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Curr Microbiol (2011) 62:325–330
DOI 10.1007/s00284-010-9694-2

Helicobacter pylori Omp18 and Its Application in Serologic Screening of Infection

Yeganeh Talebkhan · Fatemeh Ebrahimzadeh · Maryam Esmaeili · Leili Zamaninia · Azin Nahvijoo · Hossein Khedmat · Forouzandeh Fereidooni · Mohammad Ali Mohagheghi · Marjan Mohammadi

Received: 19 March 2010 / Accepted: 4 June 2010 / Published online: 23 July 2010
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Abstract *Helicobacter pylori* (Hp) is a major risk factor for gastrointestinal disorders including gastric cancer. We evaluated host serum antibody responses toward outer membrane protein18 in comparison with Urease A and B subunits. *omp18* and *ureA*-*ureB* gene fragments were PCR amplified, cloned, and expressed in *E. coli* expression system. The expressed proteins were visualized on SDS-PAGE and confirmed by immuno-blotting. Purified proteins were applied in western blotting assays in comparison with local and foreign ELISA kits. ROC curve analysis identified the optimum cut-off points for each protein. rOmp18 represented the highest rates of sensitivity (**94**%), specificity (**89**%), PPV (**97.4**%), NPV (**77.4**%), and accuracy (**93.2**%) in comparison with urease A and B subunits. These immunologic indices were in "substantial" agreement (K = 0.7) with the gold standard tests for Hp detection. This study recommends Hp conserved Omp18 as a reliable serologic marker for accurate detection of Hp infection particularly for application in population screening approaches.

Y. Talebkhan · F. Ebrahimzadeh · M. Esmaeili · L. Zamaninia · M. Mohammadi (✉)
Biotechnology Research Center, Pasteur Institute of Iran, No. 69, Pasteur Ave, 13164 Tehran, Iran
e-mail: marjan.mohammadi@pasteur.ac.ir; marjan.mohammadi2010@gmail.com

A. Nahvijoo · F. Fereidooni · M. A. Mohagheghi
Cancer Research Center, Tehran University of Medical Sciences, Tehran, Iran

H. Khedmat
Baqiyatallah Research Center for Gastroenterology and Liver Diseases, Baqiyatallah University of Medical Sciences, Tehran, Iran

Introduction

Helicobacter pylori (Hp) is one of the main gastrointestinal pathogens in human beings, which infects half of the adult population during early stages of life. Although most of the infected individuals remain asymptomatic, Hp infection is a proven causative agent for active chronic gastritis, peptic ulcers, and even gastric adenocarcinoma [1]. In developing countries such as Iran, the rate of Hp sero-positivity reaches up to 80% of the adult population [2–4] which has been associated with an increasing rate of gastrointestinal disorders especially gastric adenocarcinoma during the last decade [5]. Therefore, it is crucial to accurately detect Hp infection, preferably by non-invasive approaches.

Hp infection is accurately detected by invasive biopsy-based techniques including culture, histology, and rapid urease test (RUT). Although these techniques are more sensitive and specific, they remain costly and invasive and thus possess limited application for population screening. Non-invasive approaches including serologic assays such as ELISA and immunoblotting are, thus, the most commonly applied population screening methods. In order to identify efficient immuno-reactive proteins for incorporation into serologic screening methods, several Hp antigens have been studied, which include urease A and B subunits (UreA and UreB), cytotoxin-associated protein (CagA), vacuolating cytotoxin (VacA), as well as heat-shock proteins (HspA and HspB) [6–8].

Specific recognition of Hp outer membrane proteins by host immune system is manifested by the reduction of Omp-specific antibody titers following successful Hp eradication [9, 10]. Of these, Omp18 (HP1125) is a peptidoglycan-associated lipoprotein precursor with low sequence homogeneity with similar proteins from close bacterial species such as *Campylobacter jejuni* [11, 12].

Previous studies and our recent data demonstrating the effective role of rOmp18 in therapeutic immunization of mice [13] prompted us to evaluate host antibody responses toward this antigen and explore its application in detection of Hp infection.

Materials and Methods

Patients and Specimens

One hundred and sixty patients (87 females and 73 males), who referred for gastroscopy and provided informed consent prior to clinical sampling, were included in this study. Biopsy specimens were obtained from multiple gastric locations via endoscopy. Fasting blood samples were obtained prior to endoscopy from which sera were isolated and stored at −20°C until further analysis.

Bacterial Culture and Histopathology

Three sets of gastric antral and corpus biopsies were isolated from defined locations of the stomach. First set of biopsies was subjected to RUT (Rapid Urease Test, ChemEnzyme, Iran). The second set was homogenized and inoculated onto Hp-specific agar plates (HPSPA), supplemented with 5% defibrinated sheep blood and incubated for 3–5 days at 37°C under microaerobic atmosphere. The identities of Hp colonies were confirmed by Gram-staining, catalase, and urease tests. The third set was fixed in 10% formalin, sectioned, and stained with Giemsa, which was then blindly examined for the presence of Hp according to the updated Sydney system [14]. Patients were considered Hp-positive when at least one of the mentioned tests (culture, histology, or RUT) became positive. Hp-negative patients were defined as those with three negative test results.

Hp Sero-reactivity

Serum IgG antibodies against Hp lysates were detected by the two following assays according to the manufacturers' instructions: Trinity ELISA kit (Trinity, Ireland) and IPI ELISA kit (Pasteur Institute of Iran™). Patients were identified as Hp sero-positive if at least one of the two mentioned ELISA tests was positive, and considered sero-negative if both tests became negative.

Cloning and Expression of Recombinant Proteins

Hp Genomic DNA was extracted from Iranian isolated type I Hp strain by standard phenol–chloroform extraction method. Primer-specific PCR amplification of *ureA–ureB*-fused gene fragment (F: 5′-atcgatcatatgaaactcacccc-3′; R: 5′-aaagaattctagaaaatgctaaagag-3′) as well as *omp18* gene (F: 5′-caccaacaaggaccatagcatatgaagagatcttctgta-3′; R: 5′-ccgtcgacttacttcactaatttgacatccac-3′) was performed using Pfu DNA polymerase (Fermentas, Lithuania). After purification of the amplified products from agarose gels by Roche agarose gel extraction kit (Roche, Germany), the amplified genes were cloned in the previously digested pBluescript (SK+) cloning vector (Fermentas, Lithuania), and their identities were confirmed by sequencing with universal M13 forward and reverse primers. The recombinant cloning vectors were digested with corresponding restriction enzymes and ligated into *E. coli* expression vectors, previously digested with the same restriction enzymes. The identity of the cloned gene fragments was re-confirmed by sequencing. *E. coli* BL21 (DE3) cells were transformed with recombinant vectors and grown at 37°C in LB broth medium containing appropriate antibiotics until the optical density at 600 nm of 0.5 was reached. The expression of each r-protein was induced by 0.5 mM IPTG for 4 h. Cellular pellets were then collected by centrifugation at 9,000 rpm for 3 min. Following semi-purification of inclusion bodies, recombinant proteins were eluted from 12% sodium dodecyl sulfate polyacrylamide gel using Electro-Eluter (Bio-Rad, USA) according to the manufacturer's instructions.

Immuno-blotting of Recombinant Proteins

The three gel-eluted recombinant proteins were once again run on 12% SDS-PAGE gel and electro-transferred onto nitrocellulose membranes in a semi-dry blotting system (Bio-Rad, USA). After protein transfer, the non-specific binding sites were blocked at room temperature for 2 h using 2% dried skimmed milk in PBS with gentle agitation. The membranes were incubated in diluted (1:200) patients sera at RT for 2 h and underwent repeated washes prior to incubation with the diluted (1:2000) secondary antibody (horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG, Dako, Denmark); at RT for 1 h. Immunoreactive bands were visualized using diaminobenzidine tetrahydrochloride (DAB; Sigma, USA) as the color substrate.

Densitometric Analysis of Recombinant Proteins

Blotting strips were scanned using Bio-Rad scanner followed by computer analysis of the density of the recombinant protein bands (Quantity One, 1-D Analysis Software) (Bio-Rad, USA) (v. 4.6.3). Sero-reactivity was represented as the density of each scanned protein band which was determined as intensity/mm^2 (total intensity of all the pixels in the volume divided by the area of the volume). The density of each band was used for receiver

operating characteristic (ROC) curve analysis to determine the cut-off point, sensitivity and specificity in reference to culture, histology, and RUT as the gold standard tests for Hp detection.

Statistical Analysis

The data were analyzed by SPSS package (v. 11.5). Statistical associations were measured by Chi-square and Fisher's exact tests. ROC curve analysis of sensitivity versus 1-specificity (the false positive rate) was performed using quantitative densitometric values over a wide range of cut-off points. The analysis was performed with reference to the gold standard tests for Hp detection. The rate of agreement between different western blotting assays and reference ELISA tests was calculated using Cohen's kappa values. The calculated values were interpreted as <0 (less than chance agreement), 0.01–0.2 (slight agreement), 0.21–0.4 (fair agreement), 0.41–0.6 (moderate agreement), 0.61–0.8 (substantial agreement), 0.81–0.99 (almost perfect), and 1.0 (perfect agreement). P values below 0.05 were considered as statistically significant.

Results

Patients' Demographics

Patient' demographics, Hp infection, and serology status as well as endoscopic diagnosis are depicted in Table 1. Hp infection detected by the three gold standard tests and the overall sero-positivity were significantly associated with more severe macroscopic clinical outcomes such that the absolute majority of gastritis, PUD, and GC patients were Hp-positive as opposed to those with normal mucosa ($P < 0.05$). Hp positivity did not reveal any significant age or gender association (data not shown).

Cloning and Expression of Recombinant Proteins

A 2400-bp fragment representing fused *ureA* and *ureB* genes and a 570-bp fragment of entire *omp18* gene were amplified from every tested Hp strain by gene-specific PCR indicating their conserved nature (data not shown). The amplified PCR products were cloned and confirmed by sequencing [GenBank Accession Numbers; AY227442.1 (*ureA/ureB*) and EU130938.1 (*omp18*)]. SDS-PAGE analysis visualized the 25, 30, and 60-kDa proteins corresponding to Omp18, Urease A and Urease B subunits (Fig. 1A, C). Immunoblotting with pooled Hp-positive and Hp-negative sera confirmed the identity of expressed recombinant proteins (Fig. 1B, D).

Table 1 Patients' demographics

	Hp status		Hp sero-reactivity	
	RUT/culture/histology		ELISA	
	Negative	Positive	Negative	Positive
Number of cases	40/160	120/160	31/160	129/160
Gender (M/F)	0.83:1	0.87:1	0.78:1	0.88:1
Mean age (±SD)	45.18 (20.7)	44.3 (15.43)	43.36 (20.1)	44.61 (15.94)
Age range	17–85	16–82	20–65	20–82
Endoscopic diagnosis (%)				
Normal	20 (58.8)	14 (41.2)	18 (54.6)	15 (45.4)
NUD	12 (12.6)	83 (87.4)*	8 (8.3)	89 (91.7)*
PUD	0 (0)	6 (100)*	0 (0)	7 (100)*
GC	7 (29.2)	17 (70.8)*	5 (21.8)	18 (78.2)*

* Statistically significant, $P < 0.05$ (clinical outcomes vs. normal mucosa group)

Sero-reactivity Toward Hp Antigens

Immunological criteria of two performed ELISA tests (Trinity and IPI kits) were calculated here (Table 2) and in a previous report [15]. A "substantial" agreement was observed between these two ELISA tests with reference to gold standard tests ($K = 0.784$) (data not shown). In order to evaluate the recombinant proteins as the screening markers for the detection of Hp infection, rOmp18, rUreA, and rUreB were used in western blotting assays. ROC curve analysis using densitometric data identified the optimal cut-off points for each assay (data not shown). Serologic criteria (sensitivity, specificity, accuracy, and positive and negative predictive values) of the western blotting assays were calculated with reference to gold standard tests and compared with those of two ELISA kits (Table 2). Recombinant Omp18-based western blotting assay produced the highest rate of immunologic indices in comparison with those of rUreA and rUreB, and comparable to those of two ELISA assays.

Analysis of the rate of agreement between western blotting results (sero-positivity toward each Hp antigen) and two ELISA tests in detection of Hp sero-positivity by kappa coefficient calculation revealed the highest ("substantial") agreement for Omp18 ($K = 0.72$) in comparison with rUreA ($K = 0.33$) and rUreB ($K = 0.44$) which revealed "Fair-to-Moderate" agreements, respectively (data not shown).

Discussion

Previous cancer registry reports from Iran have indicated that the rate of gastric cancer has been increasing during

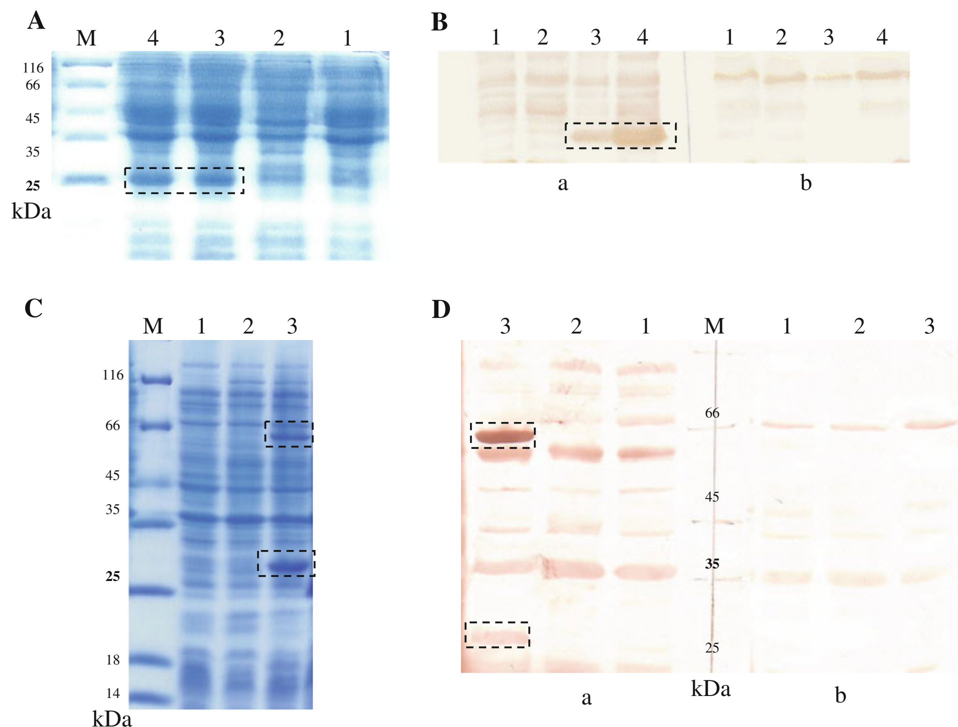

Fig. 1 **A** SDS-PAGE and **B** Western blotting analysis of expressed rOmp18 subunits using (*a*) pooled positive serum, (*b*) pooled negative serum against the following antigens; *Lanes 1 and 2 E. coli* BL21 cells lysates with empty vector (before and after IPTG induction), *lane 3 and 4 E. coli* BL21 cells lysates transformed with r-construct encoding Omp18 (after induction). **C** SDS–PAGE and **D** Western blotting analyses of expressed r-Urease subunits using (*a*) pooled positive serum, (*b*) pooled negative serum against the following antigens; *Lanes 1 and 2 E. coli* BL21 cells lysates with empty vector (before and after IPTG induction), *lane 3 E. coli* BL21 cells lysates transformed with r-construct encoding UreA and UreB (after induction). *M* protein size marker. Expressed proteins are *encircled*

Table 2 Comparison of serological criteria

Immunology criteria						
Assay types	Antigens	Sensitivity %	Specificity %	[a]PPV %	[b]NPV %	Accuracy %
a) ELISA						
Trinity	Hp lysate[c]	94.9	96.5	99	82.3	95.2
IPI	Hp lysate[d]	91.6	90.5	97.3	72.2	91.2
b) Immunoblottings						
Home made	rOmp18	**94**	**89**	**97.4**	**77.4**	**93.2**
	rUreA	94	71.5	95.2	66.6	91
	rUreB	90.5	75	96.3	53	88.7

[a] Positive predictive value

[b] Negative predictive value

[c] Originated from foreign isolated Hp strains

[d] Originated from Iranian isolated Hp strains

the past few decades [5, 16]. Hp infection by serology has been detected in the majority of the Iranian adult population reported from different provinces of the country [17, 18], similar to the rate obtained in the current study. Owing to the causative role of Hp infection in gastric carcinogenesis, and its chronic nature, accurate detection of infection by non-invasive approaches is in great demand. Despite the reported discrepancies among the worldwide applications of serological tests due to the existing heterogeneity among the Hp strains infecting various populations, these tests remain the most convenient methods in detecting Hp infection, particularly at population scale.

Amongst Hp immunogenic proteins, Urease is an essential factor in Hp colonization at gastric mucosal level, which consists of two structural subunits (UreA and UreB). Urease subunits possess highly conserved gene and protein sequences, which recommend this protein for application in serological screening approaches, [19, 20] as well as in vaccination protocols [21, 22].

Hp outer membrane proteins, on the other hand, stimulate host immune responses by inducing maturation and antigen presentation of dendritic cells [9]. These proteins may thus be proper candidates for application in population screening as well as treatment follow-up protocols [23]. The fastidious nature of Hp, along with the associated costly culture and purification methodology, calls for the development and application of recombinant target antigens in routine serologic assays. In this study, Omp18, a highly conserved outer membrane protein was produced in recombinant format, the gene sequence of which was deposited in GenBank declaring 95–96% similarity with that of reference strains, (J99 and 26695), thus reconfirming its conserved nature and its eligibility for incorporation into serologic assays.

In order to determine the efficacy of conserved Hp immunoreactive antigens in detection of Hp infection, we have primarily tested serum samples by a local ELISA kit manufactured using Iranian isolated Hp strains [15] as well as a commercially imported ELISA kit constituted of foreign Hp antigens. In this study, in accordance with our previous report [15], a substantial agreement was observed between the above mentioned two ELISA kits declaring an estimated 80% rate of infection among Iranian dyspeptic patients.

Reported variability of immunoreactivity among the Hp infected and non-infected individuals can be explained by cross reactivity of Hp antigens with other microorganisms. Owing to the conserved nature of Omp18 and low homology with its counterparts in other species, this protein has been labeled as a potentially suitable candidate for serologic assays. We, thus, compared rOmp18 with recombinant Hp UreA and UreB proteins which have been documented as conserved proteins. This analysis demonstrated the highest rate of immunologic criteria for rOmp18 in comparison with rUrease A and rUrease B, when assessed against the gold standard tests for Hp detection including bacterial culture, histology, and RUT. Western blotting assays, detecting host antibodies against Omp18, produced results with the highest rate of agreement relative to those of the local and foreign manufactured ELISA kits. These results indicate that immunoblotting against rOmp18, as a conserved protein, is a reliable and efficient approach in screening Iranian population for Hp infection with relatively high sensitivity and specificity which was comparable with the foreign and locally manufactured ELISA kits.

Previous studies demonstrating the involvement of Omp18 in the induction of host immune responses toward Hp infection through stimulation of dendritic cells and cytokine release, and the current findings lend support to the immunogenic capacity of Omp18, further recommending this protein for incorporation into serologic assays to accurately detect Hp infection, particularly for application in population-screening approaches.

Acknowledgments This study was co-funded by a grant from Pasteur Institute of Iran in support of Ph.D. dissertations and a technical assistance grant from Islamic Development Bank, Jeddah, Saudi Arabia.

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