Research Article

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

Mohaddese Mahboubi [1,*], Tahereh Falsafi [1], Majid Sadeghizadeh [2]

[1]Department of Biology, Faculty of Basic Sciences, Alzahra University, Tehran, IR IRAN
[2]Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, IR IRAN

[*]*Corresponding Author*:Mohaddese Mahboubi, Department of Biology, Faculty of Basic Sciences, Alzahra University, Tehran, IR IRAN. Tel: +98-2188058912, Fax: +98-2188058912, E-mail: mahboubi@barijessence.com

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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* $DH_5\alpha$ 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

Table 1. Specification of primers in PCR reaction

Primers	Forward (5'-3')	Reverse (5'-3')	Product size
vacA s	ATGGAAATACAACAAACACAC	CTGCTTGAATGCGCAAAC	285
vacAm	CAATCTGTCCAATCAAGCCAG	GCGTCTAAATAATTCCAAGG	642
ureC2	AACGCCCTTTCTTCTCAAGC	TTCGCTCACAAACTTATCCC	123
ureC1	TGATAAGCGCGAGCCACAAC	ACCGGAGCCACCTTATAAGC	295
cagA	ATGACTAACGAAACTATTGATC	TATCGCCAAGAGTGAATTTAG	821

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 *Bam*HI endonuclease site was 5′-CCGGATCCATGAAAAAAGCTCTCTTACT-3′ and the *oipA* reverse primer with an *Xho*I endonuclease site was 5′-CGCGGCTCGAGTTAATGTTTGTTTTTAAAGTT-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 $MgCl_2$, 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).

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 *Bam*HI and *Xho*l (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* $DH_5\alpha$ and was cultured on Luria Bertani (LB) Agar with Kanamycin (30 $\mu g.ml^{-1}$). 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-His_6 Peroxidase solution (Roche) and incubated for 1 h at 25ºC. 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

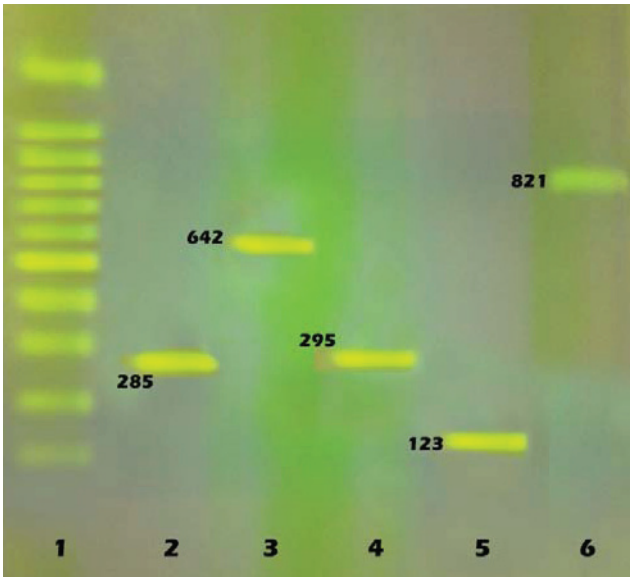

Figure 1. The results of PCR amplification *vacA* (*s* ,*m*), *cagA*, and *ureC*. Lane 1: MW standard, Lane 2: *vacAs1*, lane 3: *vacAm2*, lane 4: *ureC1*, lane 5: *ureC*2, and lane 6: *cagA* according to the Table 1

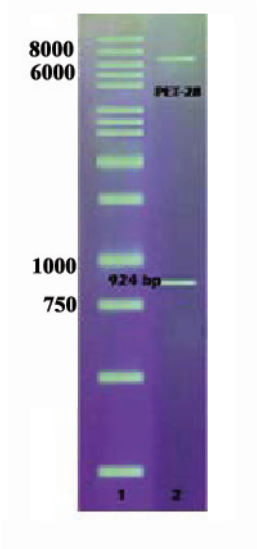

Figure 2. Representative agarose gel for demonstrating the oipA-pET28a construct after digestion by *Bam*HI and *Xho*I. Lane 1: DNA marker; Lane 2: *oipA* (924 bp) and pET-28a after digestion (5369 bp)

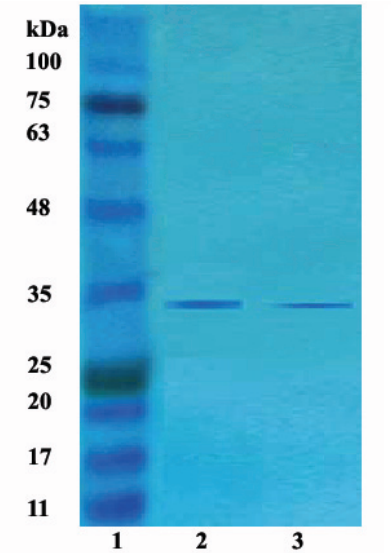

Figure 4. Western blot analysis of OipA recombinant clone with anti-OipA specific antibodies. Lane 1-prestained protein MW marker, lane 2- recombinant OipA clone, lane 3-OipA from strain S15 (strain used for cloning)

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* B8 *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.

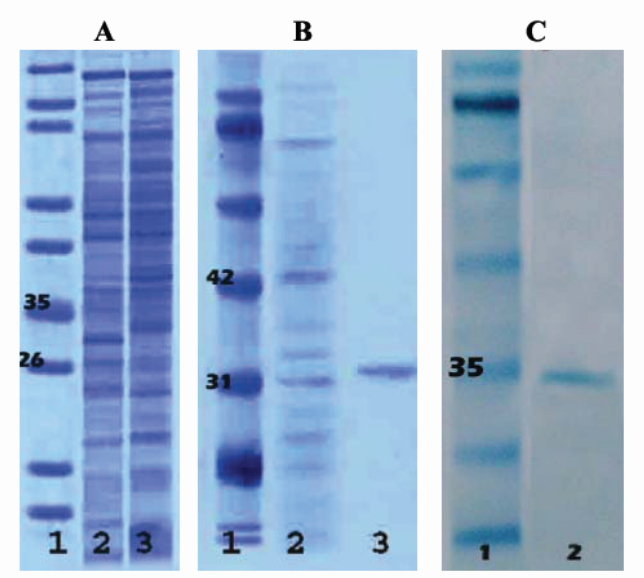

Figure 3. Protein expression in *E. coli* BL (DE3) on SDS-PAGE gel. A: Lane 1-MW marker, lane 2-control (-), lane 3-Recombinant clone. (Coomassie stained); B: Purified recombinant protein: Lane 1- MW marker, lane 2-control (-), lane 3-recombinant clone. (Coomassie stained). C: Western Blotting with anti-His-tag monoclonal antibody: Lane 1-MW marker, lane 2-Recombinant clone

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-

Table 2. The blasting of the *oipA* gene from Iranian isolates with *oipA* from Gene Bank

Description of clone gene	Query cover	Identity	Accession	Reference	bp
clone 259f5oipA	87%	95%	FJ869826	(6)	860
clone 24a3oipA	88%	95%	FJ869815	(6)	863
clone 252a2oipA	87%	95%	FJ869823	(6)	861
clone 44c4oipA	87%	95%	FJ869818	(6)	857
strain SS1oipA	89%	94%	AF233683	(4)	896
clone 254a10oipA	88%	94%	FJ869824	(6)	881
clone 44c2oipA	87%	94%	FJ869817	(6)	855
clone 259a1oipA	87%	94%	FJ869835	(6)	866
strain GI2060oipA	89%	94%	AF233682	(4)	894
clone 69a2oipA	87%	94%	FJ869819	(6)	855
clone JHP0581oipA	89%	94%	AF479754	(5)	924
clone 309a11oipA	87%	94%	FJ869830	(6)	849
clone 236c6oipA	87%	94%	FJ869820	(6)	853
clone 33a1oipA	85%	94%	FJ869816	(6)	824
clone 251i4oipA	83%	94%	FJ869822	(6)	808
clone 256c2oipA	83%	94%	FJ869825	(6)	809
clone 261a1oipA	87%	93%	FJ869827	(6)	853
strain JK35oipA	87%	92%	AF233662	(4)	886
strain JK51oipA	89%	92%	AF233667	(4)	893
clone 307c4oipA	87%	96%	FJ869829	(6)	897
clone 550c12oipA	47%	95%	FJ869832	(6)	459
clone 248f9oipA	46%	95%	FJ869821	(6)	453
clone 475a3oipA	46%	95%	FJ869831	(6)	456
clone 291c11oipA	46%	94%	FJ869828	(6)	456
clone 249a5oipA	46%	94%	FJ869834	(6)	448

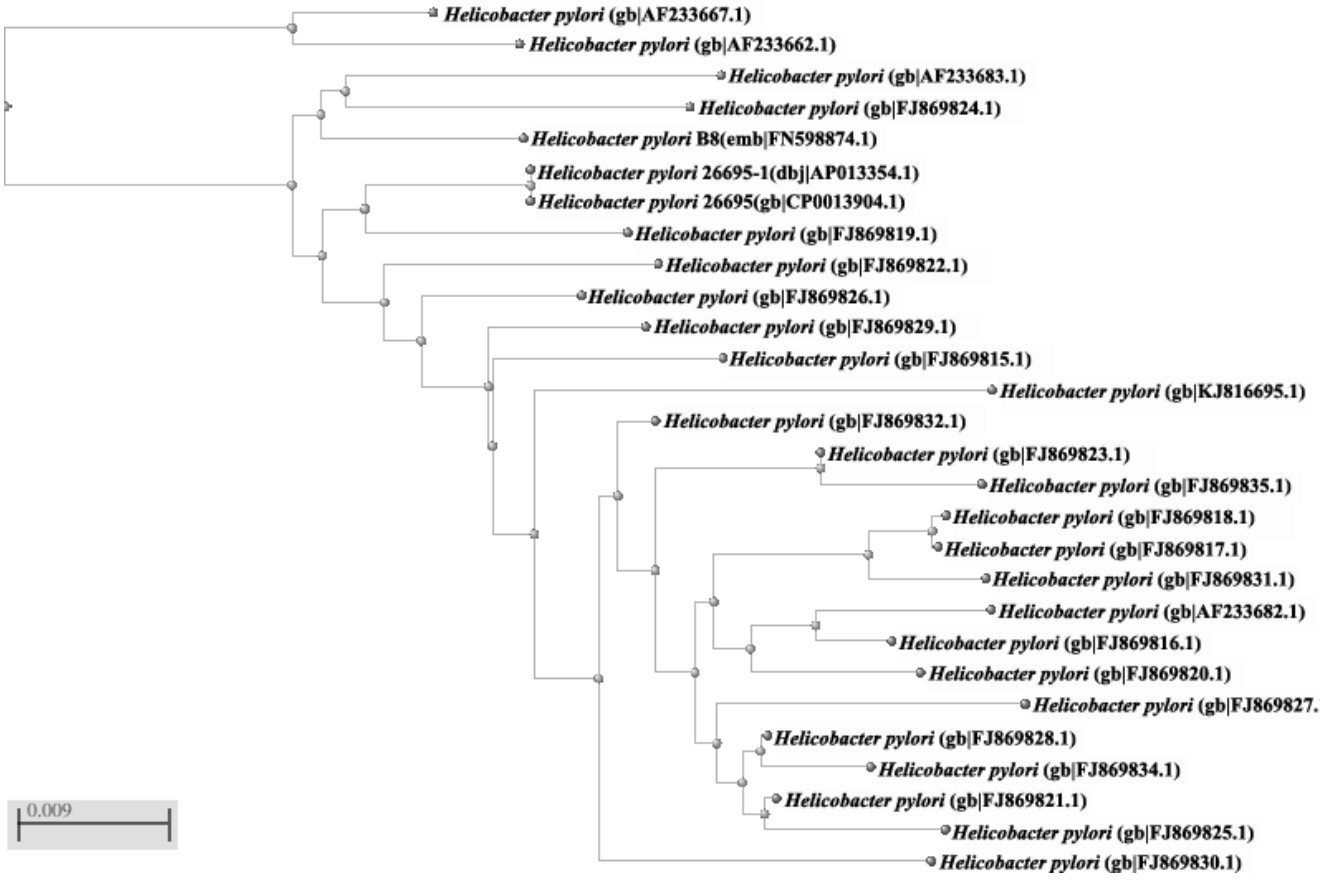

Figure 5. Phylogenic tree of the *oipA* gene from Iranian isolates (S15) with *oipA* from Gene Bank

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.

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