fold washing, the suspension was incubated at 100°C for 5 min to denature bacterial proteins. The suspension was reheated at 100°C for 2 min and then diluted in SDS-PAGE solubilization buffer. Its total protein concentration was determined by the Bradford method with bovine serum albumin as the standard (20). The aliquots were stored at -20°C for SDS-PAGE.

SDS-PAGE analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 10% PAGE in Bio-Rad Mini Protein system. Pre-stained protein molecular marker (Fermentas) was used as a standard molecular weight marker. Gels were either stained with Coomassie blue or used for immunoblotting.

Isolation of apparent 34 KDa MW OMP. An apparent 34 KDa OMP was identified on profile of several *H. pylori* strains. A strain expressing the interested protein in higher amount was selected and its OMPs were electrophorezed into a 5% SDS-Agarose gel. The slice containing an apparent 34 MW protein was cut, unstained (Tris 50 mM, SDS 3%, ethanol 10%), reduced into the small pieces (2-3mm), and then heated at 70-80°C in Tris 50 mM, EDTA 0.5 mM. After homogenization, the tube containing proteins was cooled in ice for 30 min and frozen at -80°C for two hours, then thawed. This step was repeated until near total recovery of the protein, and then its concentration was determined by Bradford method (20).

Production of monospecific polyclonal antibodies (pAbs). For obtaining pAbs against an outer membrane protein with apparent MW of 34 KDa, a white new Zealand rabbit (Razi institute, Iran), was immunized subcutaneously by purified antigen with Freund's adjuvant three times at 2 weeks intervals, and blood samples were taken two weeks after third injection. The resulting pAbs were purified using affinity chromatography (Sepharose 4B coupled with

Table 1. *H. pylori* strains used in this work.

Strain	Name	Group
S1	153	I
S2	153M	I
S3	157	I
S4	23P	I
S5	23Eh	I
S6	23	I
S7	1B	II
S8	17B	II
S9	23B	II
S10	14B	II
S11	ES1	III
S12	ES2	III
S13	ES3	III
S14	ES3M	III
S15	Moba	III

H. pylori OMPs) to obtain a monospecific pAbs against the 34 KDa protein.

Titration of sera by Indirect ELISA. The microtiter plates (Nunc) were coated with 100 µl antigen [purified OMPs antigens from *H. pylori*, 10 µg per well] in PBS overnight at 37 ° C. Plates were blocked for 2 hours at room temperature with 1% BSA in PBS, and washed with PBS containing 0.05% Tween 20. The sera were diluted to 100-fold with the addition of PBS containing 1% BSA to the wells and incubated for 2 h at 37°C. After a wash step (6 times with PBS+0.05% Tween 20), the wells were incubated with horseradish peroxidase-conjugated with goat anti-rabbit IgG (Sigma) for 1 h. After further washing, the substrate solution (TMB) was added and incubated. After 15 min, color development was stopped and absorbance was measured at 450 nm by a microplate reader (SCO GmbH). Each sample was tested in duplicate.

Immunoblotting (Dot and Western blotting). Reactivity and specificity of developed anti-*H. pylori* antibodies produced in rabbit were analyzed by Immunoblotting (Dot and Western blotting). For Dot blotting, 5 µl of antigenic suspension (used for immunization of rabbit), was absorbed into the nitrocellulose filter. After drying, the blocker solution containing 1 % BSA was added, and incubated at 37°C for 1h. After a wash step (PBS, 3 min), serial diluted sera were added and incubated for 2h, and washed three times by agitation (each 10 min) with PBS+0.05% Tween 20. Presence of immune complex was detected by addition of horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma), washing, incubation for 1 h. After further washing a substrate solution containing 3, 3', 5, 5'-tetramethyl benzidine (TMB, Sigma) was added for color development. The colored spots produced in nitrocellulose were visualized by comparing with negative controls (total antigens from *Salmonella spp.*, *Klebciella spp.*).

For Western blotting, the SDS-PAGE protein profiles were transferred onto nitro-cellulose sheets using a semidry electrotransfer apparatus (Akhtarian Co, Iran). The filters were processed according to the same protocol described above for dot blotting. The titer of sera used in this procedure was the lowest titer of sera which produced a significant absorption for *H. pylori* antigens, compared to those of negative controls.

RESULTS

In the current study, the OMPs profiles of 15 *H. pylori* strains were analyzed by SDS-PAGE. Among them, two strains demonstrating higher expression for apparent 34 KDa proteins were selected for further analysis. Table 1 demonstrates the strains used in this work. The strains namely group I, correspond to the strains stored at -70° after multiple subcultures.

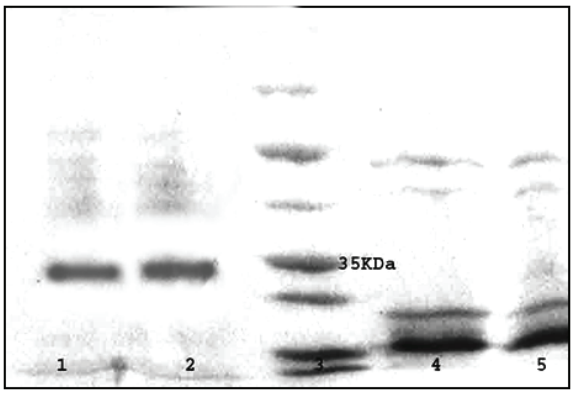

Fig. 1. SDS- PAGE of OMPs isolated from 4 various strains.
1-2: S12, and S13 (strains investigated for pAbs production), 3: MW marker, 4: S3, 5: S7

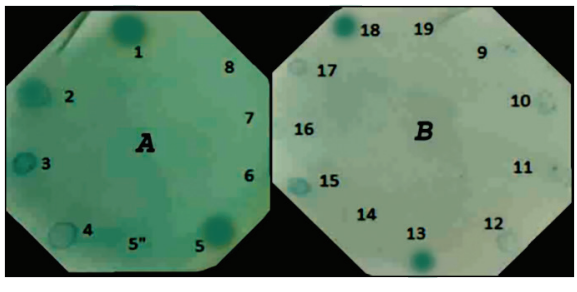

Fig. 2. Dot blot screening of various *H. pylori* strains by specific pAbs
1: Antigen used for immunization, 2:S3. 3: S13, 4: S12. 5":S11, 5: Purified recombinant OipA, 6-7: Negative controls (antigens from *Salmonella* and, *Klebciella*) 8: S2, 9:S1,10: S4, 11: S5, 12: S10, 13: S15, 14: S6, 15: S14, 16: S7, 17: S9, 18: S13, 19:S8

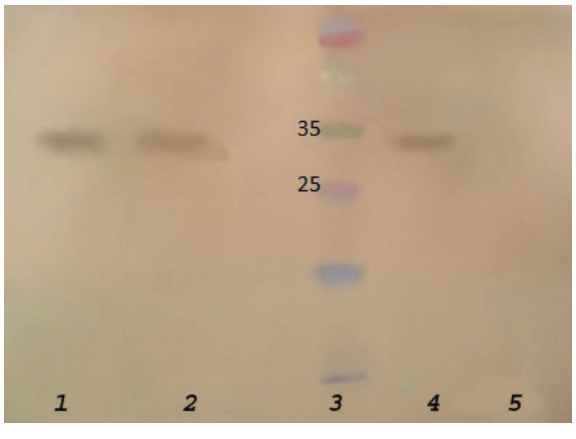

Fig. 3. Western blot analysis of *H. pylori* strains using specific anti-33 KDa pAb.
1: S13 (strain investigated for pAb production), 2: S15, 3: MW standard, 4: positive control: a recombinant OipA protein (21), 5: negative control.

Fig. 1. demonstrates the SDS-PAGE profile of four out of 15 *H. pylori* strains used in this work. those of group II were also obtained from our collection of strains stored at -70°C. The group III corresponded to the strains more recently isolated from the patients at hospital.

The serum collected from rabbit after six weeks was examined for antibodies specific for selected antigen using in-house ELISA. Titers comparison of polyclonal sera before and after affinity chromatography showed that after chromatography, its reactivity against designed antigen was more specific. The titer of sera before chromatography was about 1/3000, but after purification, lower titer was obtained. However, the ELISA values from different dilutions of purified pAbs, showed no reaction for negative controls.

Screening of strains by Dot blot showed specific reaction with *H. pylori* antigens obtained from various strains (Fig. 2)

DISCUSSION

In the current study, 15 strains were investigated for presence of 34 KDa OMP. However, in their SDS-PAGE profile, high expression of predicted 34 KDa OMP band was detectable in only two of them (Fig. 1). The majority of the clinical *H. pylori* strains investigated in this work corresponded to those that were conserved at -70° after multiple laboratory passage. It was accepted that laboratory or clinical strains which have undergone multiple passages lost potential colonization factors (21). So, lower or non-expression of 34 KDa OMP in the strains of group I would be related to multiple passages of these clinical strains in laboratory before OMP preparation. The results of this experiment revealed that purified 34 KDa antigen injected into the rabbit is highly immunogenic. Furthermore, we observed that immunological detection of the same protein by specific pAb would provide a sensitive method for detection of functional protein in various *H. pylori* strains (Fig. 2).

To understand that the OMP investigated in this work corresponded to OipA, we detected a recombinant OipA protein by our pAb in the same experimental conditions (Fig. 3). The importance of OipA, a 34 KDa outer membrane protein on gastroduodenal pathogenesis is apparently increasing (16, 22-25). In the initial stage of infection, binding via OipA would be more important since its expression explains it's switched "on" status which can initiate the inflam-

matory cascade (18, 26). By PCR-related methods, it will be difficult to predict the presence of a functional OipA protein. However, its detection by sensitive and specific immunoblot would provide a simple and accurate method for detecting expression of *oipA* as an important virulence factor in *H. pylori***.** An easier method for primary screening of *H. pylori* isolates for presence of its functional protein would be Dot blotting since it requires very smaller amount of antigen without performing SDS-PAGE and transfer.

So, the use of specific pAbs for detection of functional form of oipA in various *H. pylori* strains would be an accurate method in determining the more virulent strains of *H. pylori*.

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