

# Cloning and expression of the heterogenic vacuolating cytotoxin from an Iranian *Helicobacter pylori* strain

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## Abstract

Several reports indicate that the nonconserved genes of *Helicobacter pylori* (*H. pylori*) in particular its cytotoxin are widely heterogeneous among various geographic locations and this is manifested at the protein level ranging from 5-15% which demands access to locally deduced protein antigens for inclusion into diagnostic kits and/or inclusion as a vaccine component for the target population. We have previously demonstrated such variations via PCR-RFLP analysis between Iranian and western *H. pylori* strains. *vacA* gene from a selected strain of the most prevalent RFLP category among Iranian strains, was partially sequenced which revealed 8.3% dissimilarity with reference strains at protein level. This drastic difference prompted us to subclone the *vacA* coding region into an expression vector to produce the recombinant protein. Full sequencing of the coding region demonstrated 8-9% amino acid difference with American and German reference strains. Recombinant protein expression yielded 4% of the total *E. coli* proteins. Histidine tag allowed for purification of the recombinant VacA using immobilized metal affinity chromatography (IMAC). Identity of the recombinant protein was repeatedly confirmed by Western blot analysis using patient serum, rabbit hyper immune serum as well as anti-His monoclonal antibody.

**Keywords:** *Helicobacter pylori*, *Escherichia coli*, Recombinant, cytotoxin, *vacA*, heterogeneity, cloning, expression, purification.

## INTRODUCTION

*Helicobacter pylori*, is a microaerophilic, Gram negative, spiral shaped bacterium (Warren, 1983) which

colonizes the gastric mucosa of primates (Shuto *et al.*, 1993; Dubois *et al.*, 1994). This bacterium induces antral type gastritis, peptic and duodenal ulcers (Covacci *et al.*, 1999). Prolonged *H. pylori* infection increases the risk of gastric adenocarcinoma and gastric MALT (Mucosa-associated lymphoid tissue) lymphoma (Dubois, 1995; Parsonnet *et al.*, 1991 and Parsonnet, 1993). The rate of *H. pylori* infection in developing countries consists the majority of the adult population (Telford *et al.*, 1994) while in developed countries this rate is an approximate 20-30% (Telford *et al.*, 1994). Seroprevalence of *H. pylori* infection in Iran exceeds 80% of the sampled populations (Massarrat *et al.*, 1995).

One of the important virulence factors of *H. pylori* is the vacuolating cytotoxin, VacA (Schmitt and Hass, 1994). This protein causes damage to gastric epithelial layer and results in gastric lesions and enhances peptic ulcer disease (Telford *et al.*, 1994). Oral vaccination of mice with recombinant VacA protein gives 70% protection against live challenge (Ghiara *et al.*, 1997). In order to ensure maximum protection, the immunizing antigen should mimic that of the infecting strain. Genotyping studies worldwide have repeatedly demonstrated the existing variations among *vacA* nucleotide and amino acid sequences of the *H. pylori* strains originating from different geographic regions. Opazo *et al.* (1999) and Muller *et al.* (2002) have studied ten conserved and non-conserved genes of *H. pylori* from Chilean strains in comparison with reference strains. Despite the high strain similarities among conserved genes, these investigators noticed an estimated 7% variation in the VacA amino acid sequences of the studied strains. Similar findings were previously reported by Yang *et al.* (1997) who found near 9% diversity of *vacA* gene from Taiwanese *H. pylori*

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strains as compared to reference strains. Such diversity exists among strains within the same geographic region (Ito *et al.*, 1998) as well as among multiple strains infecting individual patients (Wang *et al.*, 2002). These and other genotyping studies (Kersulyte *et al.*, 2000; Alm and Trust, 1999 and van Doorn *et al.*, 1998) have clearly demonstrated the vast heterogenic variations in *vacA* gene among *H. pylori* strains from different geographic regions. The characteristics, pathogenicity and diversity of *H. pylori* cytotoxin have been reviewed in detail (Talebkhani and Mohammadi, 2003). Devising an effective diagnostic or preventive measure for a reportedly heterogenic organism such as *H. pylori* to be applied for a highly infected population such as Iran (Massarrat *et al.*, 1995) requires careful development of the antigenic components. In order to maximize our chances along these lines it is crucial for the detecting and/or immunizing antigen to closely mimic that of the infecting strain.

Our previous studies of *vacA* PCR-RFLP analysis of Iranian *H. pylori* strains categorized the studied strains into three main categories, one of which was significantly most prevalent (Mohammadi *et al.*, 2001). Partial sequencing of the *vacA* gene belonging to a selected Tox<sup>+</sup> (s1m1) strain of this category, confirmed the extensive amino acid diversity with reference strains and lead us to hereby report, the cloning of the *vacA* coding region followed by complete sequencing, expression and purification of the recombinant VacA protein. The resulting locally developed recombinant *H. pylori* cytotoxin with 13.5 % purity has thus been made available for measure of its diagnostic and protective capacity against a cocktail of locally infecting clinical strains of *H. pylori*.

## MATERIALS AND METHODS

**Bacterial preparation:** *H. pylori* strain, Hel155, was cultured from a biopsy sample isolated from a dyspeptic patient undergoing endoscopy, who granted informed consent. Culture conditions were as previously reported (Radcliff *et al.*, 1997). Briefly, the isolated biopsy was cultured on Brucella agar plates containing trimetoprim (5 µg/ml), vancomycin (10 µg/ml), amphotericin B (2.5 µg/ml), supplemented with 5% sheep blood. Following 3-5 days incubation under microaerobic conditions, the grown bacteria were identified as *H. pylori* by routine microbiologic assays including urease, catalase, and oxidase tests as well as wet mount and Gram staining. Molecular identity confirmation was performed by PCR amplification

of the conserved *ureC* gene of *H. pylori* as described (Labigne *et al.*, 1991).

**Chromosomal DNA isolation:** Genomic DNA was extracted from cultured Hel155 strain using standard protocols (Ausubel and Frederick, 1994). Briefly, *H. pylori* colonies were washed with PBS (pH 7.2) and collected by centrifugation at 9,000 ×g for 8 min, followed by resuspension in lysis buffer [576 µl TE buffer, 30 µl SDS (10%) and 3 µl proteinase K (20 mg/ml)] and incubation at 37°C for 1 hr. Following addition of 100 µl of 3 M NaCl, the mixture was incubated at 65°C for 10 min. The genomic DNA was extracted with Phenol: Chloroform: Isoamyl alcohol (25:24:1) and precipitated by addition of 2 volumes of ethanol and centrifuged at 13,000 ×g for 5 min. The pellet was washed with 70% ethanol and dissolved in TE buffer or deionized water and stored at -20°C for further use.

**Polymerase Chain Reaction:** Two specific primers were designed based on the sequence of *vacA* gene from a Tox<sup>+</sup> (s1m1) reference strain (NCBI GenBank Accession No: U05676); hereafter referred to as cytoF and cytoR as forward and reverse primers respectively. CytoF was matched with the beginning of P34 fragment (region following signal sequence) with *NdeI* site added to its 5' end (5'GTC ATA TGG CCT TTT TTA CAA CCG 3'). CytoR was complementary to the near end of P58 fragment with insertion of *EcoRV* recognition sequence at its 5' end (5'CAT GCT GAT TGA TAT CGA CTA AAT TAG G3'). These two primers were designed to amplify a 2534 bp fragment of the *vacA* coding region which encodes the ~95 kDa mature peptide (Manetti *et al.*, 1995).

PCR amplification was performed under the following conditions: Initial 4 min at 94°C followed by 35 cycles of 60s at 94°C, 60s at 60°C, and 150s at 72°C. The resulting PCR product was extended for a further 7 min at 72°C. DNA amplification was performed with Pwo DNA Polymerase (Roche, Germany) with a replication error rate of  $8.5 \times 10^{-6}$  errors / bp (Mc Pherson and Moller, 2000).

**Cloning and subcloning of *vacA* gene:** The PCR product was run on and extracted from a 1% agarose gel using DNA extraction kit (Fermentas, Lithuania) according to the manufacturer's instructions. The eluted product was then cloned into *EcoRV*-digested pBluescript (pBsc), SK- cloning vector (Fermentas) according to routine protocols (Sambrook and Russell, 2000). The cloned gene was identified on the basis of

*vacA* restriction maps from reference strains available in the NCBI Database and was labelled as pBs-*vac*.

The inserted gene was re-isolated from pBs-*vac* by restriction digestion using *NdeI* and *EcoRV*. The isolated gene was then subcloned into pET23a, an expression vector (Novagen, USA) which was previously linearized via digestion with *NdeI* and *HincII*. The isolated *vacA* gene from pET23a was subcloned into pET28a (Novagen, USA), containing N-terminal histidine tag following digestion with *NdeI* and *NotI*. Identity of the subcloned gene in these two expression vectors was confirmed through restriction analysis. The confirmed vectors were named pVac23a and pVac28a, respectively.

**Expression of recombinant VacA protein in *E. coli*:** pVac28a was transformed into *E. coli* BL21 (DE3) (Novagen, USA) according to the standard protocols [Sambrook and Russell, 2000]. The cells were cultured in LB (Luria Bertani) broth containing kanamycin (15 µg/ml) and incubated at 37°C on a shaker incubator till the optical density at 600 nm reached 0.5 when expression was induced by Iso propylthio β-D-galactosidase (IPTG) (0.5 mM). One milliliter aliquots were collected before and after induction of expression for SDS-PAGE analysis.

**Identity Confirmation of recombinant VacA:** The above-mentioned samples were analyzed on an 8% SDS polyacrylamide gel (Sambrook and Russell, 2000). Western blotting was performed according to standard protocols (Ausubel and Frederick, 1994) using various antisera as the source of primary antibodies. These antisera include: (a) serum from an *H. pylori* sero-positive patient (1:100 dilution) which was previously adsorbed with *E. coli* antigens according to the standard protocols (Sambrook and Russell, 2000) in order to remove cross-reacting antibodies; (b) Mouse anti-His tag monoclonal IgG (Roche, Germany) (1:2000 dilution); (c) rabbit hyper-immune polyclonal serum which was developed as described by Johnstone and Thorpe, 1996. Secondary antibodies for the mentioned first antibodies were as follows respectively: (a) HRP-conjugated rabbit anti-human IgG (DAKO, Denmark) (1:1500 dilution); (b) HRP-conjugated rabbit anti-mouse IgG (1:2000 dilution); (c) HRP-conjugated goat anti-rabbit IgG (DAKO, Denmark) (1:1500 dilution). Specific protein marker on the SDS-PAGE, was a cocktail of *H. pylori* antigens consisting the following purified proteins: CagA (110-120 kDa); VacA (87 kDa); Urease B subunit and

HSP60 (55-68 kDa); Urease A subunit (30 kDa); other proteins (20 kDa and 14 kDa). This mixture was obtained from Micro Detect, Inc., Tustin, California, USA.

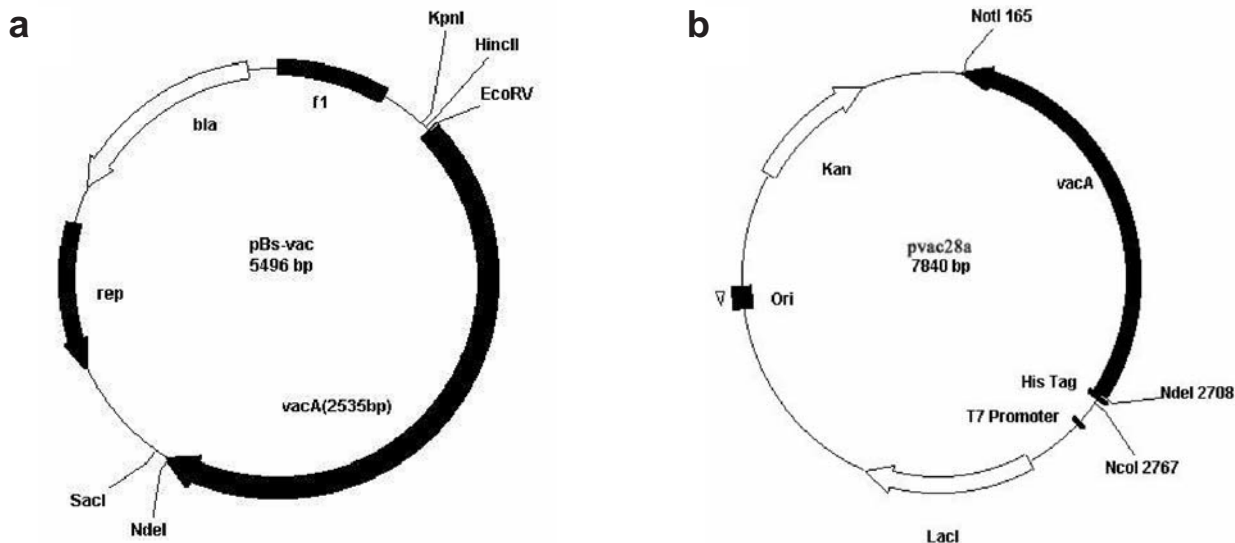
**Purification of recombinant VacA:** Semi-purification of rVacA from the inclusion bodies was performed as previously described (Sambrook and Russell, 2000). Briefly, cellular pellet was resolved in lysis buffer (300 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8) and disrupted by probe sonication (3 pulses of 30 sec each). Following the centrifugation of the sonicated suspension at 9,000 ×g for 10 min, the pellet was resolved in the above lysis buffer containing 8 M Urea. This solution was loaded on Ni-NTA resins (QIAGEN, USA) in an IMAC system and r-VacA molecules containing the N-terminal His-tag were attached to the resins and eluted from the column using an imidazole gradient.

**Gene sequencing:** The *vacA*-containing expression vector (pVac23a) was fully sequenced with T7 forward primer and internally synthesized primers (PRIM Inc. Italy).

**BLAST sequence Analysis:** Sequence similarity searches were performed via Basic Local Alignment Search Tool (BLAST) of the NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using Gene Runner (Hastings Software Inc.) and DNA Star software (DNASTAR Inc.). The following reference strains were used: U05676 (USA), NCTC11637 (Germany), NCTC11638 (USA), F38 (Japan), Z26883 (Germany), U29401 (USA).

## RESULTS

**Molecular characterization of *vacA* gene:** A clinical strain possessing the most prevalent *vacA* genotype according to PCR-RFLP analysis was chosen for amplification of the coding sequence of the gene. This was performed via PCR amplification with designed, specific primers and resulted in a ~2500 bp fragment of *vacA* gene. This segment has been reported to encode the ~95 kDa mature VacA protein (Manetti *et al.*, 1995). Cloning of the above PCR product into pBsc (SK-), a cloning vector, resulted in plasmid pBsc-*vac* (Fig. 1a). Restriction analysis through *NdeI* and *EcoRV* digestion, confirmed the identity of the cloned gene, according to the restriction maps of *vacA*



**Figure 1.** Schematic view of the cloning vector (pBluscript, pBsc, SK-, Fermentas) (a) and the expression vector (pET28a, Novagen) (b) plus *vacA* gene insert.

sequenced genes from the GenBank.

**Subcloning of *vacA* gene:** *vacA* gene was subcloned from pBsc-vac into pET23a and then into pET28a to produce the pvac28a expression vector, possessing N-terminal His.tag (Fig.1b).

**Sequence analysis and comparison.** The target clone (pET23a containing *vacA* gene) was confirmed via restriction analysis followed by full sequencing and submission to NCBI GenBank (Accession number: AY232454) (Fig. 2). Full sequencing revealed that our gene was cloned in frame with pET23a promoter, ribosome binding site, initiation codon (ATG) and sequences coding for the Histidine tag at the N-terminal region of the protein. Sequence comparison of the cloned *vacA* gene was made with other *vacA* gene sequences existing in GenBank, i.e. U05676 (USA), NCTC11637 (Germany), NCTC11638 (USA), F38 (Japan), Z26883 (Germany), U29401 (USA) through BLAST. The resulting comparison identified the entire coding region for Iranian *vacA* (2523 bp) to possess an estimated 7.8% dissimilarity with the mentioned sequences at the nucleotide level and 8.3% at the level of the predicted amino acid sequence.

**Expression of VacA protein in *E. coli*:** pVac28a which was constructed by insertion of 2523 bp fragment of *vacA* gene (encoding a ~95 kDa mature peptide) into the pET28a (expression vector) was introduced into *E. coli* BL21(DE3) and the expression of

recombinant protein was induced by IPTG. SDS-PAGE analysis of induced cells showed a band in the range of desired MW, of ~95 kDa (Fig. 3a).

Due to the presence of 6x His.tag at the N-terminal region of r-VacA, we could semi-purify the recombinant protein using Ni-NTA resins in an immobilized metal affinity chromatography procedure (Fig. 3b).

**Identity confirmation of recombinant VacA:** The primary identity confirmation of r-VacA was done via Western blotting with anti-His antibodies and the desired band was observed with an estimated molecular weight of 95 kDa (Fig. 4a). On the other hand, identity confirmation of the presence and conformation of r-VacA was done via two Western blottings. The first was performed with serum obtained from a patient infected with s1m1 strain of *H. pylori* (Fig. 4b) and the second was performed with rabbit hyper immune serum raised against *H. pylori* semi-purified antigens (greater than 50kDa), including native VacA and the desired results were observed in the range of ~95kDa (Fig. 4c).

## DISCUSSION

High prevalence of *H. pylori* infection in different populations, especially in developing countries, has clarified the critical value of detecting and therapeutic methods. Due to incomplete *H. pylori* eradication through antibiotic treatment, vaccination has been sug-

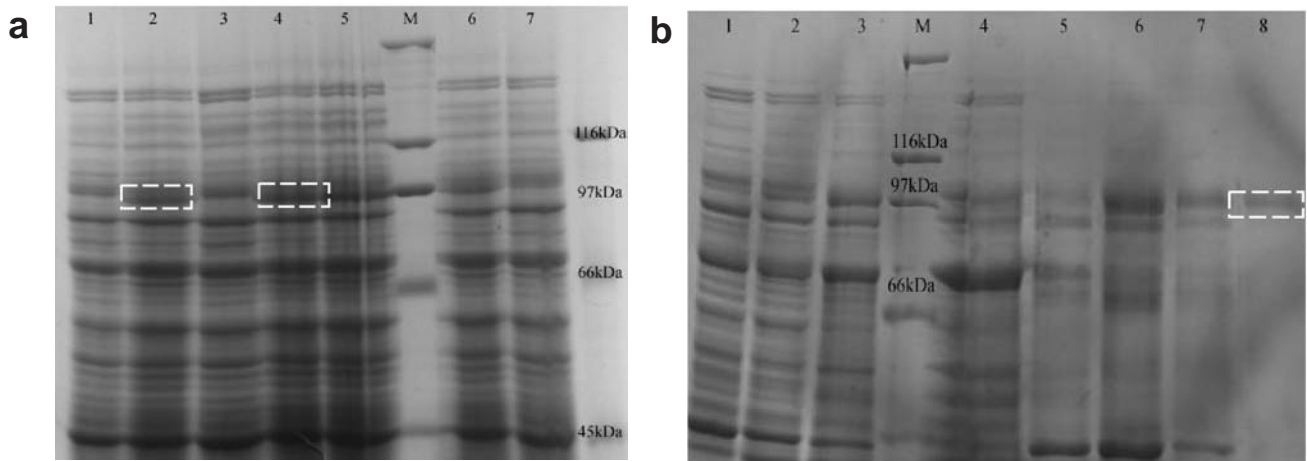
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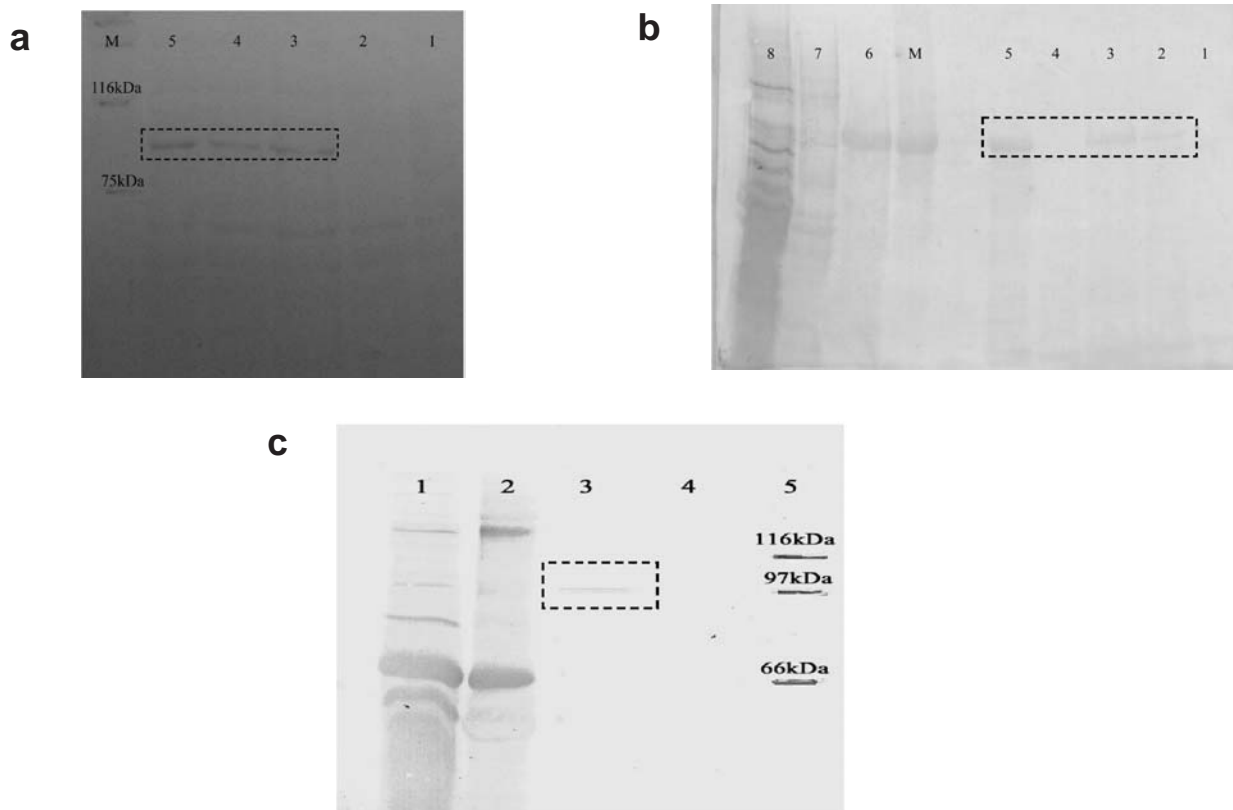
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**Figure 2.** Nucleotide and amino acid sequences of recombinant VacA (GenBank AY232454).



**Figure 3.** (a) Induction of VacA expression in *E. coli*, 1: Negative control (pET28a without *vacA* gene) (A4hrs); 2: C1, pvac28a (A4hrs); 3,4: C2, pvac28a (B, A4hrs); 5: C3, pvac28a (A4hrs); M: MW marker, 6: C4, pvac28a (A4hrs); 7: C5, pvac28a (A4hrs). (b) Purification of r-VacA, 1, 2: pvac28a (B, A4hrs); 3: supernatant of 8M Urea; M: Mw Marker; 4: Flow through; 5, 6: E1 (I, II) (50 mM Imidazol); 7, 8: E2 (I, II) (500 mM Imidazol).



**Figure 4.** Immunoblotting (a) Using anti His antibodies, 1, 2: Negative control (without pvac28a); 3: C1, pvac28a (A4hrs); 4: C2, pvac28a (A4hrs); 5: C3, pvac28a (A4hrs); M: Mw Marker. (b) Using patient serum, 1: Negative control (A4hrs); 2, 3: vac28a (B&A4hrs); 4: Supernatant of sonication; 5: Pellet of sonication; M: Rainbow marker; 6: supernatant of 8M urea; 7: MDI antigens; 8: *H. pylori* lysate. (c) Using rabbit hyper immune serum, 1: Sonicated *H. pylori* (Hel155); 2: MDI antigens; 3: Purified r-VacA (E2); 4: Negative control (without pvac28a); 5: Mw Marker.

gested as a preventive approach.

The vacuolating cytotoxin (VacA) has been approved as an effective factor involved in the pathogenesis of *H. pylori* (Tombola *et al.*, 1999 a, b; Garner and Cover 1996). On the other hand, its immunogenic properties have been demonstrated through oral administration into mice (Marchetti *et al.*, 1995; Ghiara *et al.*, 1997). Thus this protein can be a strong vaccine candidate against *H. pylori* infection.

During the year 1994, *vacA* gene was investigated by several groups in different parts of the world to identify the gene sequence and its heterogeneity between different strains of *H. pylori* (Schmitt and Hass 1994; Telford *et al.*, 1994; Phadnis *et al.*, 1994; Cover *et al.*, 1994). Most of these studies were performed to sequence the whole *vacA* gene from plasmid or Lambda DNA libraries of *H. pylori* genome.

Complex Helicobacter growth requirements, low production of native VacA from *H. pylori* cells, difficulties in native VacA purification (Cover and Blaser 1992), and finally, cytotoxic effects of native VacA on HeLa cells (Ulivieri *et al.*, 1996), limited the use of this protein in native structure as a vaccine candidate. A major study expressing recombinant VacA in *E. coli* was performed in pQE30 expression system with no peptidase cleavage site for digestion of His-tag from the final recombinant protein (Manetti *et al.*, 1995). Later in 1997, the recombinant protein was used for immunization of mice against Tox<sup>+</sup> strains of *H. pylori* thus confirming the preventive and therapeutic implications of this protein in the mouse model (Marchetti *et al.*, 1995; Ghiara *et al.*, 1997).

To determine the heterogeneity of native *vacA* gene and the deduced amino acid sequence, here, we report the cloning of *vacA* gene from a native *H. pylori* strain with s1m1 genotype, possessing the most prevalent RFLP profile (Mohammadi *et al.*, 2001), which encodes the mature VacA protein (~95 kDa), into a cloning vector for primary sequencing. In the early studies the whole *vacA* gene had not been isolated by one step procedures such as PCR but in this study, we have used a set of specific primers (according to the published nucleotide sequence of *vacA* gene from an s1m1 strain of *H. pylori* = U05676) for amplification and isolation of ~2500 bp *vacA* coding region in one step procedure with higher yield. On the other hand due to the large size of *vacA* gene fragment (~2500 bp), it was necessary to amplify this fragment by a DNA polymerase with high proof reading activity such as Pwo DNA polymerase.

The results showed about 10% heterogeneity between the reported *vacA* sequences and the Iranian

*vacA* gene which further instructs us in using the most prevalent local *H. pylori* strains for production of recombinant proteins for vaccination and diagnostic purposes. Despite the fact that the most prevalent *vacA* genotype of *H. pylori* among Iranian strains is s1m2 (Mohammadi, 2003), but we used a strain with s1m1 genotype because of the effective production of VacA protein in s1m1 genotypes of *H. pylori* (Rudi *et al.*, 1998) which is fully recognized by the host immune system.

Furthermore, we report on the expression of Iranian recombinant VacA in *E. coli* expression systems, pET23a and pET28a, designing 6x His-tag at the C- or N-terminal parts of the protein for purification through an Immobilized Metal Affinity Chromatography (IMAC) procedure. pET23a was also used previously for cloning and expression of *H. pylori* urease antigen in *E. coli* (Lee *et al.*, 1995). Using pET28a expression system, allows us to cleave the histidine tag, in case it is found necessary for proper conformation of the recombinant protein. On the other hand, another advantage of using pET28a instead of pET23 is the presence of fusion tag at N-terminal part of the multiple cloning site (MCS) which leads to RNA polymerase stability during transcription of the cloned gene. Using pET28a expression system yielded an approximately 5% r-VacA expression in *E. coli* total proteins and after semi-purification through IMAC procedure we achieved ~13% purity of r-VacA. According to the sequencing results, the high heterogeneity between recombinant VacA from the most prevalent Iranian *H. pylori* s1m1 strain and reference sequences of this protein, indicated that using local (native) genes is necessary in preparing recombinant vaccines against infections in the target population.

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## References

- Alm RA, Trust TJ. (1999) Analysis of the genetic diversity of *Helicobacter pylori*: The tale of two genomes. *J Mol Med.* 77: 834-846.
- Atherton JC, Cao P, Peek R, Tummuru M, Blaser MJ, Cover TL. (1995) Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. *J Biol Chem.* 270: 17771-17777.
- Ausubel I, Frederick M. (1994). *Current protocols in molec-*

- ular biology. John Wiley & Sons, Inc., USA.
- Covacci A, Telford JL, Del Giudice G, Parsonnet J, Rappuoli R. (1999) *Helicobacter pylori* virulence and genetic geography. *Science*, 284: 1328-1333.
- Cover TL, Blaser MJ. (1992) Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *J Biol Chem*. 267: 10570-10575.
- Cover TL, Tummuru MKR, Cao P, Thomson SA, Blaser MJ. (1994) Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. *J Biol Chem*. 269: 10566-10573.
- Dubois A, Fiala N, Heman-Ackah LM, Drazek ES, Tarnawski A, Fishbein WN, Perez-Perez GI, Blaser MJ. (1994) Natural gastric infection with *Helicobacter pylori* in monkeys: a model for spiral bacteria infection in humans. *Gastroenterology*, 106: 1405-1417.
- Dubois A. (1995) Spiral bacteria in the human stomach: The gastric Helicobacters. *Emerg Infect Dis*. 1: 79-85.
- Garner JA, Cover TL. (1996) Binding and internalization of the *Helicobacter pylori* vacuolating cytotoxin by epithelial cells. *Infect Immun*. 64: 4197-4203.
- Ghiara P, Rossi M, Marchetti M, Di Tommaso A, Vindigni C, Ciampolini F, Covacci A, Telford JL, De Magistris MT, Pizza M, Rappuoli R, Del Giudice G. (1997) Therapeutic intragastric vaccination against *Helicobacter pylori* in mice eradicates an otherwise chronic infection and confers protection against reinfection. *Infect Immun*. 65: 4996-5002.
- Ito Y, Azuma T, Ito S, Suto H, Miyaji H, Yamazaki Y, Kohli Y, Kuriyama M. (1998) Full-length sequence analysis of the *vacA* gene from cytotoxic and noncytotoxic *Helicobacter pylori*. *J Infect Dis*. 178: 1391-1398.
- Johnstone A, Thorpe P. (1996). *Immunochemistry in practice*. 3<sup>rd</sup> Ed., Blackwell Science, PP: 34-40
- Kersulyte D, Mukhopadhyay AK, Velapatino B, Su W, Pan Z, Garcia C, Hernandez V, Valdez Y, Mistry RS, Gilman RH, Yuan Y, Gao H, Alarcon T, Lopez-Brea T, Nair GB, Chowdhury A, Datta S, Shirai M, Nakazawa T, Ally R, Segal I, Wong BCY, Lam SK, Olfat FO, Boren T, Engstrand L, Torres O, Schneider R, Thomas J, Czinn S, Berg DE. (2000) Differences in genotypes of *Helicobacter pylori* from different human populations. *J Bacteriol*. 182: 3210-3218.
- Labigne A, Cussac V, Courcoux P. (1991) Shuttle cloning and nucleotide sequences of *Helicobacter* genes responsible for urease activity. *J Bacteriol*. 173: 1920-1931.
- Lee CK, Weltzin R, Thomas WDJr, Kleantous H, Ermak TH. (1995) Oral immunization with recombinant *Helicobacter pylori* urease induces secretory IgA antibodies and protects mice from challenge with *Helicobacter felis*. *J Infect Dis*. 172: 161-172.
- Manetti R, Massari P, Burrioni D, De Bernard M, Marchini A, Olivieri R, Papini E, Montecucco C, Rappuoli R, Telford JL. (1995) *Helicobacter pylori* cytotoxin: Importance of native conformation for induction of neutralizing antibodies. *Infect Immun*. 63: 4476-4480.
- Marchetti M, Arico B, Burrioni D, Figura N, Rappuoli R, Ghiara P. (1995) Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science*, 267: 1655-1658.
- Massarrat S, Saberi-Firoozi M, Soleimani A, Himmelmann GW, Hitzges M, Keshavarz H. (1995) Peptic ulcer disease irritable bowel syndrome and constipation in two populations in Iran. *Euro J Gastroenterol Hepatol*. 7: 427-433.
- Mc Pherson MJ, Moller SG (2000) *PCR, The basic from background to bench*. 1<sup>st</sup> published, BIOS Scientific Publishers Ltd., Oxford, UK.
- Mohammadi M, Doroud D, Mohajerani N, Oghalaie A, Colding H, Andersen LP. (2001) Enzymatic digestion profiles of the *Helicobacter pylori* cytotoxin and its association with peptic ulcer disease. *Int J Med Microbiol*. 291: 33.
- Mohammadi M, Oghalaie A, Mohajerani N, Massarrat S, Nasiri M, Bonnedsen M, Colding H, Andersen LP. (2003) *Helicobacter pylori* vacuolating cytotoxin and its allelic mosaicism as a predictive marker for Iranian dyspeptic patients. *Bull Soc Pathol Exot*. 96: 3-5.
- Muller I, Medina-Selby A, Palacios JL, Martinez P, Opazo P, Bruce E, Mancilla M, Valenzuela P, Yudelevich A, Venegas A. (2002) Cloning and comparison of ten gene sequences of a Chilean *Helicobacter pylori* strain with other *Helicobacter pylori* strains revealed higher variability for *VacA* and *CagA* virulence factors. *Biol Res*. 35: 67-84.
- Opazo P, Muller I, Rollan A, Valenzuela P, Yudelevich A, Garcia-de la Guarda R, Urra S, Venegas A. (1999) Serological response to *Helicobacter pylori* recombinant antigens in Chilean infected patients with duodenal ulcer, non-ulcer dyspepsia and gastric cancer. *APMIS*. 107: 1069-1078.
- Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelstein JH, Orentreich N, Sibley RK. (1991) *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med*. 325: 1127-1131.
- Parsonnet J. (1993) *Helicobacter pylori* and gastric cancer. *Gastroenterology Clinical North America*, 22: 89-104.
- Phadnis SH, Ilver D, Janzon L, Normark S, Westblom TU. (1994) Pathological significance and molecular characterization of the vacuolating toxin gene of *Helicobacter pylori*. *Infect Immun*. 62: 1557-1565.
- Radcliff F, Hazell S, Kolesnikow T, Doidge C, Lee A. (1997) Catalase, a novel antigen for *Helicobacter pylori* vaccination. *Infect Immun*. 65: 4668-4674.
- Rudi J, Kolb C, Maiwald M, Kuck D, Sieg A, Galle PR, Stremmel W. (1998) Diversity of *Helicobacter pylori vacA* and *cagA* genes and relationship to *VacA* and *CagA* expression, cytotoxin production, and associated diseases. *J Clin Microbiol*. 36: 944-948.
- Sambrook J, Russell DW (2000). *Molecular cloning*. 2Vol. 3<sup>rd</sup> Ed., Cold Spring Harbor Laboratory press, New York, USA.
- Schmitt W, Hass R. (1994) Genetic analysis of the *Helicobacter pylori* vacuolating cytotoxin: Structural



- similarities with the IgA protease type of exported protein. *Mol Microbiol.* 12: 307-319.
- Shuto R, Fujioka T, Kubota T, Nasu M. (1993) Experimental gastritis induced by *Helicobacter pylori* in Japanese monkeys. *Infect Immun.* 61: 933-939.
- Talebkhani Y, Mohammadi M. (2003) Vacuolating cytotoxin of *Helicobacter pylori*. *Iranian J Biotechnol.* 73-81: .
- Telford JL, Ghiara P, Dell'Orco M, Comanducci M, Burrioni D, Bugnoli M, Tecce MF, Censini S, Covacci A, Xiang Z, Papini E, Montecucco C, Parente L, Rappuoli R. (1994) Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *J Exp Med.* 179: 1653-1658.
- Tombola F, Carlesso C, Szabo I, de Bernard M, Reyrat J, Telford JL, Rappuoli R, Montecucco C, Papini E, Zoratti M. (1999a) *Helicobacter pylori* vacuolating toxin forms anion selective channels in planar lipid bilayers: Possible implications for the mechanism of cellular vacuolation. *Biophys J.* 76: 1401-1409.
- Tombola F, Oregna F, Brutsche S, Szabo I, Del Giudice G, Rappuoli R, Montecucco C, Papini E, Zoratti M. (1999b) Inhibition of the vacuolating and anion channel activities of the VacA toxin of *Helicobacter pylori*. *FEBS Lett.* 460: 221-225.
- Ulivieri C, Burrioni D, Telford JL, Baldari C. (1996) Generation of a monoclonal antibody to a defined portion of the *Helicobacter pylori* vacuolating cytotoxin by DNA immunization. *J Biotechnol.* 51: 191-194.
- van Doorn LJ, Figueiredo C, Sanna R, Pena S, Midolo P, Ng EKW, Atherton JC, Blaser M, Quint WGV. (1998) Expanding allelic diversity of *Helicobacter pylori* vacA. *J Clin Microbiol.* 36: 2597-2603.
- Wang J, Chi DS, Laffan JJ, Li C, Ferguson DAJ, Litchfield P, Thomas E. (2002) Comparison of cytotoxin genotypes of *Helicobacter pylori* in stomach and saliva. *Dig Dis Sci.* 47: 1850-1856.
- Warren JR. (1983) Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet.* 4: 1273.
- Yang JC, Wang TH, Wang HJ, Kuo CH, Wang JT, Wang WC. (1997) Genetic analysis of the cytotoxin-associated gene and the vacuolating toxin gene in *Helicobacter pylori* strains isolated from Taiwanese patients. *Am J Gastroenterol.* 92: 1316-1321.