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• BRIEF REPORTS •

Frequencies of the expression of main protein antigens from *Helicobacter pylori* isolates and production of specific serum antibodies in infected patients

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Abstract

AIM: To investigate the frequencies of the expression of main protein antigens of *Helicobacter pylori* (*H pylori*) isolates, such as UreB, VacA, CagA1, HpaA, NapA, FlaA and FlaB and the production of specific antibodies in sera from *H pylori*-infected patients, and to understand the correlations among the different clinical types of chronic gastritis and peptic ulcer and the infection and virulence of *H pylori*.

METHODS: *H pylori* strains in biopsy specimens from 157 patients with chronic gastritis and peptic ulcer were isolated and serum samples from the patients were also collected. The target recombinant proteins rUreB, rVacA, rCagA1, rHpaA, rNapA, rFlaA and rFlaB expressed by the prokaryotic expression systems constructed in our previous studies were collected through Ni-NTA affinity chromatography. Rabbit antisera against rUreB, rVacA, rCagA1, rHpaA, rNapA, rFlaA and rFlaB were prepared by using routine subcutaneous immunization. By using ultrasonic lysates of the isolates as coated antigens, and the self-prepared rabbit antisera as the first antibodies and commercial HRP-labeling sheep anti-rabbit IgG as the second antibody, expression frequencies of the seven antigens in the isolates were detected by ELISA. Another ELISA was established to detect antibodies against the seven antigens in sera of the patients by using the corresponding recombinant proteins as coated antigens, and the sera as the first antibody and HRP-labeling sheep anti-human IgG as the second antibody respectively. Correlations among the different clinical types of chronic gastritis and peptic ulcer and the infection and virulence of *H pylori* were statistically analysed.

RESULTS: In the 125 isolates of *H pylori*, the positive rates of UreB, VacA, CagA1, HpaA, NapA, FlaA and FlaB were 100%, 65.6%, 92.8%, 100%, 93.6%, 100% and 99.2% respectively. In the 125 serum samples from the *H pylori*-infected patients, the positive rates of antibodies against recombinant UreB, VacA, CagA1, HpaA, NapA, FlaA and FlaB were 100%, 42.4%, 89.6%, 81.6%, 93.6%, 98.4% and 92.8% respectively. *H pylori* strains were isolated from 79.6% (125/157) of the biopsy specimens, but no close correlations among the *H pylori* infection frequencies and different types of chronic gastritis and peptic ulcer could be found ($P>0.05$, $\chi^2 = 0.01$-0.87). The VacA positive rate (82.40%) in the strains isolated from the specimens of patients with peptic ulcer and the anti-VacA positive rate (54.3%) in the sera from the patients were significantly higher than those (51.5%, 32.3%) from the patients with chronic gastritis ($P<0.01$, $\chi^2 = 13.19$; $P<0.05$, $\chi^2 = 6.13$). When analysis was performed in the different types of chronic gastritis, the VacA in the strains isolated from the specimems of patients with active gastritis showed a higher expression frequency (90.0%) than those from superficial (47.9%) and atrophic gastritis (30.0%) ($P<0.05$, $\chi^2 = 5.93$; $P<0.01$, $\chi^2 = 7.50$). While analysis was carried out in the strains isolated from the specimens with superficial (93.8%) and active gastritis (100%), NapA showed a higher expression frequency compared to that from atrophic gastritis (60.0%) ($P<0.01$, $\chi^2 = 8.88$; $P<0.05$, $\chi^2 = 5.00$).

CONCLUSION: The types of chronic gastritis and peptic ulcer and their severity are not associated with *H pylori* infection frequency but closely related to the infection frequency of different virulent *H pylori* strains. The optimal antigens for developing vaccine and diagnostic kit are UreB, FlaA, HpaA, FlaB, NapA and CagA1, but not VacA.

Key words: *Helicobacter pylori*; *H pylori* infection; *H pylori* antigens; *H pylori* antibodies

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INTRODUCTION

Helicobacter pylori (*H pylori*) is confirmed to be a specific pathogen of human gastritis and gastric and gastroduodenal ulcers, and is closely associated with gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin's lymphoma[1-3]. This microbe infected at least half of the world population[4]. Vaccination is generally considered the most effective measure to prevent and control *H pylori* infection, and *H pylori*-associated diseases. For the difficulty of culture and storage of the microbe, genetic engineering vaccine seems to be the unique pathway for

developing *H pylori* vaccines[5]. Since heterogeneity of *H pylori* isolates from different areas is frequently present[6,7], the data about distribution of the main surface protein antigens of the isolates from different areas and production of the specific antibodies against the antigens in sera of *H pylori*-infected patients have important reference values for screening antigen candidates.

In the previously published data, all *H pylori* isolates could produce a surface-distributed urease comprising four subunits. Among the four subunits, subunit B (UreB) encoded by the *ureB* gene possesses the strongest antigenicity[8]. Vacuolating cytotoxin (VacA), a unique exotoxin of *H pylori*, can cause vacuolar degeneration in epithelial cell lines such as HeLa cell line[9]. Cytotoxin-associated protein A (CagA) has been demonstrated to be closely associated with the virulence of *H pylori*[10]. At the 3'-end of CagA encoding genes, there are different numbers of repeated sequences in different *H pylori* isolates, resulting in various sizes with molecular weights of 120-140 kDa[11]. Moreover, *cagA* gene carrying *H pylori* strains isolated from domestic population (>90%) is remarkably higher than that from Europe and North America populations (-60%)[6,12]. Almost all *H pylori* isolates can express *H pylori* adhesin (HpaA) which plays an important role in adhesion and colonization of the microbe[13]. Neutrophil-activating protein (NAP) of *H pylori*, a polymer composed of 10 subunits A, has an ability to induce active oxygen radicals in neutrophils, causing injury to human gastric mucosa tissue[14]. *H pylori* has a flagellum composed of two protein subunits, namely, FlaA and FlaB, which is an important pathogenic factor for *H pylori* colonization, persistent infection and inflammatory reaction[15]. Therefore, the seven proteins described above are the most important *H pylori* antigens.

In our previous studies, we constructed the prokaryotic expression systems of the seven genes mentioned above. In this study, *H pylori* strains in biopsy specimens from patients with chronic gastritis and peptic ulcer were isolated. The products expressed by the systems were collected and rabbit antisera against the recombinant proteins were prepared. By using ELISA, distribution of the antigens in *H pylori* isolates and production of antibodies against the antigens in sera of *H pylori*-infected patients were examined. Furthermore, the correlations among the different clinical types of chronic gastritis and peptic ulcer and the infection and virulence of *H pylori* were analyzed.

MATERIALS AND METHODS

Patients and specimens

Gastric biopsy specimens with positive urease for *H pylori* isolation and serum samples from 157 patients in Zhejiang Provence were collected from four hospitals in Hangzhou during January to September of 2003. None of the patients received nonsteroidal anti-inflammatory drugs, antibiotics and antacids within the previous two weeks. Of the 157 patients (112 male and 45 female; age range: 18-75 years; mean age: 43±15 years), 88 were clinically diagnosed as chronic gastritis (58 superficial, 15 active and 15 atrophy gastritis), the other 69 were clinically diagnosed as peptic ulcer (16 gastric, 46 duodenal and 7 gastric-duodenal ulcers). At the same time, serum specimens were also collected from these patients and stored at -20 ℃. Ni-NTA purication kit was purchased from BBST (Shanghai, China). DAKO (Glostrup, Denmark) and Jackson ImmunoResearch (West Grove, USA) supplied rabbit antiserum against whole cells of *H pylori*, HRP-labeling sheep anti-rabbit IgG and anti- human IgG antibodies. Agents for *H pylori* isolation and identification were purchased from BioMérieux (Marcy I'Etoile, France).

Methods

Isolation and identification of *H pylori* Each gastric biopsy specimen was homogenized with a tissue grinder and then inoculated on Columbia agar (BioMérieux) plates supplemented with 80 mL/L sheep blood, 5 g/L cyclodextrin, 5 mg/L trimethoprim, 10 mg/L vancomycin, 2.5 mg/L amphotericin B and 2 500 U/L cefsoludin. The plates were incubated at 37 ℃ under microaerobic conditions (5%O_2, 100 mL/L CO_2 and 85% N_2) for 3 to 5 d. A bacterial isolate was identified as *H pylori* according to typical Gram stain morphology, biochemical tests positive for urease (HPUT) and oxidase (TIANHE), and agglutination with the commercial rabbit antibody (DAKO) against whole cell of the microbe. All the 125 *H pylori* isolates obtained were stored in Brucella broth containing 300 g/L (V/V) glycerin at -70 ℃. *H pylori* strain NCTC11637 and *E coli* strain BL21DE3 were provided by our laboratory.

Prokaryotic expression systems and collection of target recombinant proteins The recombinant *pET32α* prokaryotic expression systems for the seven protein antigens of *H pylori* were previously constructed by our laboratory[16-19]. Molecular weights of the expressed products were 68 kDa (rUreB), 87 kDa (rVacA), 89 kDa (rCagA1, a relative conserved fragment of 2 148 bp in *cagA* gene), 29 kDa (rHpaA), 26 kDa (rNapA), 61 kDa (rFlaA) and 61 kDa (rFlaB), respectively. These recombinant proteins were collected by Ni-NTA affinity chromatography (Novagen).

Preparation of rabbit anti-sera against the recombinant proteins By using subcutaneous immunization, rabbits were weekly injected with Freund's adjuvant containing 1 mg of each of the recombinant proteins for four weeks. The rabbit sera were separated after the last immunization and titers of the sera were measured by immunodiffusion test.

Detection of bacterial protein antigens Ultrasonic supernatant from each of the 125 *H pylori* isolates was prepared with 0.05 mol/L bicarbonate buffer (pH9.6) to the final protein concentration of 50 μg/mL by ultraviolet spectrophotometry. The wells in plastic plates were coated with 0.1 mL of the protein solution and then incubated at 4 ℃ overnight. By using the self-prepared rabbit antisera (1:800-2 000 dilutions) as the first antibody, commercial HRP-labeling sheep anti-rabbit IgG (1:4 000 dilution, Jackson ImmunoResearch) as the second antibody and orthophenylene diamine (OPD) as the substrate, the A_{490} value of each of the wells was detected by an enzyme-linked immunosorbent meter after development. In this assay, similar preparations with the same protein concentration of *H pylori* strain NCTC11637 and *E coli* strain BL21DE3 were used as positive and negative controls, respectively. A tested *H pylori* ultrasonic supernatant sample was considered as positive for the corresponding antigen if its OD490 value was over the mean ±SD from the six repeated wells coated by the *E coli* strain supernatant[20].

Detection of antibodies in sera of patients By using each of the recombinant proteins with 20 μg/mL as coated antigen, the serum from each of the infected patients (1:200 dilution) as the first antibody and HRP-labeling sheep anti-human IgG (1:3 000 dilution, Jackson ImmunoResearch) as the second antibody, and OPD as the substrate, the A_{490} value of each of the wells was detected by an enzyme-linked immunosorbent meter after development. At the same time, the commercial rabbit antiserum against the whole cells of *H pylori* and six identical negative serum samples were used as positive and negative controls respectively. The positive standard in this assay was the same as described in detection of the bacterial protein antigens.

Distribution of *H pylori* isolation rates and serum antibody positive rates in different diseases Chi-square test was used to statistically analyze the differences of *H pylori* isolation rates and serum antibody positive rates in different diseases.

RESULTS

Distribution of H pylori isolation rates in different types of gastric and duodenal diseases

H pylori was found in 79.6% of the biopsy specimens (125/157) and the distribution of the isolation rates is shown in Table 1. The statistically analyzed results did not show any difference among the isolation rate distributions in different types of gastric and duodenal diseases ($P>0.05$, $\chi^2=0.01$-0.87).

Table 1 Distribution of *H pylori* isolation rates in different types of gastric and duodenal diseases

Types of diseases	Tested cases	*H pylori* isolation positve cases	Positive rate (%)
Chronic gastritis	88	68	77.3
Superficial	58	48	82.8
Active	15	10	75.0
Atrophy	15	10	75.0
Peptic ulcer	69	57	82.6
Gastric	16	12	77.3
Duodenal	46	39	84.7
Gastric + Duodenal	7	6	85.7
Total	157	125	79.6

Titers of the rabbit antisera against recombinant proteins

The results of immunodiffusion assays demonstrated that the titers of the commercial *H pylori* antibody against rUreB, rVacA, rCagA1, rHpaA, rNapA, rFlaA and rFlaB were 1:16, 1:4, 1:4, 1:8, 1:16, 1:4 and 1:4 respectively. The titers of the self-prepared rabbit antisera against rUreB, rVacA, rCagA1, rHpaA, rNapA, rFlaA and rFlaB were 1:8, 1:4, 1:4, 1:4, 1:8, 1:2 and 1:2 respectively.

Detection results of the antigens in H pylori isolates

All the *H pylori* isolates could express UreB, HpaA and FlaA. The expression rates of CagA1, NapA and FlaB in the isolates were over 90%, but that of VcaA was only 65.6% (Table 2).

Detection results of the antibodies in sera of the patients

The antibody against UreB was detectable in all serum samples of the patients. The positive rates of the antibodies against FlaA, FlaB and NapA were over 90%. The positive rate over 80% was considered to be positive for HpaA and CagA1 antibodies. The VcaA antibody positive rate was as low as 42.2% (Table 3).

Comparison of H pylori isolation rates and serum antibody positive rates in different diseases

The results of comparison among the *H pylori* isolation rates and serum antibody positive rates in the different diseases are shown in Table 4. In the specimens from chronic gastritis and peptic ulcer patients, the expressions of UreB, CagA1, HpaA, FlaA and FlaB and positive rates of their antibodies were similar to each other ($P>0.05$). But in the study, the VacA positive rate in *H pylori* strains isolated from peptic ulcer patients (82.4%) was obviously higher than that from chronic gastritis patients (51.5%) ($P<0.01$). When analysis was performed in different types of chronic gastritis, the VacA in *H pylori* strains isolated from the specimens with of patients active gastritis showed a higher expression frequency (90.0%) than those from superficial (47.9%) and atrophic gastritis specimens (30.0%) ($P<0.05$, $\chi^2=5.93$; $P<0.01$, $\chi^2=7.50$). While analysis was carried out in *H pylori* strains isolated from the specimens of patients with superficial (93.8%) and active gastritis (100%), NapA had a higher expression frequency compared to that from those with atrophy gastritis (60.0%) ($P<0.01$, $\chi^2=8.88$; $P<0.05$, $\chi^2=5.00$).

DISCUSSION

So far, a lot of protein antigens of *H pylori* have been reported. Except for the seven proteins detected in this study, attention has been paid to catalase, HSP, Ice and Bab of *H pylori*[21-23]. However, *H pylori* catalase and HSP were found to have antigens cross some enteric bacteria[21] and *H pylori* Ice and Bab were demonstrated to have mutations in isolates from different areas[22,23]. On the contrary, the genes encoding UreB, VacA, HpaA, NapA, FlaA and FlaB were relatively conserved and the sequence similarity of the proteins from different isolates

Table 2 Detection results for the expressions of UreB, VacA, CagA1, HpaA, NapA, FlaA and FlaB in *H pylori* isolates

Antigens	Cases (n)	Positive standard values (A_{490})	Value range of samples (A_{490})	Positive cases (n)	Positive rates (%)
UreB	125	0.25	0.47-1.93	125	100
VacA	125	0.21	0.27-1.73	82	65.6
CagA1	125	0.54	0.55-0.97	116	92.8
HpaA	125	0.34	0.52-1.94	125	100
NAP	125	0.36	0.36-1.23	117	93.6
FlaA	125	0.34	0.36-2.01	125	100
FlaB	125	0.30	0.31-1.78	124	99.2

Table 3 Detection results for the antibodies against UreB, VacA, CagA1, HpaA, NapA, FlaA and FlaB in sera of the patients

Antibodies	Cases (n)	Positive standard value (A_{490})	Value range of samples (A_{490})	Positve cases (n)	Positive rates (%)
Anti-UreB	125	0.25	0.27-1.97	125	100
Anti-VacA	125	0.63	0.63-1.21	53	42.4
Anti-CagA1	125	0.46	0.56-1.05	112	89.6
Anti-HpaA	125	0.28	0.32-1.96	102	81.6
Anti-NAP	125	0.43	0.54-1.38	117	93.6
Anti-FlaA	125	0.44	0.52-1.76	123	98.4
Anti-FlaB	125	0.25	0.26-1.15	116	92.8

Table 4 Distribution of the expressions of seven antigens in *H pylor* strains isolated from the biopsies and the specific antibodies in sera of *H pylori*-infected patients

Diseases	Strains/Patients (*n*)	Antigen positive (*n*) / antibody positive (*n*)						
		UreB	VacA[abc]	CagA1	HpaA	NapA[df]	FlaA	FlaB
Chronic gastritis[a]	68/68	68/68	35/22	62/59	68/56	61/61	68/66	67/60
Superficial[f]	48/48	48/48	23/18	45/43	48/41	45/45	48/48	47/44
Active[b,c,d]	10/10	10/10	9/2	9/9	10/8	10/10	10/10	10/9
Atrophy	10/10	10/10	3/2	8/7	10/7	6/6	10/8	10/7
Peptic ulcer	57/57	57/57	47/31	54/53	57/46	56/56	57/57	57/56
Gastric	12/12	12/12	9/6	11/11	12/10	11/11	12/12	12/11
Duodenal	39/39	39/39	32/21	38/37	39/31	39/39	39/39	39/39
Gastric+Duodenal	6/6	6/6	6/4	5/5	6/5	6/6	6/6	6/6
Total	125/125	125/125	82/53	116/112	125/102	117/117	125/123	124/116

The expression of VacA: [b]$P<0.01$ *vs* peptic ulcer, [a]$P<0.05$ *vs* superficial gastritis, [d]$P<0.01$ *vs* atrophy gastritis. The expression of NapA: [c]$P<0.05$ *vs* atrophy gastritis, [f]$P<0.01$ *vs* atrophy gastritis.

was as high as 90% or above[8,13-15]. Although a high level of putative amino acid sequence mutations in the *cagA* genes from different *H pylori* strains caused by various numbers of repeated sequences at the 3'-end was revealed[10,11], a 2148 bp fragment, named as *cagA1* in this study, between 67-2214 bp from 5'-end of the gene was found to have an approximate 88% similarity after analysis of the 37 *cagA* sequences registered in GenBank. All the *H pylori* strains were determined to carry *vacA* gene but only 50-60% of them were able to express VacA[24]. A great attention has been paid to VacA because this factor is the confirmed unique exotoxin of *H pylori*[25]. Therefore, the seven proteins are the common antigen candidates in *H pylori* vaccine and diagnostic kit development.

According to the results of immunodiffusion assays, the seven target proteins expressed by the prokaryotic systems could be recognized by the commercial antibody against whole cells of *H pylori* and the titers ranged 1:4-1:16. All the seven target proteins could efficiently induce rabbits to produce antibodies and the titer ranged 1:2-1:8. These data indicate that the recombinant proteins mentioned above possess qualified immunoreactivity and antigenicity.

Based on the results of ELISAs, the expression rates of UreB, HpaA, NapA, FlaA and FlaB in the 125 *H pylori* isolates were 100%, 100%, 93.6%, 100% and 99.2% respectively. The specific antibody positive rates of, UreB, HpaA, NapA, FlaA and FlaB in sera of the 125 *H pylori*-infected patients were 100%, 81.6%, 93.6%, 98.4% and 92.8% respectively. It indicates that among the seven proteins UreB is the optimal antigen, and HpaA, NapA, FlaA and FlaB are the potential antigens for developing *H pylori* vaccine and diagnostic kit. It was also found in the study that the *H pylori* isolates expressing UreB and NapA could induce the infected persons to produce detectable serum antibodies, implying the strong antigenicity of the two proteins.

It was reported that VacA was used to prepare*H pylori* vaccine[25]. However, in this study, only 65.6% of *H pylori* isolates (82/125) had an ability to express VacA and VacA antibody, the positive rate in the *H pylori*-infected patients was as low as 42.4% (53/125), revealing that VacA is not a suitable antigen for the development of *H pylori* vaccine and diagnostic kit. ELISA positive rate for CagA1 in the 125 *H pylori* isolates could reach 92.8% and the CagA1 antibody positive rate in the 125 serum samples was as high as 89.6%. The *cagA1* fragment selected could be highly conservative in different *H pylori* isolates and the expressed CagA1was also appropriately used in *H pylori* vaccine and diagnostic kit development.

Previous epidemiological investigations have shown that *H pylori* infective rate in patients suffering from chronic active gastritis and peptic ulcer was significantly higher than that in chronic superficial gastritis patients[6,26]. But in our study no close correlation between *H pylori* infection frequencies and different types of chronic gastritis and peptic ulcer could be found ($P>0.05$). In Europe and North America, CagA and VacA are usually considered indicators of stronger virulent strains of *H pylori*[27,28]. According to the results of our study, the high frequency of CagA expression (92.8%) in the strains of *H pylori* makes it impossible as an indicator for the bacterial virulence in the local area. It was found in the study that the VacA positive rate in the strains isolated from peptic ulcer patients (82.4%) was obviously higher than that from chronic gastritis patients (51.5%) ($P<0.01$) and chronic active gastritis patients (90.0%) compared to those from chronic superficial (47.9%) and atrophic gastritis patients (30.0%) ($P<0.05$, $P<0.01$). In addition, the NapA positive rate in the strains isolated from chronic superficial (93.8%) and active gastritis (100%) was remarkably higher than that from chronic atrophic gastritis patients (60.0%) ($P<0.01$, $P<0.05$) These data suggest that chronic gastritis and peptic ulcer and their severity are not associated with *H pylori* infection frequency but closely related to the infection frequency of different virulent *H pylori* strains.

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