

# Detection of *Helicobacter Pylori* Diagnostic Antigens in the Stool of Infected Patients

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## Abstract

**Background:** Some antigens of *H. pylori* are excreted into the stool of infected people. These antigens can be used to detect the infection by immunoassays such as ELISA. Our aim was to identify these antigens by immunoblotting and affinity chromatography techniques.

**Methods:** Four different antigenic preparations, namely, whole cell sonicate (WCS), outer membrane proteins (OMPs), cytoplasmic antigens (CAs) and cell surface-associated antigens (CSAAs) were obtained from *H. pylori*. Rabbit antiserum against these preparations was used to detect them in fecal antigenic extracts (FAEs) of infected patients.

**Results:** By immunoblotting, we were able to detect a 26 kDa band in the positive stool samples. Anti-OMPs acted more specifically, so, it was used to isolate the *Helicobacter pylori* diagnostic antigens (HpDAs) from the stool. More antigens (at least 4 antigens with the molecular weights of about 14, 26, 55 and 57 kDa) were isolated by affinity chromatography. But, the 26 kDa antigen had a higher concentration and was seen in almost all positive samples.

**Conclusion:** Since the 26 kDa antigen is detectable by these two techniques in all positive samples, we are confident 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.

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**Keywords** • *Helicobacter pylori* • stool antigen • diagnostic antigen

## Introduction

**H** *pylori* is one of the most common infectious agent for human beings. This bacterium colonizes the mucus layer of the stomach in almost 50% of the world population.<sup>1</sup> Chronic infection is associated with many clinical complications such as chronic gastritis, peptic ulceration and gastric cancer in some people.<sup>2</sup> Therefore, many different tests have been developed for diagnosing *H. pylori* infections.<sup>3,4</sup> These tests are divided into two general categories: a) invasive tests such as the rapid urea test and histology which require endoscopy and b) non-invasive tests such as the urea breath test and serological tests which do not require endoscopy and are quicker and easier to perform.<sup>5</sup> Recently, a new non-invasive

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test, called the *H. pylori* stool antigen (HpSA) test, has been introduced,<sup>6</sup> which diagnoses active infection by detecting *H. pylori* antigens in the stool of infected people. Nonetheless, the antigenic profile of this bacterium in the stool has not been studied comprehensively and only a few studies have paid attention to this subject.<sup>7</sup> A 59 kDa antigenic protein has also been detected as one of the antigens of *H. pylori* in the stool of infected people.<sup>8</sup> The present study was performed in order to identify the candidate antigen(s) of *H. pylori* in the stool for the diagnosis of the infection.

## Materials and Methods

### Stool samples

Feces samples were obtained from 41 patients (22 males with mean age of 37.5 years and 19 females with mean age of 44.8 years), with different gastrointestinal diseases who referred to the Digestive Diseases Research Center (DDRC) of Dr. Shariati Hospital, Tehran, Iran for upper gastrointestinal endoscopy. The samples were then stored at -20°C until use. The diagnosis of infection was based on the rapid urease test (RUT) and serology. Eighteen out of the total of 41 patients had a positive result of the tests, while nine of them were negative on both tests. The results of these tests were contradictory in the other 14 patients. The last group was excluded from the study and the stool samples of the two first groups were selected (consisted of 15 males with mean age of 31.5 years and 12 females with mean age of 46 years) for further analysis. Each patient gave informed consent after receiving a full explanation of the purpose of the study.

### Preparation of fecal antigenic extracts (FAEs)

Extraction of the antigenic content of the patients' stool was carried out according to a new protocol based on the works of other investigators.<sup>9-11</sup> 10-30 grams of the stool was taken from each patient and frozen at -20°C until further analysis. Ten grams of the stool samples was homogenized in phosphate buffered saline (PBS) PH 7.2 (Containing 1mM PMSF, 1mM Benzamidine and 5mM EDTA to inhibit proteases) at the final concentration of 30% (w/v). The suspension was shaken vigorously for 5 minutes and then centrifuged for 30 minutes at 5000g, 4°C. The supernatant was treated with alkyl trimethyl ammonium bromide (Sigma-Aldrich, USA) to remove the mucins (100 µl of 10% solution of this cationic detergent for one gram of the wet weight of the stool). The mixture was shaken gently for 10 minutes and centrifuged for 15 minutes at 5000g, 4°C. The supernatant was mixed with

Silicon dioxide (Merck, Germany), 10% (w/v), to remove the lipids. Silicone dioxide was removed by centrifugation of the suspension for 5 minutes at 1500g, 4°C. Finally, the upper phase was isolated and dialyzed against 4 liters of PBS and used as fecal antigenic extract.

### Bacterial isolates and culture conditions

Eleven *H. pylori* isolates were prepared from the biopsy samples of the patients with different gastrointestinal disorders. The biopsy samples were transferred to the laboratory in a transport medium (normal saline containing 0.6% agar) and cultured on Brucella agar (Merck, Germany) supplemented with 5% defibrinated sheep blood and a mixture of vancomycin (10 µg/ml), trimethoprim (5 µg/ml) and polymyxin B (5 µg/ml). The plates were incubated in micro-aerobic condition (5% CO<sub>2</sub>) at 37°C for 5-10 days. *H. pylori* colonies were identified by typical gram-negative morphology, biochemical tests,<sup>12</sup> and sub-cultured on the non-selective medium. Bacterial cells were scraped from the plates and suspended in PBS pH 7.2. They were washed three times with PBS and the final pellet was stored at -70°C until use.

### Preparation of different *H. pylori* antigenic fractions

Four different antigenic preparations, namely, whole cell sonicate (WCS), outer membrane proteins (OMPs), cytoplasmic antigens (CAs) and cell surface-associated antigens (CSAA) were obtained from *H. pylori*. CSAA fraction or hydrolysate was prepared by the Phadnis method.<sup>13</sup> The whole cell sonicate was prepared by treating the bacteria with ultrasonic waves (Ultra sonicator MSE, output 14 micron, 50% duty cycle for 10 times, each time 30 seconds). Intact cells were isolated by centrifugation at 5000g, 10 minutes, 4°C. Cytoplasmic antigens were prepared by ultracentrifugation (150000g, 45 minutes, 4°C) of the whole cell sonicate. The supernatant, which contained CAs, was isolated and stored at -20°C after concentration (1 mg/ml) and dialysis against PBS. OMPs were prepared according to the method of Baik et al.<sup>14</sup>

### Production of rabbit antiserum against antigenic fractions

Eight white New Zealand rabbits were purchased from Razi Serum and Vaccine Research Institute, Karaj, Iran. The animals were immunized with different *H. pylori* antigenic fractions according to the standard protocols. Primary immunization (150µg) was carried out on day 0 (subcutaneously); the first and the second recall immunizations (50 µg) were carried out 4 and 8 weeks later (intramuscularly),

respectively. Seven days after the last recall injection, serum of the rabbits was collected and frozen at  $-20^{\circ}\text{C}$  until use.

#### *Electrophoresis and immunoblotting*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of fecal extracts was performed according to the method of Laemmli,<sup>15</sup> in 10% resolving gel. The proteins were stained with silver nitrate according to the method described by Rabilloud.<sup>16</sup> The resolved proteins were transferred to PVDF or a nitrocellulose membrane by tank blotting.<sup>17</sup> The blotting membranes were blocked with PBS PH 7.5 containing 1% BSA, for one hour and then incubated with an appropriate dilution of the primary antibody (rabbit antiserum against different *H. pylori* antigenic preparations) for 1 hour. The membranes were washed three times with PBS containing 0.05% tween 20 (PBST), then incubated for 1 hour in a proper dilution of Goat anti-rabbit IgG conjugated to Horse Radish Peroxidase (Tebsun, Tehran, Iran). The membrane was again washed three times with PBST and incubated in substrate solution (20 mM Tris-HCl pH 7.5 contained 0.9% NaCl, 0.1%  $\text{H}_2\text{O}_2$ , 0.5mg/ml Diaminobenzidine or DAB) until the development of reactive bands. Finally the membrane was washed with distilled water and then dried.

#### *Isolation of H. pylori antigens from FAEs by affinity chromatography*

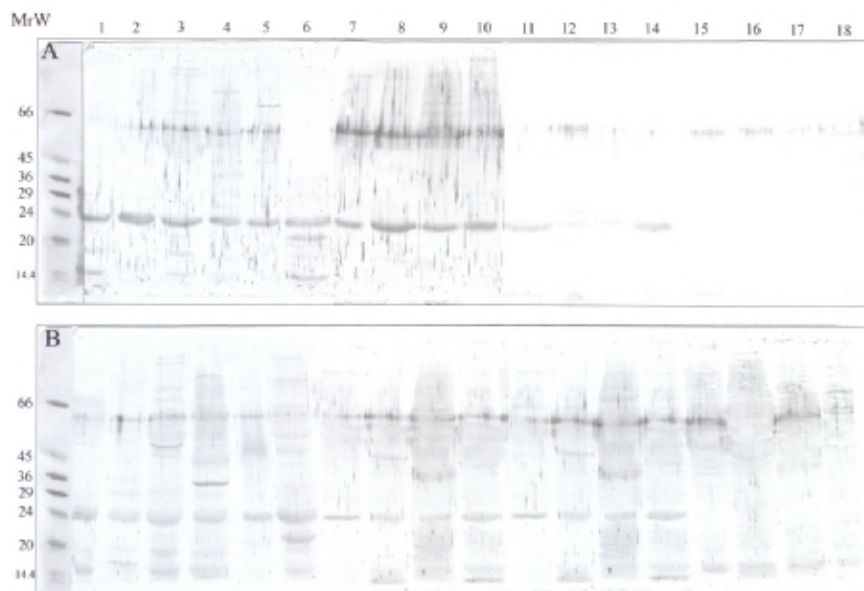
Rabbit anti-*H. Pylori* OMPs was used to prepare an affinity column. The IgG fraction of

this antiserum was isolated by ion exchange chromatography,<sup>18</sup> and then adsorbed with FAE of some negative stool samples to eliminate non-specific reactions. The affinity column was prepared by conjugation of IgG fraction of rabbit antiserum to CNBr-activated Sepharose 4B (Pharmacia Biotech, Sweden) according to the instructions of the manufacturer. FAEs of four negative and seventeen positive stool samples were analyzed by this method.

## Results

#### *Detection of H. pylori antigens in feces by immunoblotting*

Fecal antigenic extracts of 14 positive and 4 negative samples were reacted against the four different antisera (anti-WCS, anti-OMPs, anti-CAs and anti-CSAAs) by immunoblotting. Anti-CSAA reacted in a non-specific manner with the positive and negative stool samples (data not shown). So, this antigenic preparation, called hydrolysate by Phadnis et al, was not a good antigenic fraction for serological studies and therefore, the antiserum against it was omitted from the study. Anti-CAs and anti-WCS reacted with antigenic extracts of stool samples in a similar manner (figure 1). Both antisera reacted with a 24-26 kDa antigen in the positive samples and non-specifically with a 61 kDa protein band in all samples (positive and negative). These antisera also reacted with other protein bands in some positive samples. For example; anti-WCS reacted with 14 (figure 1A, lanes 1 and 3 and 6),



**Figure 1:** Reactivity of the rabbit anti-*H. pylori* whole cell sonicate (A) and anti-cytoplasmic antigens (B) toward stool samples. MrW, molecular weight markers; Lanes 1-14, positive stool samples; lanes 15-18, negative stool samples. A 26 kDa band was detected specifically in all positive samples by both antisera. These antisera reacted non-specifically with the 61 kDa band in both positive and negative samples. Anti-CAs reacted toward a low molecular weight antigen (14 kDa) in both positive and negative stool samples.

20 (figure 1A, lane 6), 75 (figure 1A, lane 4) and 70 kDa (figure 1A, lane 5) bands. In some positive samples, Anti-CAs also reacted with some non-frequent bands at the 14 (figure 1B, lanes 1, 3, 4, 10, 12, and 14), 20 (figure 1B, lane 6) and 33 kDa (figure 1B, lane 4) positions. The 14 kDa band was also detected in the negative samples (figure 1B, lanes 15-18). It should be noted that the 24-26 kDa antigens are the major antigens of *H. pylori* which are excreted into the stool and are detectable specifically by anti-CAs and anti-WCS.

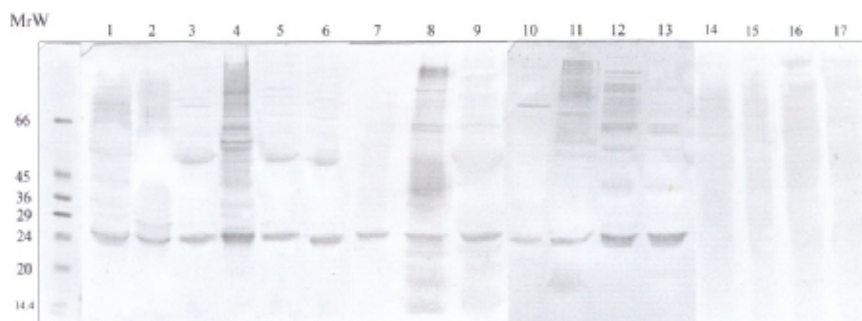
Anti-OMPs also reacted with a protein band at 24-26 kDa position in the positive samples (figure 2, lanes 1-13). But in contrast to the other two antisera, it did not react with the 61 kDa antigen in the negative stool samples (figure 2, lanes 14-17). This antiserum reacted with some other antigens with the approximate molecular weight of 14 (figure 2, lane 8), 55 (lanes 11 and 12), 61 and 77 (lanes 4, 8 and 12) and 71 kDa (lane 10) in the positive samples.

*Isolation of HpDAs from the stool by affinity chromatography*

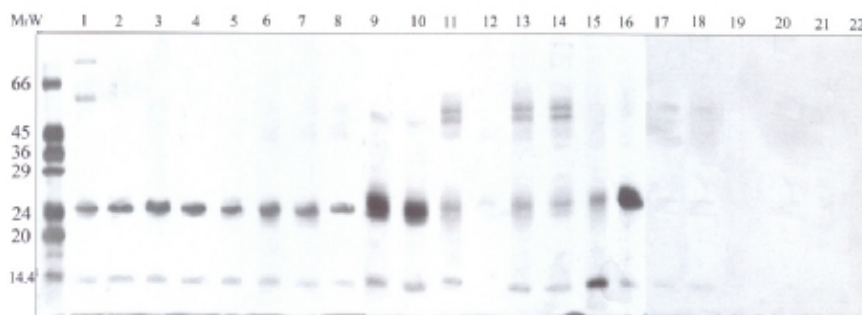
Anti-OMPs was used to isolate HpDAs from the stool samples by affinity chromatography. Ten grams of each 18 positive samples and 4 negative samples was processed according to the above-mentioned protocol. All the extracts were first passed through a control affinity col-

umn which contained normal rabbit IgG to eliminate cross reacting materials which might bind non-specifically to IgG. The adsorbed FAEs were then applied to a column contained anti-OMPs IgG. In the case of negative samples, the eluate of the affinity column did not have any absorption at 280 nm. For certainty, all fraction of the eluate were collected and lyophilized to concentrate possible trace or extreme low-quantity antigens. The resulting preparations were analyzed by SDS-PAGE and the gel was stained by the sensitive silver method. As figure 3 (lanes 19 to 22) shows, we were not able to isolate any antigen from the negative samples.

In the case of the positive samples, the eluate was collected and concentrated and then analyzed by SDS-PAGE. Four bands with the approximate molecular weights of 14, 26 (figure 3, lanes 1-18), 55 (lanes 9-14) and 57 kDa (lanes 11, 13, 14, 17, and 18) were isolated from the positive stool samples. Low molecular weight antigens (14 and 26) were seen in almost all the positive samples, but only some of these samples contained the other antigens. Concentration of the 26 kDa antigen was higher than that of the other antigen, so it is a major antigen of *H. pylori* which is detectable in the stool samples. Molecular weight of this major antigen was ranged between 24 and 26 kDa in different samples.



**Figure 2:** Reactivity of the rabbit anti-OMPs toward the stool samples. MrW, molecular weight markers; Lanes 1-13, positive stool samples; and lanes 14-17 negative stool samples. A 26 kDa band was detected specifically in all positive samples. This antiserum did not react against the 61 kDa band in both positive and negative samples. But it reacted toward the other antigens with the molecular weight of 55, 61, 71 and 77 kDa.



**Figure 3:** The antigens isolated by affinity chromatography from 4 negative and 18 positive stool samples were electrophoresed in 10% resolving gel and stained with silver nitrate. MrW, molecular weight markers; Lanes 1-18 isolated antigens from the positive samples; Lane 19-22, negative samples. All the positive samples contain 14 and 26 kDa antigens, but 55 and 57 kDa antigens are only seen in some positive sample (lanes 9-14 and 17-18).

In some samples, it appeared as two distinct antigens with MW of 24 and 26 kDa (figure 3, lanes 13, 17 and 18). So, there may be two different antigens with similar but not identical molecular weights.

## Discussion

Despite the availability of some stool antigen tests, the complete antigenic profile of *H. pylori* in the stool of infected people is not yet known and it is desirable to elucidate. A bacterium such as *H. pylori* has many antigens which are localized in different parts of the organism like outer membrane and cytoplasm. To better identify and determine the sub-cellular origin of the excreted antigens of *H. pylori* in the stool of the infected people, we adopted a reductionism approach. A mixture of coccoid and spiral-shaped *H. pylori* cells was sub-fractionated to obtain simpler antigenic preparations. The antisera against these fractions were developed in rabbit and were used to analyze and isolate *H. pylori* antigens which are excreted into the stool.

Anti-CSAAs, when used as detecting antibody in blotting experiments, reacted non-specifically with the positive and negative stool samples, so it was used no more. The other three antisera reacted specifically with the 26 kDa antigen in all positive samples. This band was generally not seen in the negative stool samples and is the only band that could differentiate between the positive and negative stool samples. This antigen has been introduced as a conserved antigen of *H. pylori*.<sup>19</sup> Based on Lindholm's work, this protein is seen in higher titers in shaken broth cultures and its quantity is comparable in different strains when they are cultured in the same way. Release of this antigen into the culture medium was also seen in our experiments, because anti-CSAAs reacted with an antigen with similar molecular weight in FAEs of the positive stool samples (data not shown).

Anti-OMPs reacted in a more specific manner with the positive samples. This antiserum recognized the 26 kDa antigen in all samples; however, this band was faint in some samples, which is probably due to low infection load or other unknown factors. Hook-Nikanne and his colleagues in an attempt to characterize the major antigens of different strains of *H. pylori* from all over the world, identified a 25 kDa antigen as one of the major antigens of many strains despite their geographic diversity. This antigen has been introduced as a protein which is associated with urease-HSP B complex (other antigens have molecular weight of 60, 56, and 31 kDa).<sup>20</sup>

The 61 kDa antigen, recognized by anti-WCS and anti-CAs antisera, is an antigen with

a great degree of cross reactivity. This band is probably the antigen, introduced by Hook-Nikanne as one of the major components of *H. pylori* and is seen in many strains. It is probably the large subunit of the urease or HSP60 of *H. pylori*, both of which have been reported to cross react with similar antigens of other species.<sup>21</sup> Other studies have shown that the medium-sized antigens of *H. pylori* (43-66 kDa) are responsible for cross reactivity with other bacterial species.<sup>22,23</sup> So, the results of our immunoblotting experiments are compatible with the previous works.

Collectively, two general conclusions were made based on the results of the immunoblotting experiments. First, all the antisera could detect the 26 kDa antigen in the positive stool samples. Second, among the four different antisera, anti-OMPs did not cross react with the 61 kDa antigen but reacted with the 26 kDa antigen specifically. It can be concluded that the cross reacting material is not present in OMPs preparation and is a cytoplasmic protein which reacts with anti-WCS and anti-CAs antisera. But this conclusion poses a conflict. One may ask why all the four antisera react with this antigen. There are two answers to this question. One possibility is that the 26 kDa antigen is a cytoplasmic protein which is also co-purified with the outer membrane. This phenomenon has been observed in many situations. For example, urease of *H. pylori*, as cytoplasmic antigen, is excreted into the culture medium and adsorbed to the bacterial surface,<sup>13</sup> and is co-purified with the OM. Another possibility is that the specified band at the 26 kDa position is composed of two antigens with similar molecular weight. One antigen which is recognized by anti-WCS and anti-CAs antisera, originates from cytoplasm, and the other is a membrane antigen which is recognized by anti-OMPs and is specific to *H. pylori*.

Based on the results of our immunoblotting experiments, anti-OMPs was chosen to isolate *H. pylori* specific antigens from the stool samples. Two major antigens with the molecular weight of 24-26 and 11.5-14.5 kDa were isolated from almost all positive samples. The 26 kDa antigen was composed of two distinct bands closely near each other in some samples. So, this band might not be a single antigen and may include two different antigens with similar molecular weights.

The presence of specific low molecular weight antigens (19, 25 and 26 kDa) in the outer membrane of *H. Pylori* which could be used for vaccination and diagnosis has been shown by other investigators.<sup>24</sup> Zheng Jiang has introduced OMP26 as one of the specific antigens of *H. pylori* which can be used as a

good antigen for vaccination.<sup>25</sup> Lepper PM has introduced low molecular weight proteins (24, 26, 30 and 33 kDa) as highly specific antigens and that the patients' serum reactivity against them can be considered as a criterion for *H. pylori* infection.<sup>26</sup>

So far, only two studies have been done by Suzuki et al for isolating some *H. pylori* antigens from the stool of infected people,<sup>7,8</sup> in which they isolated an antigen with molecular weight of 59 kDa, identified as catalase. A similar antigen with the molecular weight of 57 kDa was isolated from the positive samples in the present research, but, this similarity may be probably only at the molecular weight level. In addition, this antigen was only isolated from some of the stool samples. So, our results are not compatible with those of Suzuki et al. This discrepancy may have resulted from the methodological factors or the presence of different *H. pylori* strains which were used as source of the antigen. In addition, FAEs were prepared by different methods and different types of antibodies were used (polyclonal in our study, monoclonal by Suzuki et al). So, the 59 kDa antigen can not be considered as a proper diagnostic antigen at least in the Iranian patients.

We were also able to isolate another major antigen with the molecular weights ranging from 11.5 to 14.4 kDa from almost all the positive samples. Molecular weight variability is probably due to molecular trimming in gastrointestinal tract by hydrolytic enzymes. The quantity of this antigen was less than the 26 kDa antigen. A similar antigen has been identified by Hook-Nikanne et al as a major band of different *H. pylori* strains<sup>20</sup>. There is not much information about this antigen although it is one of the major antigens of *H. pylori* which is excreted into the stool and can be isolated by affinity chromatography.

Other antigens with the molecular weights of 55 and 57 kDa were also isolated from some of the positive samples in the present study. But, they could not be considered as good diagnostic antigens based on the criteria offered by Kimmel<sup>1</sup>. Especially, these antigens are not found in all of the positive samples.

## Conclusion

Although several diagnostic kits have been developed for diagnosing *H. pylori* infection, it is for the first time that many *H. pylori* antigens, especially the 26 kDa antigen, are isolated from the stool of infected people. This antigen has been introduced as a *H. pylori* specific antigen in the recent published works.<sup>25</sup> It is recommended to purify the 26 kDa antigen from the bacterium, and produce monoclonal antibody against it. The

potential of this antibody can be assessed in enzyme immunoassays to detect *H. pylori* infection.

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