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Extraction of the Outer Membrane Proteins of *H. pylori* and Evaluation of Their Presence in Stool of the Infected Individuals

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ABSTRACT

***Helicobacter pylori* infections are accompanied with the release of some antigenic material into the stool. Outer membranes proteins (OMP) are among the major antigens of bacteria, which in comparison with other bacterial constituents have less cross-reactivity. In this study, OMP of *H. pylori* were isolated from 11 clinical isolates and rabbit antiserum against them was used to detect possible antigens of *H. pylori*, which are released into the stool. We used immunoblotting and affinity chromatography to detect such antigens in fecal antigenic extracts of infected individuals. By immunoblotting, we were able to detect a 26 kDa band under reducing and non-reducing conditions, but many more antigens (at least 5 antigens with molecular weights of about 14, 26, 52, 57.5 and 66 kDa) were isolated by affinity chromatography. The 26 kDa antigen had a higher concentration and is seen in nearly all positive samples. Since the 26 kDa antigen is detectable by these two techniques, we suggest that this antigen is one of the major antigens of *H. pylori* which is released into the stool and can be considered as a candidate diagnostic antigen to be used in diagnostic kit development.** *Iran. Biomed. J. 8 (2): 83-88, 2004*

Keywords: *H. pylori*, Outer membrane proteins (OMP), Stool, SDS-PAGE, Affinity chromatography

INTRODUCTION

Helicobacter pylori is a Gram-negative, spiral-shaped and microaerophilic bacterium which is recently discovered [1]. This bacterium colonizes the mucus layer of stomach in nearly 50% of world population. *H. pylori* is an etiological agent of active chronic gastritis and peptic ulceration. In addition, this bacterium may be an initiation factor of gastric cancer and mucosa-associated lymphoid tissue lymphoma. Once acquired, *H. pylori* persists within its host for years, possibly lifetime, if left untreated [2]. Therefore, it is important to characterize the *H. pylori* components contributing to its colonization, persistence and pathogenic potential. In addition, because of the high rate of infection, diagnosis of infection has become very important. There are many different methods for diagnosis of *H. pylori* [3-5]. *H. pylori* infections are usually diagnosed by taking biopsy specimens obtained by endoscopy followed by rapid urease test, histology, and culture and non-invasive methods, such as detection of serum antibody response or by urea breath test (UBT) [6]. Serological tests have widely been used in epidemiological and post treatment studies, mostly by enzyme immunoassays. These assays are rapid and easy to perform and can be automated. The sensitivity of such tests is remarkably high, but they lack high specificity due to the antigenic cross-reactivity with other bacterial species [7]. Urea breath test is a highly sensitive and specific test for detection of *H. pylori*, but it is expensive and not readily available to the majority of the general practitioners. Thus a non-invasive and sensitive method that detects *H. pylori* infection and

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eradication of the organism after treatment is desirable.

H. pylori antigens are released into the stool [8] and enter into the circulation of infected individuals [9]. Currently, there is no test available commercially to detect these antigens in the circulation, but enzyme immunoassays (EIA) for detection of *H. pylori* antigens in the stool have been developed. These tests include an EIA that uses polyclonal rabbit antibody (Premier Platinum HpSA; Meridian Diagnostics Inc.) and an EIA that uses a cocktail of monoclonal antibodies (mAb) (FemtoLab *H. pylori*; Connex GmbH). The Premier Platinum HpSA assay has a low specificity, but the FemtoLab EIA have been shown to be reliable tools for non-invasive diagnosis of *H. pylori* infection. However, the *H. pylori* antigen profile in feces that is recognized by the polyclonal antibodies or mAb remains uncertain and would be of interest to elucidate [10].

Outer membrane proteins (OMP) of *H. pylori* are among the antigens that are released into the stool and have less cross-reactivity with other bacterial OMP [11]. So, we decided to identify OMP of *H. pylori* that are released into the stool of infected individuals and can be used as more specific *H. pylori* stool antigens (HpSA) to develop a diagnostic kit. In the present study, we describe an optimum method for preparation of OMP of *H. pylori* and evaluate their presence in the stool of infected individuals by immunoblotting and affinity chromatography.

MATERIALS AND METHODS

Culture of bacteria. The *H. pylori* strains examined in this study were isolated from biopsies, which obtained from stomach of patients with different gastrointestinal disorders. Of the patients undergoing gastroscopy, one specimen from antrum was cultured. The specimens were transported to laboratory in sterile normal saline containing 0.6% agarose, inoculated onto selective blood agar plates and incubated under microaerophilic conditions for 3-5 days [12]. Colonies were identified as *H. pylori* by morphology and biochemical tests: catalase and urease [13]. The bacterium was cultured on Brucella agar (Merck, Germany) containing 5% defibrinated sheep blood (Razi Serum and Vaccine Research Institute, Karaj, Iran) in an atmosphere containing 5% (vol/vol) carbon dioxide for 48 hours.

Isolation of outer membrane proteins. Two different methods were used for isolation of OMP: Method 1 was based on Rosenbusch work [13] which ended to a low yield. Method 2 was based on Schnaitman [14] procedure with some modifications. Bacteria (1 g) was washed three times with cold PBS. The bacterial pellet was resuspended in 10 ml Tris.HCl (0.050 M) pH 7.8 containing EDTA (1 mM/L) and phenylmethylsulfonylfluoride (PMSF) (1 mM/L). The bacterial suspension was sonicated 8 cycles (Soniprep 150 MSE). Each cycle was carried out for 45 seconds with a 2-minute interval between each cycle. Unbroken cells were pelleted by centrifugation at 1,500 ×g for 15 minutes. The bacterial pellet was resuspended in 10 ml of Tris.HCl (0.050 M) pH 7.8 and then DNAse and RNAse (Sigma-Aldrich, USA) were added to a final concentration of 0.1 mg/ml. The suspension was incubated at 37°C for 2 hours. After incubation, the supernatant was ultracentrifuged at 150,000 ×g and 4°C for 45 minutes. Cell wall pellet was solubilized in 10 ml of 2% Sarcosine (Sigma-Aldrich, USA) and incubated for 30 minutes at room temperature. Outer membrane was pelleted by ultracentrifugation, supernatant was discarded and the pellet was washed gently with PBS and solubilized in 1 ml of PBS pH 7.8 containing 1 mM PMSF (Sigma-Aldrich, USA).

Patients. The patient sample consisted of 41 patients (22 males with the mean age of 37.5 years and 19 females with the mean age of 44.8 years) with different gastrointestinal diseases. The diagnosis of infection was based on rapid urease test (RUT) and serology. Of these patients, a total of 18 had a positive result of the two tests and nine of them were negative on both tests. The result of these tests was contradictory in other patients. The last group was excluded from the study and the stool specimens of the two first groups were selected for further analysis. The selected group was consisted of 15 males with mean age of 31.5 years and 12 females with mean age of 46 years. Each patient was given informed consent after receiving a full explanation of the purpose of the study.

Rabbit antisera. Four White New Zealand rabbits were purchased from Razi Serum and Vaccine Research Institute, Karaj, Iran. Animals were immunized with 150µg of *H. pylori* OMP according to the standard protocols [15]. Primary immunization was carried out on day 0; the first and the second recall immunizations were carried out on 4 and 8 weeks later, respectively. Ten days after two recall injections, sera of the rabbits were collected and were frozen at -20°C until used.

Preparation of fecal antigenic extracts. Extraction of protein content of patients stool was carried out according to a protocol based on the works of other investigators with some modifications [16-19]. A few grams of stool were taken from each patient and transferred to laboratory as soon as possible. Stool samples were frozen at -20°C until further analysis. The stool (5 g) was homogenized in PBS-I solution to a final concentration of 30% of wet weight of stool (wt/vol). PBS-I solution was prepared from PBS and a cocktail of antiproteases (1 mM PMSF, 1 mM Benzamidine, 5 mM EDTA). The suspension was shaken vigorously at 5000 ×g and 4°C for 5 minutes and then centrifuged for 30 minutes. The pellet was discarded and supernatant was treated with Mixed Alkyltrimethylammonium Bromide (Sigma-Aldrich, USA) to remove mucins. For one gram of wet weight of stool, 100 λ of 10% solution of this cationic detergent was used. The resulting supernatant was shaken gently for 10 minutes and then centrifuged at 5000 ×g and 4°C for 15 minutes. The pellet was discarded and the supernatant was mixed with Silicon dioxide (Merck, Germany), 10% (w/v), to remove lipids. Silicone dioxide was pelleted by centrifugation at 1500 ×g and 4°C for 5 minutes. The upper phase was isolated and dialyzed against 4 liters of PBS and used as fecal antigenic extract.

SDS-PAGE and immunoblotting. SDS-PAGE was performed under reducing and non-reducing conditions based on Laemmli method [20]. Resolving gel concentration was adjusted to 10%. Proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane according to Towbin method [21].

Affinity chromatography. Rabbit anti-*H. pylori* OMP was used to prepare an affinity column [15]. The column was prepared by attaching the IgG fraction of rabbit antiserum to CNBr-activated Sepharose 4B (Pharmacia biotech, Sweden). Eighteen positive stool samples were analyzed by this method.

RESULTS

Isolation of H. pylori OMP. Methods 1 and 2 were performed under the same conditions and on the same crude material. The main difference between these two methods is at the final steps. Method 1 has more steps and the final product is purer than the product of method 2 because some impurities such as peptidoglycan and LPS that are associated with outer membrane are removed by lysozyme and Sulfabetaine treatment. But the yield of method 1 is less than method 2 and only 6 bands are detectable by silver staining of the gel (Fig. 1, lane 2). Method 2, which is simpler than method 1, gives a better result and the pattern of the electrophoretic bands is compatible with the reported patterns [22], but the product is less purer and contaminated with impurities such as LPS and peptidoglycan (lane 3). As seen in Figure 1, the proteins of the outer membrane of *H. pylori* have two molecular weights.

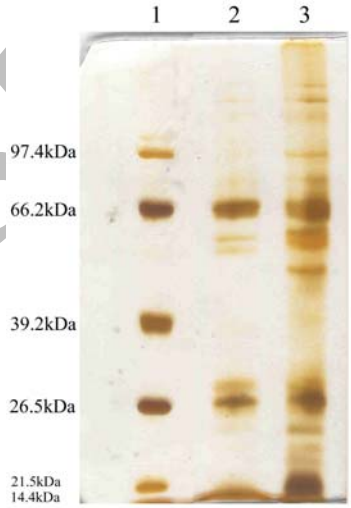

Fig. 1. Antigenic profile of the outer membrane proteins (OMP) of *H. pylori*. Proteins were separated on a SDS-PAGE gel (10%) and stained with silver nitrate. Lane 1, molecular weight markers; lane 2, OMP which were extracted by method 1 and Lane 3, OMP which were extracted by method 2.

Immunoblotting analysis of fecal antigenic extracts. Immunoblotting analysis was carried out on 11 positive stool specimens and 9 non-infected control samples. SDS-PAGE was carried out on reducing and non-reducing conditions. In both cases, a band of about 26 kDa was appeared in nearly all positive stool samples (Fig. 2). This band was seen only in two negative samples with less reactivity.

There were also other bands in some positive samples, but the band detected by diamino benzidine (DAB) in the area corresponding to 26 kDa was considered to be the most possible candidate for diagnostic test development. This band reacted strongly with rabbit anti-*H. pylori* OMP.

Fig. 2. Immunoblotting of positive stool samples. Lane 1, molecular weight markers; Lanes 2-12, positive stool samples; and lane13, outer membrane proteins of *H. pylori*. A 26-kDa band were detected in all positive samples.

Affinity chromatography of fecal antigenic extracts. The eluate of the affinity column had only one peak, which was subjected to SDS-PAGE to analyze its antigenic content. Eighteen positive and 4 negative samples were analyzed by this method. There were not any bands in negative samples (Fig. 3), but at least five bands with molecular weight of about 14, 26, 52, 57.5 and 66 kDa were isolated from positive stool samples (Figs. 4 and 5). Nearly all positive samples contained 14 and 26 kDa antigens, but only some of them had 52, 57.5 and 66 kDa antigens. The 66-kDa antigen is seen in five positive samples (Fig. 3, lines 6 and 7; Fig. 5, lanes

Fig. 3. The fractions isolated by affinity chromatography from four negative and two positive stool samples were electrophoresed in 10% resolving gel and stained with silver nitrate. Lane 1, molecular weight markers; Lanes 2-5, isolated fractions from negative samples. Lane 6 and 7, isolated fractions from positive samples. Both positive samples contain 14, 25, 27 and 66 kDa antigens.

4, 6 and 8), but it is less frequent (28%) than 14 and 26 kDa antigens (100%). The 52 kDa band is seen in 3 positive samples (Fig. 5, lanes 2, 5, and 6) and 57.5 kDa antigen is seen in five positive samples (Fig. 4, lane 2; Fig. 5, lanes 2, 4, 5 and 6).

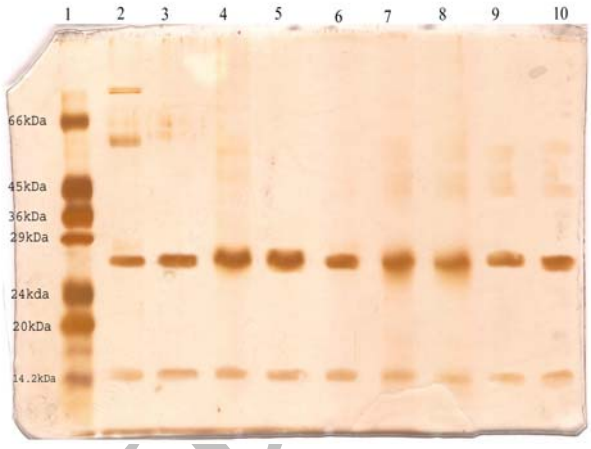

Fig. 4. The fractions isolated by affinity chromatography from nine positive stool samples were electrophoresed in 10% resolving gel. Lane 1, molecular weight markers; Lanes 2-10, isolated fractions from positive samples. The bands were revealed by silver staining. Most samples contain only 14 and 26 kDa antigens.

DISCUSSION

Some antigens of *H. Pylori* are released into the stool infected individuals that are called *H. pylori* stool antigens (HpSA). It has been tried to develop diagnostic kits to detect *H. pylori* infection by examining stool samples. The first kit of this kind was developed by Meridian Diagnostics Inc. (USA) based on affinity purified anti-*H. pylori* polyclonal antibody. The main drawback of this kit was its low specificity, because of the cross-reactivity of the anti-*H. pylori* antibodies with other antigens in the stool [8].

OMP of bacteria have less cross-reactivity with each other in comparison to the other bacterial constituents [11]. So, we decided to identify OMP of *H. pylori* that are released into the stool of the infected individuals and can be used as more specific HpSA to develop a diagnostic kit.

Rabbits were hyperimmunized with extracted OMP and their serum was used in immunoblotting and affinity chromatography techniques.

When the fecal antigenic extract of infected patients analyzed by immunoblotting technique, a distinctive band was appeared in positive samples with a molecular weight of about 26 kDa (Fig. 2).

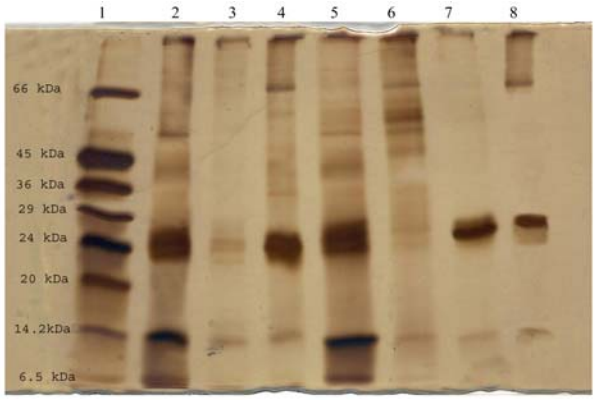

Fig. 5. The fractions isolated by affinity chromatography from seven positive stool samples were electrophoresed in 10% resolving gel. Lane 1, molecular weight markers; Lanes 2-8, isolated fractions from positive samples. The bands were revealed by silver staining. Most samples contain only 14 and 26 kDa antigens, but some samples contain 52, 57.5 and 66 kDa antigens.

This band is generally not seen in negative stool samples, but a weak similar band with less intensity is seen only in the two negative samples (Fig. 2, lanes 3 and 7). This band is the only one that can differentiate between positive and negative stool samples. Positive stool samples are also contained other bands that have not a consistent position on the blot and can not be considered as diagnostic antigens.

The final goal of this study was to extract the diagnostic antigen from *H. pylori* itself, but it was not possible to reach this goal based only on immunoblotting because the identified molecule may be a fragment of a larger molecule that is attacked by luminal proteases. As can be seen in Figure 2, there is not an equivalent band at lane 13, which is the lane of OMP. So, it is possible that the source molecule has been attacked by proteases in the intestinal tract.

Based on this fact, we decided to extract possible *H. pylori* OMP that are released into the stool by affinity column contained rabbit anti-*H. pylori* OMP. Fecal antigenic extracts were first passed through an affinity column which contained normal (non-immunized) rabbit serum, to eliminate various cross-reactivity antigens that may interact with rabbit serum proteins such as IgG.

SDS-PAGE analysis of the eluate of the test column showed that nearly all positive samples contain two molecules with molecular weight of 14 kDa an 26 kDa (Figs. 4 and 5). As we mentioned earlier, the 26 kDa antigen is also detectable by immunoblotting technique. So, this molecule is a common antigen that is identifiable by these two techniques. However, positive samples contain other bands with molecular weight of 52, 57.5 and 66 kDa. The 66 kDa antigen is seen in five positive samples (Fig. 3, lines 6 and 7; Fig. 5, lanes 4, 6 and 8), but it is less frequent (28%) than 14 and 26 kDa antigens (100%). The 52 kDa band is seen in 3 positive samples (Fig. 5, lanes 2, 5, and 6) and 57.5 kDa antigen is seen in five positive samples (Fig. 4, lane 2; Fig. 5, lanes 2, 4, 5 and 6). The 57.5 kDa antigen has the nearest molecular weight to the molecule that is recently recognized in the positive stool samples by Suzuki *et al.* [10]. These investigators have claimed that the isolated molecule is the catalase of *H. pylori* with a molecular weight of 59 kDa. We don't know the exact relationship between the 57.5 kDa antigen in our positive stool samples and the 59 kDa antigen isolated by the above-mentioned researchers. Catalase is mainly a cytoplasmic antigen, while the 57.5 kDa antigen is isolated by anti-OMP. Therefore, they may not be identical.

The difference between positive stool samples, with regard to 52, 57.5 and 66 kDa antigens, is justifiable by the fact that there are some strains of *H. pylori*, and different individuals may be infected by different strains [1]. Therefore, the antigenic profile of *H. pylori* in stool may not be the same in all positive samples. One implication of our results is that the 26 kDa antigen is a more common antigen than other isolated antigens by affinity technique and it can be regarded as a diagnostic antigen. It is necessary to isolate the corresponding molecules from *H. pylori* by an antiserum against the isolated antigens from the stool. These antigens can then be separated by techniques such as gel filtration or preparative SDS-PAGE. It would be possible to evaluate their diagnostic performance by developing ELISA based on antibodies against each of them.

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