Cloning and Sequence Analysis of Gene Encoding OipA from Iranian Patients with Helicobacter pylori Infection

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Background: Outer inflammatory protein A (OipA) is one of the important adhesins of H. pylori and a valuable candidate for vaccine development. Its gene is under “on-off” switch status which correlates with OipA protein expression.

Objectives: We aimed to obtain a recombinant OipA clone (with “on” status) from an Iranian clinical isolate.

Materials and Methods: A clinical H. pylori isolate demonstrating high expression for an outer membrane protein (OMP) with an apparent MW of 33-35 kDa was selected. oipA specific primer was designed according to oipA sequences from B8 strain. The purified PCR-product was sequenced and submitted to Gene Bank. The pET-28a plasmid and E. coli DH5α were used for cloning and transformation. The recombinant plasmid was transferred to E. coli BL21 (DE3). Extracted proteins were purified and presence of OipA was confirmed by western blotting using both anti His-tag monoclonal antibody and anti-OipA specific antibody.

Results: The sequence of the oipA gene and the MW of the purified recombinant OipA protein consisted on 924 bp and 33-35 kDa, respectively. Its identity with other published oipA genes was 92-96%; highest identity was observed with that of a Mexican oipA clone, obtained from a H. pylori strain associated with severe symptoms.

Conclusions: Recombinant oipA clone obtained in this work, may be a functional oipA gene with “on” status, associated with more severe outcomes of H. pylori infection.

Keywords: Cloning; Helicobacter pylori; Iran; OipA

1. Background

Helicobacter pylori is a human pathogen that infects half of the world’s population and causes gastritis, gastric and duodenal ulcers, gastric cancer, and Mucosa Associated Lymphoid Tissue lymphoma. Adhesion of H. pylori to gastric epithelial cells may be the first important step in the pathogenesis of H. pylori infection (1). Together with CagA and VacA that are two well-described virulence factors of H. pylori, OipA (outer inflammatory protein A) is an important virulence factor which plays role in the special interaction with host cell membrane and favors production of a proinflammatory cytokine, IL-8 (2).

Numerous studies on OipA have demonstrated that the oipA gene expression into a 33-35 kDa protein is regulated by the slipped-strand repair mechanism that changes the number of CT dinucleotide repeats in the 5’-region of the oipA gene. Thus, a switch “on” status is functional and switch “off” is nonfunctional (3, 4).

The oipA gene has been cloned from various regions of the world, including South America, North America, Europe, Asia, East Asia and Japan (4-6). However, molecular comparison of the recombinant clones has demonstrated some polymorphism in the variable regions of the oipA gene suggesting geographical location related differences among them. Furthermore, Torres-Morquecho et al., (2010) have found the significant positive selection acting on variable regions of oipA, that could be related to differences in its risk for gastroduodenal diseases among different human populations (6).

There is no study from Iran that has reported cloning the full gene of oipA. In a work performed by Teymournejad et al., in Iran (2013), not only a universal laboratory H. pylori strain (26695) have been used for molecular cloning, but also the sequences of their clone contained only 783 bp, and the related protein had 30 kDa (7). Yamaoka et al., (2000), the leading
investigator working in this field, has reported that functional OipA protein had 34 kDa weight (4) and the full gene of oipA had almost 930 bp.

2. Objectives
Considering the important role of OipA in the pathogenesis of H. pylori infection and its variability among the strains isolated from various geographical regions, we aimed to obtain a recombinant OipA from a strain isolated from the Iranian patients. For selection of strain, we have taken into consideration two important factors: selection of strain from patients with H. pylori related symptoms, as well as OipA expression ability of the strain.

3. Material and Methods
3.1. Isolation and Identification of H. pylori Strains
To isolate the H. pylori strain, antral biopsies obtained from the patients suffering from the symptoms of H. pylori infection was processed according to the previously described protocol (4). The protocol was approved by the local Bioethics Committee. Written informed consent was obtained from all the patients prior to the participation in the study. In brief, the strains were routinely cultivated on Brucella agar (Biolife; Albimi, Italiana S. r. L. Viale Monza 272-20128 Milano-Italia) supplemented with 5% sheep blood, polymyxin-B (8 mg.l-1), amphotericin (2 mg.l-1) and vancomycin (6 mg.l-1) and were incubated for 3-7 days under microaerophilic conditions (10% CO 2 and 95% humidity) at 37ºC. Identification of H. pylori strains were performed by morphology of the colonies, Gram staining, biochemical tests (oxidase, rapid urease, and nitrate) and PCR amplification of H. pylori 16SrRNA and ureC.

3.2. Selection of Strain for Cloning
Clinical H. pylori strains were screened for the presence of a 33-35 kDa protein on SDS-PAGE profiles of OMPs. OMPs were prepared according to the previously described method (8). A strain (S15), demonstrating high expression for a protein with an apparent MW of 33-35 kDa, was selected for cloning of the oipA gene. For this strain, the vacA/cagA genotype/status was also determined according the previously determined PCR primers and PCR protocols (5, 9).

3.3. Amplification of oipA Gene
H. pylori DNA was obtained using the DNA extraction kit (SinaClone, Iran) according to the manufacturer’s instructions. The primer sequence was designed according to the available genome sequence of oipA from H. pylori B8 strain (Table 1). The oipA forward primer with BamHI endonuclease site was 5'-CCG-GATCCATGAAAAAAGCTCTTTACT-3' and the oipA reverse primer with an XhoI endonuclease site was 5'-CGCGGCTCGAGTTAATGTTTTTTAAGTT-3'. The PCR reaction was performed in 25 μl mixture containing the DNA template (100 ng), 0.2 mM of each dNTP, 0.4 μM of each primer, 2 mM MgCl2, 0.5 U Taq DNA polymerase and PCR buffer (1x) (SinaClone, Iran). Following denaturation at 95ºC (2 min), the fragment was amplified through 30 cycles as follows: 95ºC (30 s), 65ºC (45 s), 72ºC (90 s); the extension was continued at 72ºC for 10 min. Aliquots of the PCR product were electrophoresed in a 1% (W/V) agarose gel in 0.5× Tris-borate-EDTA buffer (90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA), and stained in DNA green safe (Sinaclone, Iran) at 4 ppm and predicted 924 bp was visualized under UV.

3.4. Sequence Analysis
Before cloning, the PCR product of the oipA gene was sequenced (Macrogen, Korea) and the results of sequencing were submitted to GenBank that were accepted under accession number KJ816695 (http://www.ncbi.nlm.nih.gov/nuccore/KJ816695).

Table 1. Specification of primers in PCR reaction

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>vacA s</td>
<td>ATGGAAATACAAACACAC</td>
<td>CTGCTTGAAATGGCAAAAC</td>
<td>285</td>
</tr>
<tr>
<td>vacAm</td>
<td>CAATCTGCTCCAAACACAC</td>
<td>GCGTCAATAATCCGAAAG</td>
<td>642</td>
</tr>
<tr>
<td>ureC2</td>
<td>AAGGCCCCTTCTTCTCAAGC</td>
<td>TTTGCTCAACAACTTACCC</td>
<td>123</td>
</tr>
<tr>
<td>ureC1</td>
<td>TGATAAGGCGGAGGACCAAC</td>
<td>ACGGAGCCCACCTTAAAGC</td>
<td>295</td>
</tr>
<tr>
<td>cagA</td>
<td>ATGACTAACGAAACTATTGATC</td>
<td>TATCCGCAAGAGTGAATTAG</td>
<td>821</td>
</tr>
</tbody>
</table>

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Sequence comparison between oipA gene obtained in this work and those previously obtained in other parts of the world was also performed.

3.5. Construction and Purification of Recombinant oipA

To construct a recombinant plasmid containing the oipA gene, the PCR product was purified with the Ambiclean kit (Vivantis Technologies Sdn Bhd, Malaysia). The pET-28a containing His tag sequences and purified PCR product were both digested by two endonucleases including the BamHI and XhoI (Thermo Scientific, USA) to make cohesive ends. The resulting fragments of pET-28a and oipA were ligated by T4 DNA ligase (Thermo Scientific, USA). The pET-oipA construct was transformed into E. coli DH5α and was cultured on Luria Bertani (LB) Agar with Kanamycin (30 μg.ml⁻¹). The recombinant clones were isolated and the recombinant plasmid was extracted for further verification by PCR and restriction enzyme digestion.

In order to obtain a better expression, the extracted recombinant plasmid construct was transformed into E. coli BL21 (DE3). Induction was performed in LB broth by 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside).

3.6. Purification and Identification of Recombinant OipA Protein

OipA protein was purified by Ni-NTA affinity chromatography (Novagen, USA) and its presence was confirmed by Western blotting using anti-His-tag monoclonal antibody. For this purpose, the purified protein was separated by 12% SDS-PAGE and was transferred onto a PVDF membrane. The membrane was blocked with blocking solution containing albumin 1% for 1 h at 25°C. The blot was directly treated with 100 mU.ml⁻¹ Anti-His₆ Peroxidase solution (Roche) and incubated for 1 h at 25°C. The blot was washed with washing solution (3×, each 5 min). Predicted desired band was detected by TMB (3, 3, 5, 5-Tetramethylbenzidine) as substrate.

3.7. Production of Monospecific Polyclonal Antibodies (pAbs) Against OipA and Specific Identification of Recombinant OipA

For this purpose, a white New Zealand rabbit (Razi institute, Iran), was immunized, subcutaneously by purified antigen (OipA) with Freund’s adjuvant three times at 2 week intervals, and blood samples were taken two weeks after third injection. The resultant pAbs were purified using affinity chromatography (Sepharose 4B coupled with H. pylori OMPs). For Western blotting, the SDS-PAGE containing proteins were transferred onto nitro-cellulose sheets using a semi-dry electrotransfer apparatus (Akhtarian Co, Iran). The filters were processed according to the protocol previously described (10).

4. Results

The selected clinical H. pylori strain (S15), which was isolated from a patient with severe gastritis expressed a protein with an apparent MW of 33-35 kDa. Its vacA/cagA genotype/status determined by PCR was s1m2 vacA and cagA positive, respectively (Figure 1). The product of the PCR amplification for the oip-A gene was 924 bp. The result of restriction digestion of recombinant pET-28a plasmid confirmed the presence of the oip-A gene (Figure 2). The result of sub-cloning into E. coli BL (DE3), as well as its PCR product was similar to those of E. coli DH5α. The purified protein from the OipA expression vector E. coli BL (DE3) showed an apparent MW of 34 kDa (Figure 3 A-B).

The purified OipA protein was detected by anti-His-tag monoclonal antibody in Western blotting and confirmed the presence of 34 kDa protein (Figure 3C). Specific identification of recombinant OipA with anti H. pylori OipA by Western blotting was demonstrated in Figure 4.

The DNA sequences of the oipA gene from this
Iranian isolate was compared with 25 oipA DNA sequences (E-value=0). Identity of Iranian oipA genes with other published oipA genes in GeneBank was in the ranges 92-96% (Table 2, Figure 5). Identity of oipA gene obtained in the present work with that of JHP0581 oipA gene from China (AF479754), was 94% (5). Its identity with the oipA from Japan and USA was 92-94% (AF233660-AF233683), respectively (4) and with that of the Mexican (FJ899816-FJ899835) was 93-96% (6). Also, its identity with oipA from H. pylori strain and standard 26695 H. pylori strain were 94% and 95%, respectively. The CT repeat of the Iranian oipA gene had five CT repeat at the 5′ end of the strand.

5. Discussion
To obtain a protective vaccine against H. pylori infection, numerous investigators have evaluated antigenic properties of H. pylori virulence factors including urease, catalase, VacA, CagA, NapA, GroES, AlpA, BabA, HpaA and SOD recombinant proteins (11-16). Among them, OipA may be one of the most important as it is present in 97.5% and 70% of patients with peptic ulcer and chronic gastritis, respectively (4). Furthermore, it was demonstrated that expression of oipA by H. pylori, enhances the level of IL-8 in serum by increasing the neutrophil infiltration in the gastric mucosa (17). However, there are many unanswered questions about its in-vivo expression status, polymorphism and regulation. It was suggested that the isolates containing the cag pathogenicity island (cagA positive status), typically also have oipA with functional status “on”, probably for their inter-related regulation (18). For this purpose, in the present work, a strain with cagA positive status was selected for molecular cloning (Figure 1). Furthermore, production of a functional OipA protein requires intact signal region of the gene and the
absence of any mutation downstream of the gene. For this purpose and for molecular cloning, we selected a strain expressing well the 33-34 kDa OMP. In addition, to evaluate the association of oipA status with H. pylori related clinical symptoms; we used a local clinical strain isolated from patients with severe clinical symptoms. Successful cloning of entire oipA gene able to express the 33-34 kDa protein would explain that this is the first work obtaining a functional oipA clone in Iran (Figures 2-4). By comparison of DNA sequence of this Iranian clone with the sequences of other oipA genes cluster, high grade similarity was observed with Mexican oipA and with that of Chinese oipA to a lesser extent (Table 2, Figure 5). The oipA polymorphism, which is well documented by Torres-Morquecho et al. (2010), has demonstrated a possible relationship to the severity of H. pylori-related symptoms and outcomes. This relationship is observed more in the isolates from south regions (6). The fact that the strain used in this work has been isolated from a patient with more severe symptoms, association of this 33-34 kDa protein with more severe outcome of H. pylori infection in Iranian population may also be suggested. Furthermore, presence of more identity between oipA of this work and that of Mexican, would explain its similar role in the risk for gastroduodenal diseases among different human populations. However, comparison of complete oipA gene sequences from multiple Iranian patients may better clarify this suggestion.

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Conflict of interest
The authors have no substantial financial or commer-
cial conflicts of interest with the current work or its publication.

Authors’ Contributions

Mahboubi M and Falsafi T, designed the study and wrote the manuscript, also provided vital analytical tools; Mahboubi M performed the majority of experiments; Sadeghizadeh M, participated to analytical tools and critically reading of the manuscript.

References


Figure 5. Phylogenic tree of the oipA gene from Iranian isolates (S15) with oipA from Gene Bank


