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World J Gastroenterol 2004;10(23):3464-3469
World Journal of Gastroenterology
 ISSN 1007-9327

Diagnosis of *Helicobacter pylori* infection and diseases associated with *Helicobacter pylori* by *Helicobacter pylori* outer membrane proteins

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Supported by the Basic Research Fund of Science and Technology Committee of Chongqing, [2002] 18-86; National Natural Science Foundation of China, No.30371318
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Received: 2003-10-15 **Accepted:** 2003-12-06

Abstract

AIM: To examine the serological response of patients with upper gastrointestinal diseases and *Helicobocter pylori* (*H pylori*) infection to two *H pylori* outer membrane proteins (OMPs) (M_r18 000 and M_r26 000) acquired by gene recombinant technique, and to determine the diagnostic significance of serological tests derived from these OMPs.

METHODS: Recombinant vectors encoding the two *H pylori* OMPs were used to transform and express in BL21 (DE3) *E.coli*. After purification with Ni^{2+}-NTA agarose resin, colloid gold kits were prepared with purified recombinant proteins to detect *H pylori* infection and *H pylori*-associated diseases by the immunity-marker technology. We selected 150 patients with *H pylori* infection and digestive symptoms without previous treatment, including chronic gastritis (n = 60), duodenal ulcer (n = 30), gastric ulcer (n = 30), and gastric cancer (n = 30). As controls, 33 *H pylori*-negative healthy volunteers were also recruited. Serum samples were collected from all subjects, and the antibodies to specific proteins of *H pylori* were tested with the colloid gold test kits. The sensitivity, specificity and accuracy of the colloid gold tests were evaluated, by using the combination of standard diagnostic methods (^{13}C urea breath test and bacteria culture) and classic enzyme-linked immunosorbent assay (ELISA) as reference.

RESULTS: After purification with Ni^{2+}-NTA agarose resin, the purity of recombinant fusion proteins was about 95%. The recombinant fusion proteins were recognized by the specific monoclonal antibodies against the two *H pylori* OMPs, as demonstrated by the ELISA. Of the 150 serum samples from patients infected with *H pylori* 141 (94.0%) responded positively to the recombinant protein with M_r26 000, while the seropositive rates were 95.0%, 96.7%, 96.7% and 90.0% for patients with *H pylori*-associated chronic gastritis, duodenal ulcer, gastric ulcer, and gastric cancer respectively. The sensitivity, specificity, and accuracy of the colloid gold kit with M_r26 000 protein were 94.0%, 97.0%, and 94.5%, respectively. Compared with the classic ELISA, bacteria culture and ^{13}C urea breath test results in detecting *H pylori*-infection, there was no significant difference (P>0.05). For the colloid gold kit with M_r18 000, the seropositive rates were 52.0%, 40.0%, 40.0%, 53.3% and 86.7%, respectively, in *H pylori*-infected patients, and those with *H pylori*-associated chronic gastritis, duodenal ulcer, gastric ulcer, and gastric cancer. There was a significant difference (P<0.05) in seropositivity between patient with gastric cancer (86.7%) and those with other diseases (43.3%).

CONCLUSION: The two colloid gold kits derived from the recombinant OMPs are useful tools either for detecting *H pylori* infection, or for, predicting *H pylori*-associated gastric malignancy.

Jiang Z, Huang AL, Tao XH, Wang PL. Diagnosis of *Helicobacter pylori* infection and diseases associated with *Helicobacter pylori* by *Helicobacter pylori* outer membrane proteins. *World J Gastroenterol* 2004; 10(23): 3464-3469
http://www.wjgnet.com/1007-9327/10/3464.asp

INTRODUCTION

Since the initial report of an unidentified curved bacillus located on the gastric epithelium of patients with chronic active gastritis, the discovery of *Helicobocter pylori* (*H pylori*) and its association with a number of gastrointestinal diseases has revolutionized gastroenterology. It has attracted the interest of scholars in gastroenterology and microbiology. Infection of gastric mucosa with *H pylori* could be found in approximately 50% of the world population[1], its association with peptic ulcer disease, chronic gastritis, mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma has been well documented over the past two decades[2-19]. Moreover it was also found in some extradigestive diseases[20-36]. The direct evidence of carcinogenesis was recently demonstrated in an animal model[37,38], so this organism has been recently categorized as a class I carcinogenetic factor by the World Health Organization. It is obviously important to detect and eradicate *H pylori* infection.

The routine detecting methods including invasive and non-invasive tests have differences in sensitivity and specificity, each with their indication and characteristics in clinical practice[39-48]. Because of great quantity of serum samples especially in epidemiological studies, enzyme linked immunosorbent assay (ELISA) is the widely used test[49-52]. Antigens used in ELISA are divided into three kinds. The first is the total cell of microbacteria sonicated by ultrasonic wave, which is easy to be confused with *Helicobacter*, campylobacter, or a diverse range of other bacteria and incur to intercourse response with each other in detecting *H pylori* infection. The second is the partly purified antigens, with greatly increased specificity and decreased intercourse response, but *H pylori* could not be cultured in great quantity without special apparatus and conditions. The last is the recombinant purified antigen by gene recombinant technique. To date, many genes of OMP of *H pylori* have been amplified by scholars with polymerase chain

reaction from *H pylori* chromosomes and inserted into the compatible sites of expression vectors by using T_4 DNA ligase. Moreover recombinant vectors could be expressed in *E.coli*[53-62], but a few reports are available on lower-molecular-mass OMP application to the detection of *H pylori* infection and diagnose *H pylori*-associated diseases. In order to acquire a great amount of purified 18 000, 26 000 OMP of *H pylori*, we constructed recombinant vectors containing genes encoding with M_r18 000, 26 000 OMP of *H pylori* expressed in *E.coli* respectively and identified the antigenicity of expressed products[63]. So we prepared the colloid gold kits with purified recombinant proteins by antigen-antibody reaction and gold-marked technique to determine whether they were capable of detecting *H pylori* infection and *H pylori*-associated diseases.

MATERIALS AND METHODS

Material

Well-characterized strains of BL21/pET32a (+) /Omp_{26}, BL21/pET32a (+) /Omp_{18} were constructed, expressed and identified by the Department of Microbiology. The expression fusion proteins were recognized by the corresponding monoclonal antibody with M_r18 000, 26 000 OMP of *H pylori* and the animal's serum immunized with recombinant fusion proteins respectively. After purification using Ni^{2+}-NTA agarose resin columniation, the purity of recombinant fusion proteins was about 95%. ^{13}C urea breath test was purchased from Headway Company, Ni^{2+}-NTA agarose resin columniation was obtained from QIAGEN Company, ultrasonic liquid (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, PH 7.0), abluent (50 mmol/L phosphate, 300 mmol/L NaCl, 20 mmol/L imidazole, pH 7.80) and lavation (50 mmol/L phosphate, 300 mmol/L NaCl, 250 mmol/L imidazole, pH 7.80) were provided by the Institute of Viral Hepatitis of Chongqing University of Medical Sciences.

Sample collection

Sera were collected from 150 patients with gastrointestinal symptoms and *H pylori* infection in the Outpatient Clinic of the Gastroenterology Department of the University Hospital during Jan. 2002 to Dec. 2002 including 60 cases of gastritis, 30 cases of gastric ulcer, 30 cases of duodenal ulcer and 30 cases of gastric cancer diagnosed by gastroscopy. Sera from 31 healthy volunteers without *H pylori* infection were collected as control. All testee were forbidden to take H_2-antagonists, corticosteriods, proton pump inhibitors and antibiotics within 4 wk.

Expression of recombinant plasmid

Single bacterial colonies (BL21/pET32a (+) /Omp_{18}, BL21/pET32a (+) /Omp_{26}) were picked and cultured respectively in 2 mL LB broth containing 100 mg/L of ampicillin, at 300 r/min at 37 ℃ overnight. On the next day, BL21 *E.coli* strains containing recombinant plasmids were grown until mid-log phase (Absorbence at 600 nm = 0.5 to 1.0), and then induced to express recombinant fusion proteins in 100 mL LB by adding 1 mmol/L IPTG for 4 h. Following induction, bacteria were harvested by centrifugation at 12 000 r/min for 15 min, and stored at -20 ℃ for SDS-PAGE analysis.

Immunoblot analysis of recombinant fusion protein

Due to C end of recombinant fusion antigens with six histidines, recombinant fusion antigens were purified with Ni^{2+}-NTA agarose resin. Briefly, 500 mL of cultivated bacteria suspension was prepared, centrifuged, resuspended with the buffer liquid (50 mmol/L phosphate, 300 mmol/L NaCl, pH 7.0), and sonicated by ultrasonic wave with the energy of 600 W×35% for 40 min, and ultracentrifuged for 15 min at 10 000 *g* at 4 ℃. The sonicated recombinant fusion antigens were purified using Ni^{2+}-NTA agarose resin with abluent (50 mmol/L phosphate, 300 mmol/L NaCl, 20 mmol/L imidazole, pH 7.80) and lavation (50 mmol/L phosphate, 300 mmol/L NaCl, 250 mmol/L imidazole, pH 7.80), and quantified. The antigenicities of expressed recombinant fusion proteins were determined by immunoblotting. Following electrophoretic transfer of SDS-PAGE-separated (150 g/L acrylamide) recombinant fusion proteins to 0.45 μm pore size PVDF membrane, and after a 30-min wash in tris-saline blotting buffer, antigen-impregnated PVDF strips were incubated with the sera from patients infected with *H pylori* and anti-Omp_{18} or anti-Omp_{26} antibody for 2 h at RT. After washed, the proteins were detected by incubating the strips in alkaline phosphatase-conjugated goat anti-man IgG and alkaline phosphatase-conjugated goat anti-mice IgG antibody for 1 h at RT.

Colloid gold test of H pylori OMP

With gold marker and special antigen-antibody reaction technique, *H pylori* infection could be detected using the antigen-antibody-antigen method. The recombinant fusion proteins were impregnated in nitrocellulose membrane (NC) as a detecting strip, the recombinant fusion proteins were marked with gold as a colored reagent by which the special antibody of patient serum to *H pylori* could be detected in seconds. The criteria of the test were as follows: Negative (-): antigen-impregnated NC membrane was not recognized by patient serum, non-special antibody to *H pylori* in patient serum, so there was only an aubergine strip in mass-control district, and not in detecting district simultaneously. Positive (+): antigen-impregnated NC membrane was recognized by patient serum, special antibody to *H pylori* in patient serum. In mass-control and detecting district, there were aubergine strips. If there was no aubergine strip in mass-control district, errors might occur in experiment course. The colloid gold test paper is shown in Figure 1.

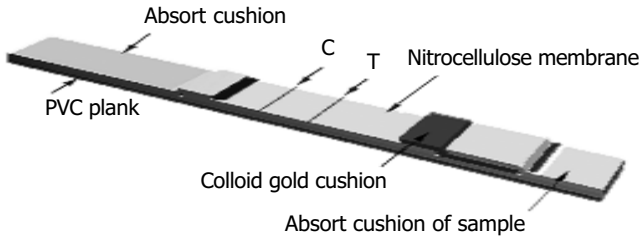

Figure 1 Colloid gold test paper.

Diagnostic criteria of the test

The sensitivity, specificity and accuracy of the colloid gold kits were evaluated on the basis of the serum ELISA results taken as reference with combined of standard diagnostic methods (^{13}C urea breath test, bacteria culture as the gold standard). Patients were defined as *H pylori* infection if one out of two validated tests of ^{13}C urea breath test and culture was positive, and as non-*H pylori* infection if two tests were negative. Patients infected with *H pylori* were determined as false negative if the colloid gold kits were negative; patients without *H pylori* infection were determined as false positive if the colloid gold kits were positive. Based on the above results, the applied value of the colloid gold kit in clinical practice was evaluated.

RESULTS

Analysis of recombinant fusion protein

After pET32a (+) /Omp_{18} and pET32a (+) /Omp_{26} were transfected into BL21 *E.coli* strains, the strains with high expressions of fusion proteins were selected and grown respectively until mid-log phase (Absorbence at 600 nm = 0.4 to 0.6), and then

induced to express recombinant fusion proteins by adding of 1 mmol/L IPTG for 4 h. Following induction, bacteria were harvested by centrifugation at 12 000 *g* for 5 min, resuspended in protein-buffer and seethed for 5 min. Their molecular mass was M_r38 000 and 46 000 respectively by 150 g/L SDS-PAGE gel analysis. After the recombinant bacteria were sonicated by ultrasonic wave and ultracentrifuged (10 000 *g*, 15 min, 4 ℃), the levels of soluble fusion proteins in the supernatant were about 18.96% and 26.38% of total cellular protein respectively. After purification by Ni^{2+}-NTA agarose resin columniation, the purity was about 95%. Recombinant fusion proteins were all recognized by the corresponding monoclonal antibody with M_r18 000, 26 000 OMP of *H pylori* and the animal's serum immunized with recombinant fusion proteins respectively. The results showed recombinant fusion proteins could provide excellent antigenicity.

Detection of H pylori infection by colloid gold kit

Sera from 150 patients with gastrointestinal symptoms and *H pylori* infection including 60 cases of gastritis, 30 cases of gastric ulcer, 30 cases of duodenal ulcer and 30 cases of gastric cancer examined by gastroscopies, and sera from 33 healthy volunteers without *H pylori* infection were assayed using the colloid gold kits with M_r18 000, 26 000 respectively (Table 1, Figure 2). The results were as follows. Ninety-four percent of patients infected with *H pylori* showed response to recombinant protein with M_r26 000, while 95%, 96.7%, 96.7% and 90.0% of patients with *H pylori*-infected chronic gastritis, gastric ulcer, duodenal ulcer, and gastric cancer showed responses (Table 2). There was no significant difference between the conventional examined methods and the colloid gold kits (P>0.05), indicating that the prevalence of infection diagnosed by both methods was similar. To specific recombinant protein with M_r18 000, 52.0% of patients showed response, while 40.0%, 40.0%, 53.3% and 86.7% of patients with *H pylori*-infected chronic gastritis, gastric ulcer, duodenal ulcer, and gastric cancer respectively showed responses (Table 3). Moreover there was a significant difference (P<0.05) in the detecting rates of *H pylori* infection between patients with gastric cancer (86.7 %) and those with other diseases (43.3%). Based on the classic ELISA, bacteria culture and ^{13}C urea breath test results, the sensitivity, specificity, and accuracy of the colloid gold kit with M_r26 000 protein were 94.0%, 97.0%, and 94.5%, respectively.

Table 1 Detecting results of *H pylori* infection using colloid gold kit with M_r18 000, 26 000 OMP

Methods	Testee	Positive	Negative	False positive	False negative
Routine methods	183	144	32	1	6
M_r26 000 protein	183	141	32	1	9
M_r18 000 protein	183	78	33	0	72

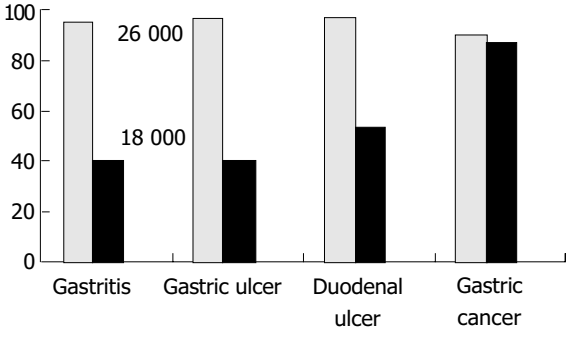

Figure 2 Detecting results of diseases infected with *H pylori* using colloid gold test kits with M_r26 000, 18 000 OMP.

Table 2 Detecting results of diseases infected with *H pylori* using colloid gold kit with M_r26 000 OMP

Disease	Patients	yr	Positive	Negative
Gastritis	60	50.3±15.9	57	3
Gastric ulcer	30	57.3±13.2	29	1
Duodenal ulcer	30	46.5±14.2	29	1
Gastric cancer	30	64.7±17.4	27	3

Table 3 Detecting results of diseases infected with *H pylori* using colloid gold kit with M_r18 000 OMP

Disease	Patients	yr	Positive	Negative
Gastritis	60	50.3±15.9	24	36
Gastric ulcer	30	57.3±13.2	12	18
Duodenal ulcer	30	46.5±14.2	16	14
Gastric cancer	30	64.7±17.4	26	4

DISCUSSION

M_r18 000, 26 000 OMP of *H pylori* are commonly expressed in all *H pylori* strains examined so far. Furthermore, no cross-reaction has been shown when antibodies (polyclonal and monoclonal) to low-molecular outer membrane proteins were used to screen closely related species of *Helicobacteria*, campylobacteria, or a diverse range of other bacteria[64]. In our study, *H pylori* 18 000, 26 000 OMP were successfully expressed in *E.coli* with good antigenicity. With marked gold and special antigen-antibody reaction technique, *H pylori* infection could be detected by the antigen-antibody-antigen method. Recombinant fusion proteins were impregnated in NC membrane as a detecting strip, and marked with gold as a colored reagent by which the special antibody to *H pylori* could be determined in seconds. At first the recombinant proteins were prepared in large quantities, purified and regulated. Due to the diameter size of grained gold would affect directly the result of test, the diameter of grained gold was adjusted to 40-60 nm, at the same time the concentration of antibody was adjusted in order to acquire steady and reliable products marked with gold. A test detecting IgG antibodies to *H pylori* was thus constructed.

Sera were collected from 150 patients with gastrointestinal symptoms and *H pylori* infection and from 33 healthy volunteers without *H pylori* infection used as controls during one year. The detecting results using colloid gold kit were as follows. Ninety-four percent of patients infected with *H pylori* showed response to recombinant protein with M_r26 000, 95.0%, 96.7%, 96.7% and 90.0% of patients with *H pylori*-infected chronic gastritis, gastric ulcer, duodenal ulcer, and gastric cancer, showed responses to specific proteins with M_r26 000 respectively, There was no significant difference between the routine examined methods and colloid gold kits (P>0.05). 52.0% of patients infected with *H pylori* showed response to recombinant protein with M_r18 000, while 40.0%, 40.0%, 53.3% and 86.7% of patients with *H pylori*-infected chronic gastritis, gastric ulcer, duodenal ulcer, and gastric cancer respectively, showed responses to specific proteins with M_r18 000, moreover there was a significant difference (P<0.05) in the detecting rates of *H pylori* infection between gastric cancer (86.7 %) and other diseases (43.3%). The results showed that colloid gold kits with M_r26 000 proteins of *H pylori* could be used as a conventional examination method. Based on the classic ELISA, bacteria culture and ^{13}C urea breath test results, the sensitivity, specificity, and accuracy of the rapid test kit with M_r26 000 protein were 94.0%, 97.0%, and 94.5%, respectively, and a significant association was found between the serologic response to M_r18 000 OMP antigen and malignant outcome of *H pylori* infection. The two colloid gold kits with

M_r26 000, 18 000 proteins of *H pylori*, could be used to detect *H pylori* infection and *H pylori*-associated diseases, and to predict the risk of peptic ulcer or malignancy. The results mentioned above were consistent with those reported[65-67].

All strains of *H pylori* could express low-molecular-mass OMP, which stimulates the body to produce corresponding antibodies, moreover the antibodies produced are related to the corresponding molecular size of antigens and immuno-status of the body. Decrease of the antibodies was associated with the corresponding molecular size of the antibodies. So the results showed that the responses of patients infected with *H pylori* to recombinant proteins with M_r26 000 were stronger (94.0%) than that (52.0%) to recombinant proteins with M_r18 000, while the growth of gastric cancer was associated with much more factors and stages. All pathogenic factors may act on the pre-carcinoma stage alone or together with each other in the model of chronic gastritis-atrophic gastritis-intestinal metaplasia-atypical hyperplasia-gastric cancer. Chua *et al*.[68] compared the seroprevalence of antibodies with various *H pylori* antigens in Singaporeans with gastric adenocarcinoma and the normal Singaporean population using both conventional immunoglobulin (Ig) G ELISA and Western blot immunoassay, and found that strains of *H pylori* including antigens with M_r19 500 and seronegative antigens with M_r35 000 could provide their potential for carcinogenesis. Immunoreactive species-specific M_r19 500 OMP of *H pylori* is actually Lpp20, while its actual molecular mass is 18 000 OMP. So *H pylori* strains based on carcinogenic potential could provide a basis for selective surveillance and eradication therapy. Low-molecular-mass OMP could lead to the 12th gene mutation of C-Ha-ras, and amplify p^{21} protein expressed by ras gene and c-met protein could also be overexpressed. Therefore the detecting rate of low-molecular-weight OMP of *H pylori* in gastric cancer is higher than other OMP. In our study, the detecting rate of M_r26 000 OMP of *H pylori* in gastric cancer was similar to that of M_r18 000 OMP. The results showed low-molecular-weight OMP could be used, not only in vaccine target candidates, but also in detection of gastric cancer in highly risk patients with *H pylori* infection. In a nutshell, the colloid gold kit constructed with M_r18 000, 26 000 OMP of *H pylori* could detect anti-*H pylori* IgG-antibody. Compared with other methods, this method not only provides a rapid, simple and painless test, but also gives a reliable specificity, especially in diagnosis of gastrointestinal tumors. The colloid gold kit based on marked gold and special antigen-antibody reaction technique is a rapid diagnostic kit, only a few minutes are required to complete an assay, and no special instruments are needed. Compared with other immunoassay techniques, the colloid gold kit has following advantages. It is based on the antigen-antibody reaction on membranes, its detecting time is much shorter than ELISA, at the same time colloid gold is red in color, so there is no need to add other colored reagents, moreover, the products marked with gold are more stable than those marked with enzyme. The colloid gold kit evaluated in our study enables a simple, rapid, noninvasive, and accurate diagnosis of *H pylori* infection, and is an ideal test method for screening patients with gastrointestinal tumors.

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Edited by Wang XL and Xu JY **Proofread by** Xu FM