PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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A study of recombinant protective *H. pylori* antigens

Zheng Jiang, Xiao-Hong Tao, Ai-Long Huang, Pi-Long Wang

Zheng Jiang, Xiao-Hong Tao, Pi-Long Wang, Department of Gastroenterology, the First Affiliated Hospital, Chongqing University of Medical Sciences, Chongqing 400016, China
Ai-Long Huang, Institute of Viral Hepatitis, Chongqing University of Medical Sciences, Chongqing 400010, China
Correspondence to: Dr. Zheng Jiang, Department of Gastroenterology, the First Affiliated Hospital, Chongqing University of Medical Sciences, Chongqing 400016, China. jzh053@mail.china.com
Telephone: +86-23-68891218
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Abstract

AIM: **To construct a recombinant vector which can express M_r26000 outer membrane protein (OMP) from *Helicobacter pylori* (*Hp*), and to obtain the vaccine protecting against *Hp* infection and a diagnostic reagent kit quickly detecting *Hp* infection.**

METHODS: **The gene encoding the structural M_r26000 outer membrane protein of *Hp* was amplified from *Hp* chromosomal DNA by PCR, and inserted in the prokaryotic expression vector pET32a (+), which was transformed into the Top10 E. *coli* strain. Recombinant vector was selected, identified and transformed into BL-21(DE3) E. *coli* strain. The recombinant fusion proteins were expressed. The antigenicity of recombinant protein was studied by ELISA or immunoblotting and immunized Balb/c mice.**

RESULTS: **The gene of M_r26000 OMP was amplified to be 594 base pairs, 1.1% of the cloned genes was mutated and 1.51% of amino acid residues was changed, but there was homogeneity between them. The recombinant fusion protein encoded objective polypeptides of 198 amino acid residues, corresponding to calculated molecular masses of M_r26000. The level of soluble expression products was about 38.96% of the total cell protein. After purification by Ni-NTA agarose resin columniation, the purity of objective protein became about 90%. The ELISA results showed that recombinant fusion protein could be recognized by patient serum infected with *Hp* and rabbit serum immunized with the recombinant protein. Furthermore,Balb/c mice immunized with the recombinant protein were protected against *H.pylori* infection.**

CONCLUSION: **M_r26000 OMP may be a candidate vaccine preventing *Hp* infection.**

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INTRODUCTION

Helicobacter pylori (*Hp*) is a microaerophilic, spiral and gram-negative bacillus first isolated from human gastric antral epithelium in 1982. It is recognized as a human-specific gastric pathogen that colonizes the stomachs of at least half of the world's population[1]. Most infected individuals are asymptomatic. However, in some subjects, the infection is associated with the development of peptic ulcer, gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin's lymphoma[2-11]. Furthermore, this organism was recently categorized as a class I carcinoma by the World Health Organization[12], and direct evidence of carcinogenesis was recently demonstrated in an animal model[13,14]. Immunization against the bacterium represents a cost-effective strategy to reduce the incidence of global gastric cancer and would also have a major impact on *H. pylori*-peptic ulcer disease[15]. The selection of antigenic targets is critical in the design of an Hp vaccine. To date, this area is scarcely touched upon. The majority of studies focused on the urease enzyme, heat shock protein, VacA, and so on[1,16-19],but not M_r26000 outer membrane proteins. So, in this study, the recombinant plasmid of *H. pylori* M_r26000 outer membrane protein genes was constructed,and expressed for development of *Hp* vaccine.

MATERIALS AND METHODS

Materials

A well-characterized strain, *H. pylori* (*Hp*), was afforded by the Department of Microbiology, Chongqing University of Medical Sciences. Top10, BL21 E.coli strains and pET32a(+) plasmid were presented by the Institute of Viral Hepatitis of Chongqing University of Medical Sciences. Restriction enzymes (HindIII, BamHI) and T_4 DNA ligase were purchased from Promega, TagDNA polymerase was produced by Immunology Department of the former Beijing Medical University. Isopropyl-β-D-thiogalactopyranoside (IPTG), dNTP and oligonucleotide primers were obtained from Sigma Chemical Co. and so on.

Cloning of Hp M_r26000 OMP gene

Oligonucleotide primers were designed to amplify *H. pylori* open reading frame (ORFs) of M_r26000 outer membrane protein based on the published genome sequence[20]. The primers were designed with a *Bam*HI site incorporated into the 5' end and a *Hin*d III site at the 3' end as follows(5'-3'): GCGGATCCATGTTAGTTACAAAA CTTGCC (forward) and AAGCTTAATGGAATTTTCTTT (reverse). Genomic DNA prepared from Chongqing *H. pylori* strains was used as the template in the PCR. The PCR cycle consisted of 30 cycles of denaturation at 94℃ for 60s, annealing at 58℃ for 45s, with an extension step at 72℃ for 90s. Products were visualized on $10g \cdot L^{-1}$ agarose gel and purified using a PCR purification kit. After digestion with the restriction enzymes BamHI and Hind III simultaneously, the purified products were cloned into the compatible sites of the expression vectors pET32a(+) using T_4 DNA ligase at a molar ratio of 4:1 at 4℃ overnight.

Fifty μL Top10 incubated at 37℃ overnight was added into 2mL Luria-Bertani broths and routinely grew at 37℃, and shaken at $300r \cdot min^{-1}$ for 4h. When optical density at 600nm was 0.5, it was ultracentrifuged at $10000r \cdot min^{-1}$ at room temperature (RT) for 2min. The resulting deposits were suspended with $100mmol \cdot L^{-1}$ $CaCL_2$ 150μL and incubated at 0℃ for 2h. Ten μL connected products(aboved) was resuspended and incubated at 0℃ for 30min, at 42℃ for 2min and at 0℃ for 2min respectively. At last, it was incubated at 37℃ at $180r \cdot min^{-1}$ for 30min after the addition of 1mL LB broth, 200μL was collected and spred onto an LB plate containing $100mg \cdot L^{-1}$ ampicillin as the selectable marker and incubated at 37℃ overnight.

Extraction and expression of recombinant plasmid

The next day, the single cloned bacterial drop was selected, and cultured in 2mL LB broth containing 100mg·L^{-1} ampicillin at 37℃ overnight at 300r·min^{-1}, then recombinant plasmids were extracted and screened with plasmid extraction kit according to the manufacturer's instruction, in the meantime, identified by PCR and restriction enzyme digestion. The recombinant plasmids were selected and transformed into competent BL21(DE3) E.*coli* strains using standard procedures. BL21 E.*coli* strains containing recombinant plasmid were grown until mid-log phase (optical density at 600nm= 0.5 to 1.0), and expression of the fusion proteins was induced by addition of 0.5-4.0mmol·L^{-1} IPTG for 4h. Following induction, the bacteria were harvested by ultracentrifugation at 12000r·min^{-1}, resuspended in protein-buffer and seethed for 5min. Total protein was electrophoresed on SDS-PAGE gel and stained with coomassie.

Immunoblotting analysis

Briefly, the M_r26000 OMP was purified using Ni-NTA agarose resin after bacteria were cultured and broken down cy microwave with the energy of 600W×35% for 40min, ultracentrifuged (10000g, 15min, 4℃), and then quantified. *H.pylori* M_r26000 outer membrane protein-specific antibody was produced following subcutaneous immunization of the New Zealand rabbits, while age-matched control rabbits were immunized with PBS as described previously[17]. Serum antibody specificity was determined by ELISA or immunoblotting following electrophoretic transfer of SDS-PAGE-separated (150g·L^{-1} acrylamide) *H. pylori* M_r26000 outer membrane protein to 0.45μm pore size PVDF membrane. After a 30min wash in Tris-saline blotting buffer, antigen-impregated PVDF strips were incubated with the rabbit sera for 2h at RT. After washing, bound rabbit antibodies were detected by incubation of the strips in alkaline phosphatase-conjugated goat anti-rabbit IgG antibody for 1h at RT.

Prophylactic immunization

Six- to eight-week-old mice were immunized three times by subcutaneous immunization using emulsified M_r26000 OMP with Freund's adjuvant at intervals of 1, 14 and 21 days respectively, to produce antibody responded to M_r26000 outer membrane protein. The dose consisted of 1mL (100mg·L^{-1}) of purified M_r26000 OMP and 1mL complete Freund's adjuvant. Thereafter, the dose consisted of 0.5mL OMP and 0.5mL incomplement Freund's adjuvant. Age-matched control mice were immunized with PBS. The antibody titers in immunized mice were monitored by ELISA with purified fusion protein. Mice were challenged with a single dose of 10^8 *H. pylori* organisms 7 days after the last immunization. Twenty-eight days after challenge, the mice were killed by cervical dislocation. The stomach of each animal was removed,bisected longitudinally, and pinned out. Full-thickness tissue was taken from the antrum-body area of one-half of each stomach and placed into 0.2mL of urease test medium. Urease activity in the sample, identified by a distinctive color change in the medium, was assessed after 24h incubation at RT. The remainder of the stomach was fixed in 100mL·L^{-1} buffered formalin and embedded in paraffin. Longitudinal sections, stained with a modified May-Grunwald Giemsa stain, were scanned by full length under light microscopy. Mice were considered protected or not according to the previously report[17].

Statistical analysis

The Student test was used to evaluate the presence or absence of experimental infection in test and control animals as well as the anti-M_r26000 outer membrane protein response to immunization. *P* values <0.05 were considered as statistically significant.

RESULTS

PCR amplification of H. pylori M_r26000 OMP gene

According to the literature, the gene encoding the M_r26000 outer membrane protein, was amplified by PCR with Chongqing *H. pylori* strain's chromosomal DNA as the templates. The cloning products were electrophoresed and visualized on 10g·L^{-1} agarose gel(Figure 1). It revealed that M_r26000 OMP DNA fragment amplified by PCR contained a gene of approximately 594 nucleotides, which was compatible with the previous reports[21].

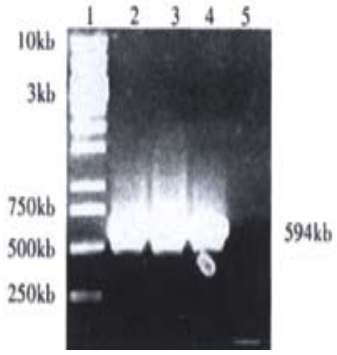

Figure 1 Ten g·L^{-1} agarose gel electrophoreses of M_r26000 OMP DNA fragment amplified by PCR from *Helicobacter pylori*. Lane1: Nucleotide marker; Lane2-4: PCR products; Lane 5: Negative control.

Identification of recombinant plasmid by restriction enzyme digestion

The recombinant plasmids pET32a(+) were all digested by *Hind*III or *Bam*HI, and by *Hind*III and *Bam*HI simultaneously, then digestive products were visualized on 10g·L^{-1} agarose gel eletrophoreses (Figure 2). It demonstrated that recombinant plasmid contained the objective gene.

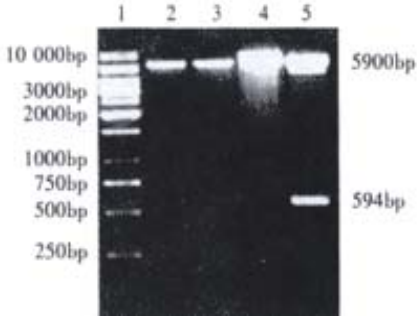

Figure 2 The identification of recombinant plasmid by restriction enzyme digestion. Lane1:Nucleotide marker; Lane2: pET32a(+) / *Hin*dIII; Lane3: pET32a(+) / *Hin*dIII, *Bam*HI; Lane4: Recombinant plasmid / *Hin*dIII; Lane5: Recombinant plasmid / *Hin*dIII, *Bam*HI.

Sequence analysis of cloned M_r26000 OMP nucleotide

The nucleotide sequence of the cloned genes inserted in pET32a(+) was analyzed by automated sequencing across the cloning junction, using the universal primer T_7. The results were: the cloned genes contained 594 nucleotides with a promoter and a start codon coding a putative protein of 198 amino acid residues with a calculated molecular mass of M_r26000. As compared with previously reports, 1.1% of the cloned genes were mutated, and 1.51% amino acid residues were changed. The homogeneity was about 98% between them. The cloned gene and mutative protein sequences were published in GenBank (AY 033499).

Analysis of the recombinant fusion protein

Following recombinant vector transformed into BL21 *E. coli* strains,

the fusion protein was amply expressed. Its molecular mass was M_r 46000 by 150g·L^{-1} SDS-PAGE gel analysis (the expression of the pET32a(+) vector, M_r20000). After the recombinant bacteria broken down by microwave and ultracentrifuged (10000r·min^{-1}, 15min, 4℃), the level of soluble fusion protein in the supernatant was about 38.96% of total cell protein. After purification by Ni-NTA agarose resin columniation, the purity of objective protein was about 90% (Figure 3).

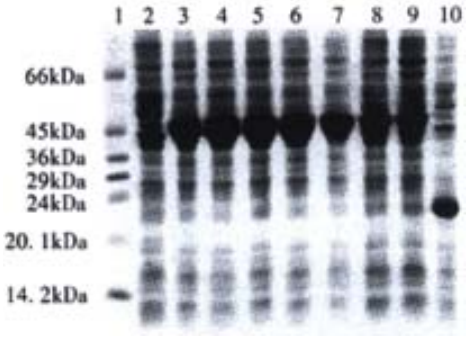

Figure 3 150g·L^{-1} SDS-PAGE analysis of the fusion protein expressed in BL21(DE3). Lane1: Molecular weight marker; 2Lane: BL21 after 4h induction with IPTG; Lane3-9: BL21/recombinant vector expression after 4h induction with 0.5,1,1.5,2,2.5,3,4mmol·L^{-1} IPTG respectively; Lane10: BL21/pET32a(+) vector expression after 4h induction with IPTG.

Antigenicity study of recombinant fusion protein

Sera were obtained from persons infected and not infected with *H. pylori* respectively. The recombinant fusion protein was recognized by the *H. pylori* positive sera, not recognized by the *H. pylori* negative sera, while the expressed protein of BL21/pET32a(+) not recognized by the *H. pylori* positive sera; the recombinant fusion protein was also recognized by the rabbit sera immunized with M_r26000 OMP, however the expressed protein of BL21/pET32a(+) not recognized by the rabbit sera immunized with M_r26000 OMP.

Prophylactic efficacy with H. pylori M_r26000 OMP

Subcutaneous immunization with *H. pylori* OMP and FA(Freund's adjuvant) conferred immune protection against *H. pylori* challenge in 19 (95%) of 20 mice. In contrast, 15 (100%) of 15 naive control animals were infected with *H. pylori*. These differences were statistically significant (P<0.05). The protection from infectious challenge was correlated with serum antibody reactivity to M_r26000 OMP by immunoblotting. Similar reactivity was absent in the sera collected from same animals prior to immunization, while sera from mice sham immunized with PBS and FA failed to display similar immune responsiveness.

DISCUSSION

The outer membrane is a continuous structure on the surface of gram-negative bacteria and an asymmetric bilayer with phospholipids in the inner monolayer and the bulky glycolipid lipopolysaccharide (LPS) in the outer monolayer, in bacterial pathogens, has bilateral particular significance as a potential target for protective immunity and avoiding the host's immune system. Outer membrane vaccines have been used with considerable success to induce protection against a number of organisms, including *H. pylori* the heat shock protein, urease A, B and so on. M_r26000 OMP is a low molecular mass Hor protein belonging to family I of *H. pylori*[20]. An earlier study showed that it was commonly expressed in all *H. pylori* strains examined so far. Furthermore, no cross-reaction is shown when antibodies (polyclonal and monoclonal) to *H. pylori* low-molecular outer membrane protein are used to immunoscreen closely related species of helicobacter, campylobacter, or a diverse range of other bacteria. *Hp* low molecular outer membrane protein is unique.

In our study, 1.1% of the cloned genes was mutated, 1.51% of amino acid residues was changed as compared with other reports[20]. The reasons of difference might be summarized as follows: (1)*H. pylori* chromosomal DNA as templates were different; (2) there is heterogeneity among strains; and (3) *H. pylori* was provided with the ability of transformation, which could lead to *H. pylori* variated and genome reseted[22]. But there was homogeneity between them. The purified recombinant M_r26000 OMP antigen could be recognized by the sera of patients infected with *H. pylori* and rabbit sera immunized with the recombinant protein. Moreover, in animal model, Balb/c mice immunized with the recombinant fusion protein were protected against *H. pylori* infection. These were consistent with previous reports[23-26]. While being an immunogenic marker, M_r26000 OMP showed a high sensitivity and specificity[27]. Moreover, a significant association was found between the serologic response to M_r26000 antigen and malignant outcome of *H. pylori* infection[28-31]. So the serum test for detecting antibody with low molecular weight proteins of *H. pylori* could be useful for identifying *H. pylori*-infected patients at risk of peptic ulcer or malignancy. The results showed that M_r26000 OMP is not only an immunogenic marker for detecting *Helicobacter pylori* infection and gastric carcinoma, but also a true vaccine candidate.

In addition to constructing the recombinant vector,we also tried to seek live carriers, because antigen delivery systems can influence the immune response qualitatively as well as quantitatively. Immunization via the mucosal route offers the advantage that it has the potential to stimulate both mucosal immunity and systemic immunity. It is simple, safe and can be used for the immunization of large population groups. Another advantage is the existence of the common mucosal immune system which induced protective immune responses at one mucosal site to be expressed at another[32]. So live carriers on oral route are ideal vaccine delivery systems and are being increasingly used to express large amounts of protective recombinant antigens. We are investigating live carriers to provide a mucosal vaccine vector to deliver M_r26000 OMP to antigen-presenting cells on the mucosal surface.

REFERENCES

1 Michetti P, Kreiss C, Kotloff K, Porta N, Blanco JL, Bachmann D, Herranz M, Saldinger P, Corthesy-Theulaz I, Losonsky G, Nichols R, Simon J, Stolte M, Ackerman S, Monath TP, Blum AL. Oral immunization with urease and Escherichia *coli* heat-labile enterotoxin is safe and immunogenic in *Helicobacter pylori*-infected adults. *Gastroenterology* 1999; 116: 804-812
2 Suganuma M, Kurusu M, Okabe S, Sueoka N, Yoshida M, Wakatsuki Y, Fujiki H. *Helicobacter pylori* membrane protein 1: a new carcinogenic factor of *Helicobacter pylori*. *Cancer Res* 2001; 61: 6356-6359
3 Nakamura S, Matsumoto T, Suekane H, Takeshita M, Hizawa K, Kawasaki M, Yao T, Tsuneyoshi M, Iida M, Fujishima M. Predictive value of endoscopic ultrasonography for regression of gastric low grade and high grade MALT lymphomas after eradication of *Helicobacter pylori*. *Gut* 2001; 48:454-60
4 Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med* 2001; 345: 829-32
5 Casella G, Buda CA, Maisano R, Schiavo M, Perego D, Baldini V. Complete regression of primary gastric MALT-lymphoma after double eradication *Helicobacter pylori* therapy: role and importance of endoscopic ultrasonography. *Anticancer Res* 2001;21(2B): 1499-1502
6 Morgner A, Miehlke S, Fischbach W, Schmitt W, Muller-Hermelink H, Greiner A, Thiede C, Schetelig J, Neubauer A, Stolte M, Ehninger G, Bayerdorffer E. Complete remission of primary high-grade B-cell gastric lymphoma after cure of *Helicobacter pylori* infection. *J Clin Oncol* 2001; 19:2041-2048
7 Kate V, Ananthakrishnan N, Badrinath S. Effect of *Helicobacter pylori* eradication on the ulcer recurrence rate aftersimple closure of perforated

duodenal ulcer: retrospective and prospective randomized controlled studies. *Br J Surg* 2001; 88: 1054-1058
8 Zhuang XQ,Lin SR. Progress in research on the relationsh between *Hp* and stomach cancer. *Shijie Huaren Xiaohua Zazhi* 2000; 8:206-207
9 Yu XE, Zhao AX, Wei DL, Du JZ. Relationship between *Helicobacter pylori* infection and gastric cancer. *World J Gastroenterol* 1998; 4(Suppl 2): 96
10 Gao HJ, Yu LZ, Bai JF, Peng YS, Sun G, Zhao HL, Miu K, L¨¹ XZ, Zhang XY, Zhao ZQ. Multiple genetic alterations and behavior of cellular biology in gastric cancer and other gastric mucosal lesions: *H. pylori* infection, histological types and staging. *World J Gastroenterol* 2000; 6:848-854
11 Yao YL, Zhang WD. Relation between Helicobacter and gastric cancer. *Shijie Huaren Xiaohua Zazhi* 2001; 9:1045-1049
12 Goto T, Nishizono A, Fujioka T, Ikewaki J, Mifune K, Nasu M. Local secretory immunoglobulin A and postimmunization gastritis correlated with protection against *Helicobacter pylori* infection after oral vaccination of mice. *Infect Immun* 1999; 67:2531-2539
13 Watanabe T, Tada M, Nagai H, Sasaki S, Nakao M. *Helicobacter pylori* infection induces gastric cancer in Mongolian Gerbils. *Gastroenterology* 1998; 115:642-648
14 Honda S, Fujioka T, Tokieda M, Satoh R, Nishizono A, Nasu M. Development of *Helicobacter pylori*-induced gastric carcinoma in Mongolian Gerbils. *Cancer Res* 1998; 58:4255-4259
15 Hatzifoti C, Wren BW, Morrow JW. *Helicobacter pylori* vaccine strategies-triggering a gut reaction. *Immuno Today* 2000; 21: 615-619
16 Kotloff KL, Sztein MB, Wasserman SS, Losonsky GA, DiLorenzo SC, Walker RI. Safety and immunogenicity of oral inactivated whole-cell *Helicobacter pylori* vaccine with adjuvant among volunteers with or without subclinical infection. *Infect Immun* 2001; 69: 3581-3590
17 Keenan J, Oliaro J, Domigan N, Potter H, Aitken G, Allardyce R, Roake J. Immune response to an 18-kilodalton outer membrane antigen identifies lipoprotein 20 as a *Helicobacter pylori* vaccine candidate. *Infect Immun* 2000; 68: 3337-3343
18 Dubois A, Lee CK, Fiala N, Kleanthous H, Mehlman PT, Monath T. Immunization against natural *Helicobacter pylori* infection in nonhuman primates. *Infect Immune* 1998; 66: 4340-4346
19 Ikewaki J, Nishizono A, Goto T, Fujioka T, Mifune K. Therapeutic oral vaccination induces mucosal immune response sufficient to eliminate long-term *Helicobacter pylori* infection. *Microbiol Immunol* 2000; 44:29-39
20 Alm RA, Bina J, Andrews BM, Dolg P, Hancock REW, Trust TJ. Comparative genomics of *Helicobacter pylori*: analysis of the outer membrane protein families. *Infect Immun* 2000; 68: 4155-4168
21 Toomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, Mckenney K, Fitzegerald LM, Lee N, Adams MD, Hickey EK, Berg DE, Gocayne JD, Utterback TR, Peterson JD, Kelley JM, Cotton MD, Weldman JM, Fujll C, Bowman C, Watthey L, Wallin E, Hayess WS, Borodovskys M, Karp PD, Smiths HO, Fraser CM, Venter JC. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 1997; 388:739-747
22 Zhong P, Wu KX, Xu FH, Xu CD, Xu JY. Coning and characterization of Hp cagA gene sequences derived from Chinese strains. *Shijie Huaren Xiaohua Zazhi* 1999;7: 669-672
23 Li MF, Ling Z, Ma AY, Zhao JH, Sun JX, Yu SZ, Wu XF. Cloning, expression and immunogenic of *Hp* UreB gene. *Shijie Huaren Xiaohua Zazhi* 1999; 7:596-600
24 Saldinger PF, Porta N, Launois P, Louis JA, Waanders GA, Bouzourene H, Michetti P, Blum AL, Corthesy-theulaz IE. Immunization of Balb/c mice with Helicobacter Urease B induces a Thelper 2 response absent in Helicobacter infection. *Gastroenterology* 1998;115: 891-897
25 Ghiara P, Ross M, Marchetti M, Tommaso AD, Vindingni C, Ciampolini F, Covacci A, Telford J, Magistris MTD, Pizza M, Rappuoli R, Giudice GD. Therapeutic intragastric vaccination against *Helicobacter pylori* in mice eradicates an otherwise chronic infection and confers protection against reinfection. *Infect Immun* 1997; 65:4996-5002
26 Kleanthous H, Myers GA, Georgakopoulos KM, Tibbitts TJ, Ingrassia JW, Gray HL, Ding R, Zhang ZZ, Lei W, Nichols R, Lee CK, Ermak TH, Monath TP. Rectal and intranasal immunizations with recombinant Urease induce distinct local and serum immune responses in mice and protect against *Helicobacter pylori* infection. *Infect Immun* 1998; 66:2879-2886
27 Yang WH, Lin SR, Wang LX, Ding SG, Jin Z. The relation between the pathogenetic facters of *Helicobacter pylori* and gastric diseases. *J Beijing Med Univ* 2000;32:34-38
28 Shiesh SJ, Sheu BY, Yang SB, Tsao HJ, Lin XZ. Serologic response to lower-molecular-weight proteins of *Helicobacter pylori* is related clinical outcome of *Helicobacter pylori* infection in Taiwmn. *Dig Dis Sci* 2000;45:781-788
29 Mitchell HM, Hazell SL, Li YY, Hu PJ. Serological response to specific *Helicobacter pylori* antigen: antibody against cagA antigen is not predictive of gastric cancer in a developing country. *Am J Gastroenterol* 1996; 91:1785-1789
30 Raymond J, Sauvestre C, Kalach N, Bergeret M, Dupont C. Immunoblotting and serology for diagnosis of Helicobacter pylori infection in children. *Pediatr Infect Dis J* 2000; 19:118-121
31 Lu JJ, Perng CL, Shyu RY, Chen CH, Lou Q, Chong SKF, Lee CH. Comparison of five PCR methods for detection of *Helicobacter pylori* DNA in gastric tissues. *J Clin Microbiol* 1999; 37:772-774
32 Hasan UA, Abai AM, Harper DR, Wren BW, Morrow WJW. Nucleic acid immunization: concepts and techniques associated with third generation vaccines. *J Immunol Method* 1999; 229:1-22

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