African Journal of Biotechnology Vol. 11(31), pp. 8038-8044, 17 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.051
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Oral vaccination with attenuated *Salmonella choleraesuis C500* expressing recombinant UreB and CagA antigens protects mice against *Helicobacter pylori*

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Accepted 8 March, 2012

Helicobacter pylori **are well known as the major gastro-duodenal pathogen of peptic ulcer disease and gastric cancer. Recombinant** ***H. pylori*** **vaccine comprising a single subunit antigen can only induce immune response with limited protection efficiency. Development of oral vaccine would be a new effective strategy for the prevention of** ***H. pylori*** **infection. In this study, the protective effect of** ***H. pylori*** **multicomponent vaccine consisting of UreB and CagA subunit antigens was constructed and investigated in mice. The UreB and CagA gene of** ***H. pylori*** **were inserted into the plasmid pYA3493 and expressed in attenuated** ***Salmonella choleraesuis C500*****. The UreB and CagA producing strains were then administered orally to mice, contracting to Whole-cell vaccine against** ***H. pylori SS1*** **infection, and the immune response was assessed by mice immunity IgG ELISA and** ***H. pylori SS1*** **attack. Noticeable IgG response was induced in the sera of mice orally immunized with** ***S. choleraesuis C500*** **strain consisting of UreB and CagA subunit antigens. Mice vaccinated orally were significantly protected against gastric** ***Helicobacte*****r infection following a challenge with** ***H. pylori*** **strain SS1. Orally vaccination with the expression of UreB-CagA could prevent gastric infection with** ***H. pylori.***

Key words: *Helicobacter pylor*, orally vaccination, UreB and CagA subunit antigens, mice.

INTRODUCTION

Helicobacter pylori is a Gram-negative bacterium, specialized in the colonization of the stomach (Warren and Marshall, 1983), and well known as the major gastro-duodenal pathogen of peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma, and gastric cancer (Blaser, 1995; Asaka, 1994; Graham, 1992; Michetti, 1997). Adhesion to the gastric epithelium and the host responses take the crucial role in the pathogenesis of *H. pylori* infections.

Several *H. pylori* virulence factors have been identified, including the urease, the vacuolating cytotoxin (VacA), and the cytotoxin-associated gene A antigen (CagA) (Covacci, 1993; Cover, 1996, Cover and Blaser, 1992; Hirai, 1994; Tee, 1995).

Urease activity of *H. pylori* could produces ammonia, which buffers the pH of its immediate surroundings within the stomach and facilitates the organism's nitrogen metabolism at neutral pH as well as protecting it from acid damage at low pH (Williams, 1996). Urease might also help to recruit neutrophils and monocytes in the inflamed mucosa and to activate production of pro-inflammatory cytokines (Harris, 1996). Moreover, urease (including subunit proteins UreA and UreB) is one of the main antigens recognized by the human immune response to *H. pylori,* and UreB seems to be more

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protective than UreA (Ferrero, 1994). Thus, the UreB has been used in vaccination trials to prevent infection with *H. pylori* in mice (Del Giudice, 2001). Vaccine has been applied successfully as a potential therapeutic strategy for prevention and treatment. Recently, human *H. pylori* oral subunit vaccine (UreB LTB) has been approved for use in China, its anti-*H. pylori* infection protective time is one year and protective rate is 72.1%. However, the establishment of long-term oral immunity for protection against *H. pylori* infection has not yet been reported.

The CagA is encoded in a pathogenicity island known as 'Cag PAI' and is deeply correlated with the severity of *H. pylori*-related diseases. The CagA is frequently associated with cytotoxin production. CagA-positive *H. pylori* infection elicits large amounts of IL-8 production from epithelial cells and IL-8 induces cellular infiltration into themucosa and consequently leads to the activation of anti-bacterial immune responses (Brandt, 2005; Sharma, 1998). Several studies have suggested that CagA is a useful marker for the most virulent strains that are associated with peptic ulcer, atrophic gastritis and adenocarcinoma (Figura, 1989; Crabtree, 1992; Blaser, 1995). They also found that $CagA^+$ *H. pylori* (with CagA gene) caused more severely damage to $CagA^-$ *H. pylori* and knockout of its CagA gene could prevent damage to the cell junctions of gastric epithelial cells (Keates S, 1999).

In 90% of *H. pylori*-infected patients treated with antibiotics and strong acid suppressor drug, such as proton pump inhibitors, its eradication is successful (Rappuoli, 1999). However, failure of *H. pylori* eradication treatment has recently increased due to the proliferation of antibiotic-resistant strains (Murakami, 2002; Kotloff, 2001; Nystrom and Svennerholm, 2007). Vaccination against *H. pylori* is therefore one of the most effective ways to control *H. pylori* infection and, indeed, administration of oral bacterial antigens can protect mice against *H. pylori* infection (Marchetti, 1998). Recently, attenuated and nonpathogenic bacteria have been developed as mucosal vaccine delivery vehicles (Thole, 2000; Seegers, 2002; Nouaille, 2003; Wells and Mercenier, 2008). The risk of nonpathogenic bacteria is low and advantageous, particularly for the children, the elderly and immunocompromised individuals. In addition, as mucosal delivery vehicles, recombinant bacterial vaccine vectors offer several practical advantages, including avoidance of culturing large quantities of pathogens, no need to purify antigenic components or subunits. The use of oral routes for immunization against infective diseases is desirable due to easy administration and high compliance rates, and mucosal surfaces are the portals of entry for *H. pylori*.

In generally, plasmids can be stably maintained in bacteria through the use of antibiotic selection genes encoded on the plasmid. However, antibiotics and their resistance genes are not desirable due to biosafety and regulatory concerns. Recent studies have demonstrated that the asd based host-plasmid balanced lethal system could overcome the problem of the use of antibiotic resistance gene. Zhao (2008) used △crp and △asd double deleted strain *C500*/pYA3493 balanced lethal system to express filamentous hemagglutinin and pertactin antigens gene and obtain the ideal efficacy.

Previous studies have shown that animals can be protected from *H. pylori* infection by immunization. In this study, we expressed UreB and CagA in the *C500*. The recombinant attenuated *S. choleraesuis* were used for oral immunization of mice, contracting to Whole-cell vaccine against *H. pylori SS1*.

MATERIALS AND METHODS

Bacterial strain, plasmid and culture

H. pylori Sydney strain 1 (SS1) was kindly provided by Professor Yong Xie (Nanchang University, Jiangxi, China). The *H. pylori* strain was cultured at 37°C under microaerophilic conditions on Columbia agar plates containing 10% sheep blood.

This strain was grown in Brucella Broth containing 10% fetal bovine serum with gentle shaking at 37°C for 72 h under microaerophilic conditions. After cultivation for three days, the live bacteria were centrifuged at 5000×g for 15 min and washed three times with phosphate-buffered saline (PBS, pH 7.4).

pYA3493 plasmid and strain of *χ6097* were kindly provided by Curtiss R 3rd(2002) at Department of Biology, Washington University. *S. enterica sv. Choleraesuis C500* strain with △crp △asd double deletion was obtained from Dr. Guo Aizhen (2006).

Construction of UreB/CagA DNA vaccine

A pair of primers termed FP and RP were designed according to UreB gene and CagA gene of *H. pylori SS1* in GenBank AF508016 and AF247651 as follows: UreB: FP:5-gAA TTC ATg AAA AAg ATT AgC AgA AAA gA-3'; RP:5'-gTC gAC TTg CCA AgT TCT AgT gAT AA TTC-3'. CagA: FP：5' -ACg CgT CgA CAT gAC TAA CgA AAC CgT CgA -3'; RP: 5'-AAg CTT TCT TAC AAg gAT TCA TCA AAC ACg-3'.

UreB: EcoRI and SalI; CagA: SalI and HindIII recognition sites were introduced into the 5,-terminal of FP (underlined) and RP (underlined), respectively, to facilitate cloning. The polymerase chain reaction (PCR) was done under the following conditions: preheating at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 40 s, and extension at 72°C for 1 min (CagA genes) or 1.5 min (UreB gene). The PCR products were analyzed by agarose gel electrophoresis and recovered using a TaKaRa agarose gel DNA purification kit (China). The fragments were then ligated into the TaKaRa pMD18-T simple vector and sequenced at the Nucleic Acids Facility (Sangon, Shanghai, China). The obtained plasmids was named pMD18-T-CagA and pMD18-T-UreB and both plasmids were transformed into Competent cells DH5a.

Preparation of UreB/CagA DNA vaccine

The plasmid pMD18-T-UreB was digested with EcoRI and SalI to obtain the UreB fragment. The purified UreB fragment was ligated into the EcoRI-SalI digested pYA3493 expression vector, resulting in pYA3493-UreB and then transformed into Competent cells *E. coli x6097*. The plasmid pMD18-T-CagA was digested with SalI and HindIII to release the CagA fragment. The purified CagA fragment

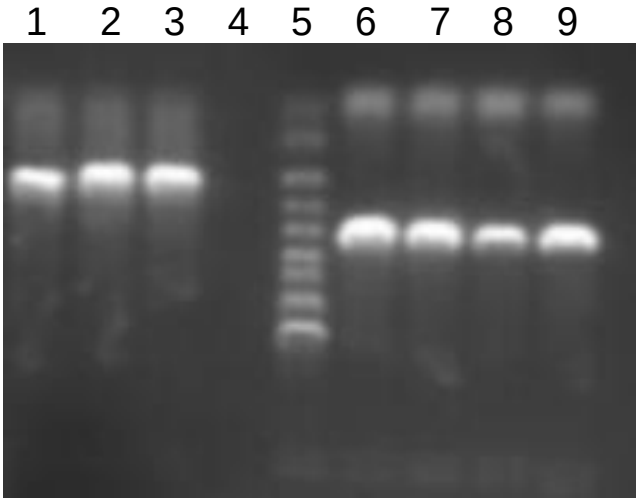

Figure 1. The target fragment of UreB and CagA gene amplified from *H. pylori* strain SS1 DNA. Lane 5, 1 kb DNA marker; lanes 6-9, the target amplification fragment of UreB gene (1134bp) from genomic DNA of *H. pylori* strain SS1; lane 4: blank control; lanes1-3, the target amplification fragment of CagA gene(480bp) from genomic DNA of *H. pylori* strain SS1.

was ligated into the SalI-HindIII digested pYA3493-UreB, resulting in pYA3493-UreB-CagA and then transformed into Competent cells *χ6097*. Thus the plasmid pYA3493-UreB-CagA was purified and transformed into *Salmonella choleraesuis C500* strain by electroporation, and SDS-PAGE and western blot analysis were then carried out (Because the ligated production was more easily transformed into Competent cells *χ6097* by heat shock than *Salmonella choleraesuis C500* strain by electroporation).

Immunization and sample collection

Female Kunming mice aged 7 weeks were used in this experiment. One-week acclimatization period was given before immunization. Before immunization, feed and water were withheld for 8 h, and blood samples were taken from the cutting-down tails. 50 mice were randomly divided into 5 groups: inoculated orally into 0.2 mL of *C500* expressing pYA3493-UreB-CagA; *C500* with pYA3493; *C500*; PBS and injection into 0.2 mL (2×10^9) of *H. pylori SS1* with adjuvant. Oral doses of 1×10^{10} $CFUmL^{-1}$ were administered via an intragastric gavage. Before immunization, the 0.2 mL $NaHCO_3$ (7.5%) were administered via an intragastric gavage to the mice. Immunizations were performed on days 0, 7, 14, 21 and 28, and serum samples were taken at intervals of 7 days or 14 days and then centrifugalizated and stored at -20°C until use. Food and water were given 2 h after immunization.

Detection of UreB-specific serum IgG

ELISA plates were coated overnight at 4°C with 1 mg/mL recombinant purified UreB. Three-fold serially diluted serum samples starting from 1: 400 were applied onto the plates and incubated for 1 h at 37°C. Serum IgG were detected by peroxidase-labeled goat anti-mouse IgG. Endpoint titers were determined as the reciprocal of the dilution factor of sample yielding background levels of OD450 nm. The ELISA results were evaluated using positive/negative (P/N) OD ratios. Symbols of P and N indicated the OD value of the tested samples and negative control samples, respectively (Yang, 1998; Ebel, 2002). The ratio of the OD of known positive and known negative sera is termed as positive/negative (P/N) ratios. A P/N value higher than 2.0 and OD value over 0.3 was determined as comprehensive positive standard for the ELISA, otherwise, negative (Han, 2008).

Challenge of vaccinated mice

The cultured *H. pylori SS1* were harvested and resuspended in phosphate-buffered saline (PBS) to yield a concentration of 1×10^{10} $CFUmL^{-1}$. Vaccinated mice were challenged with 0.2 mL (2×10^9) *H. pylori SS1* instilled into stomach once every day for three days. Food and water were given 2 h after the challenge.

Assessment of *H. pylori* colonization of the mouse

Four weeks after receiving the challenge, mice were sacrificed by spinal dislocation. The stomachs and duodenum were washed twice in sterile 0.9% NaCl, and the pylorus, liver, lung, duodenum and spleen from mice were assessed by the hematoxylin-eosin (H.E.) staining. For *H. pylori SS1* quantitative culturing, the stomach samples were weighed, and homogenized in PBS, serially diluted in PBS and plated onto Columbia agar plates containing 10% sheep blood with antibiotics.

Statistics

All analyses were performed using SAS system 8.1 Software. The OD value and the data of bacterium were compared by a Student's T-test, expressed as mean ± SD. P values of <0.05 were considered to be significant.

RESULTS

Amplification of UreB gene and CagA gene by PCR

The target fragment of UreB gene and partial CagA gene with the expected size amplified from DNA template of *H. pylori* stain SS1 is shown in Figure 1. The nucleotide sequences of UreB gene and partial CagA gene were completely similar to UreB and CagA gene of *H. pylori* SS1 in GenBank AF508016 and AF247651. Proteins were extracted and assayed by Western blotting after SDS-PAGE, using monoclonal antiserum to UreB and CagA (Santa Cruz Biotechnology). The results indicated that rUreB was produced in the supernatant of pYA3493-ureB-CagA (Figures 2 and 3).

Antibodies assay

Groups of mice were immunized orally with *C500*, which expresses the UreB-CagA protein. Control mice were vaccinated in the same way with pYA3493 strains of *C500, C500,* PBS and injection *H. pylori SS1*. The level of IgG in the serum was significantly higher ($P<0.001$) in mice immunized with *C500* that expresses the UreB-CagA protein and injection *H. pylori SS1* (Figure 4).

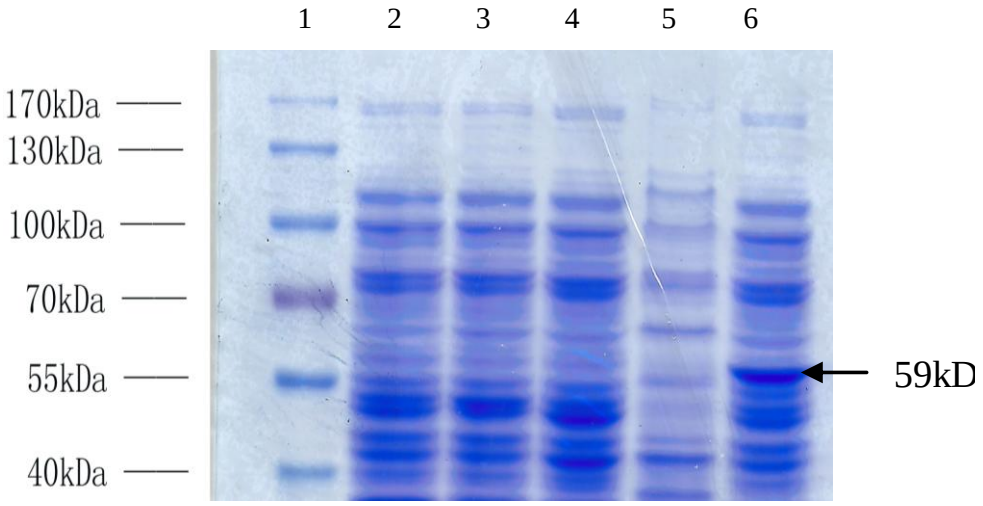

Figure 2. Analysis of recombinant UreB-CagA expression with SDS-PAGE. Lane 1, Protein marker; Lanes 2- 4, *C500* with pYA3493; Lane 5, *C500*; Lane 6, *C500* with pYA3493-UreB-CagA (59kD).

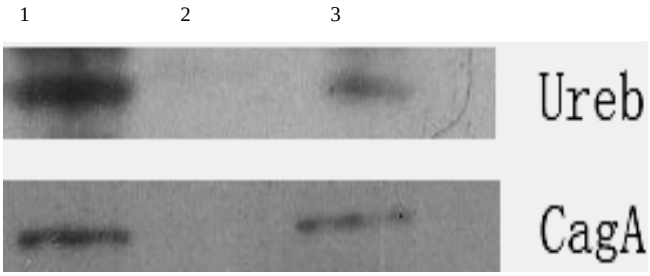

Figure 3. Western blot analysis of UreB and CagA in transgenic bacterium. Lane 1, Western blotting of *C500* with pYA3493-UreB-CagA; Lane 2, western blotting band of *C500*; Lane 3, western blotting band of *C500* with pYA3493.

Immunohistochemical analysis

Compared with the control group, histological analysis pointed that the liver, lung, spleen, duodenum and pylorus had no lesions. But the immunohistochemical analysis showed that the anti-UreB antibody was positively expressed in both the duodenum and pylorus immunized orally with C500 that expresses the UreB-CagA protein. On the contrary, there was no positive reaction in the control groups (Figure 5).

H. pylori SS1 colony counts in different tissues

Four weeks after inoculation, all of the Kunming mice were immunized positively by colony counts. The positive immunized group includes orally inoculated *C500* with pYA3493-UreB-CagA and injection of *H. pylori SS1* vaccine, which was whole-cell vaccine of *H. pylori SS1* with adjuvant. The negative immunized group includes orally inoculated *C500* with pYA3493, *C500* and PBS (Table 1). The negative immunized mice had a range of *H. pylori* colonization from 5.4×10^4 to 7.3×10^4 bacteria g^{-1} stomach. The immunized mice with vaccine inoculated orally with C500 expressing pYA3493-UreB-CagA and by injected whole-cell of *H. pylori SS1,* were significantly different in protection between the groups of mice immunized with the negative control groups ($^aP<0.01$).

DISCUSSION

The infection of *H. pylori* is deeply associated with the development of peptic ulcer disease and cancer in the host stomach. In developing countries, the high incidence of gastric cancer, high *H. pylori* reinfection rates, antibiotic resistance and high cost of antibiotic treatment make vaccination a special attractive intervention (Ramirez, 1997; Lahaie, 2002). One alternative to induce immune responses in the mucosal surfaces is by introducing the vaccine antigen orally, where the antigen is recognized and processed by specialized immune cells situated at the gastrointestinal tract surfaces (Backert, 2000). *S. choleraesuis C500* can be a tool in the construction of oral subunit vaccines. *C500* is an avirulent vaccine strain attenuated by chemical methods, which is highly immunogenic and safe and has been used widely (Qiao, 2005; Zhao, 2008; Man, 2009). In this study, all mice immunized with *C500* survived, and no clinical signs were observed in the immunized mice during the entire experimental period.

This report shows that oral vaccination with recombinant *C500* UreB-CagA producer strains prevented gastric infection with *H. pylori*. The aim of this study is to modulate the immune responses to UreB- CagA as

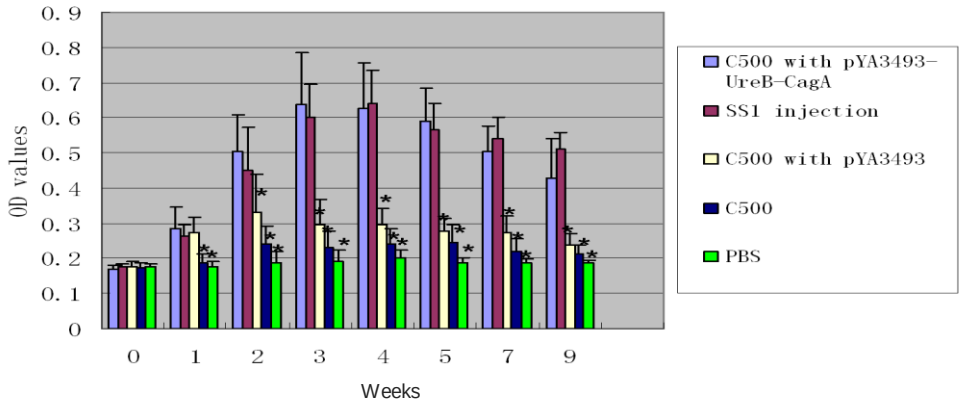

Figure 4. Dynamics of anti-UreB antibodies levels detected in mice. ELISA titers of sera from mice on days 0, 7, 14, 21, 28, 35, 49 and 63 (*P<0.01).

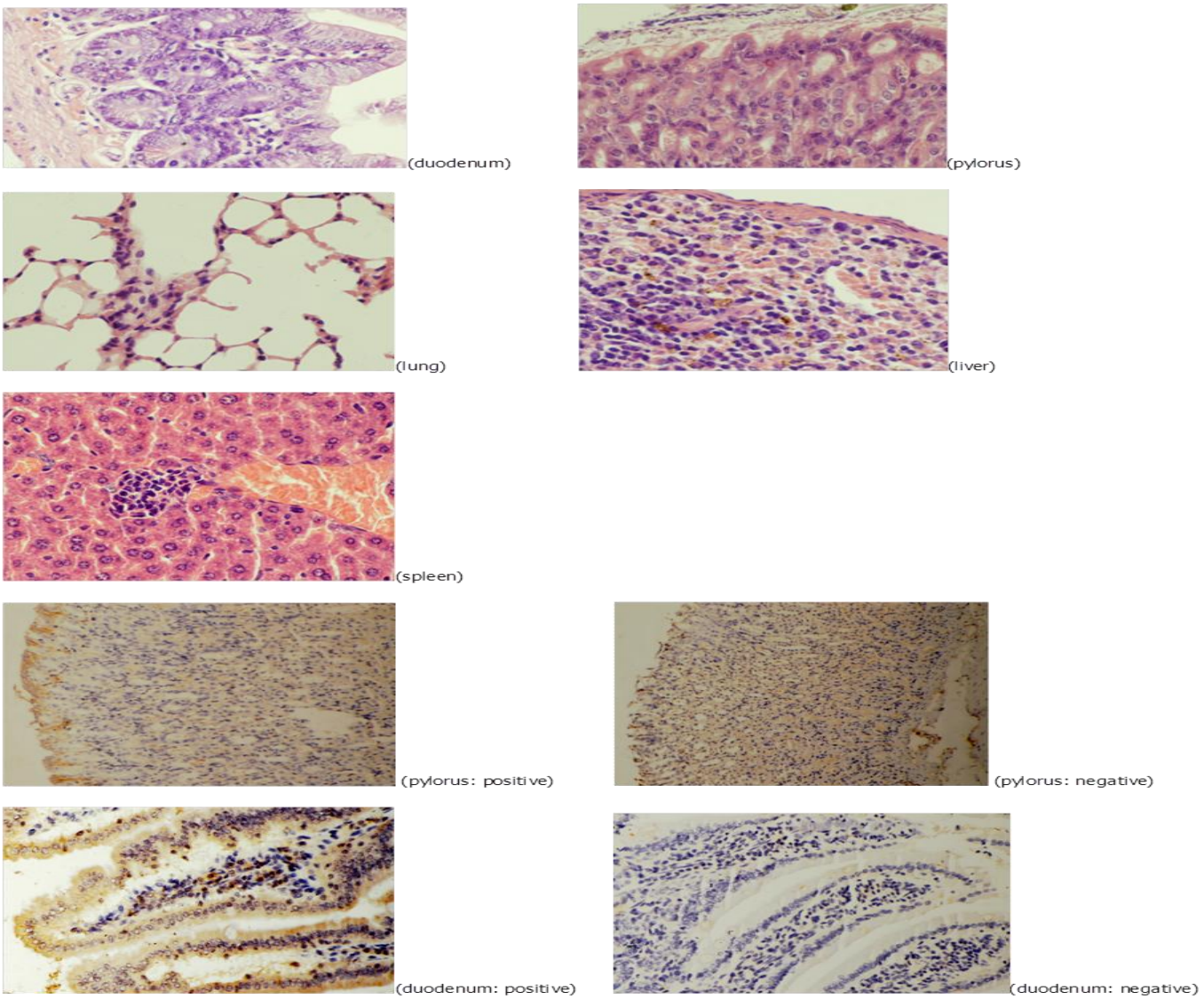

Figure 5. Paraffin section of duodenum(a), pylorus(b), lung(c), liver(d) and spleen(e) by the hematoxylin-eosin staining. Immunohistochemical investigation of the pylorus (f:positive, g:negative) and duodenum(h:positive,i:negative).

Table 1. *H. pylori SS1* colony counts in different tissues (Means ± SD) ([a] $P<0.01$, [b] $P>0.05$).

Tissue	*C500* with pYA3493-UreB-CagA (×102/g)	*C500* with pYA3493 (×102/g)	*C500* (×102/g)	PBS (×102/g)	*H. pylori SS1* injection (×102/g)
Pylorus	9±4	354±104.1[a]	338±105.8[a]	576±131.1[a]	4±2.3[b]
Duodenum	2±0.66	191±38.1[a]	230±38.1[a]	252±102.1[a]	2±0.9[b]
Total	**11**	**540**	**570**	**730**	**6**

the protein model and recombinant *C500* strains as the vehicle. The rUreB/CagA-producing *C500* strain was constructed to produce rUreB-CagA in an extracellular location and rUreB/CagA could be detected in the supernatant of *C500* by Western blotting experiments. Injection whole-cell vaccine and Oral inocula of 2×10^9 recombinant *C500* expressing UreB-CagA were given on days 0, 7, 14，21 and 28 to mice elicited anti-UreB serum antibody responses that were significantly different from those observed in the control groups (inoculated orally *C500* with pYA3493, *C500* and PBS). Following oral immunization, a rapid increase in anti-UreB was detected in sera, but it declined on day 35. The IgG levels of whole-cell vaccine injection of *H. pylori SS1* with adjuvant was lower than the oral vaccine. At the same time, the number of SS1 of tissues in the group which received injection was less than that of the group which received oral immunization ($P>0.05$). Our results indicate that oral vaccination could elicit mucosal immune responses in the intestine and duodenum, and the *H. pylori SS1* colony counts were significantly lower in the negative control group ($P<0.01$), but similar in amount with the positive control group ($P>0.05$).

However, we can conclude that immunization with live recombinant *C500*-expressing UreB-CagA subunits reduced *H. pylori* colonization compared to the negative control mice, suggesting that a protective immune response had been induced in the mice.

These results suggest that oral administrations of *H. pylori*-antigen with rCTB are safe and do not cause an anaphylactic reaction. This result was compatible with previous reports (Eiji Kubota et al., 2007). From our assessments, mucosal immunization by oral administration may be a better way of suppressing proliferation of *H. pylori.*

Furthermore, the results obtained from the work of Lee et al. (2001) showed that there was no protective effect against *H. pylori* after *H. pylori* strain *SS1* challenge, although the antigen-specific serum IgG titers were detected in mice immunized with recombinant *L. lactis* constitutive expression of the *H. pylori* UreB gene. Our results suggest that UreB-specific IgG was both necessary and sufficient to prevent gastric infection with *H. pylori* and this is because the *H. pylori SS1* colony counts were significantly lower than the negative control group ($P<0.01$)

In conclusion, we have shown that the recombinant pYA3493-UreB-CagA is effective as a vaccination for immunization against *H. pylori* infection since mucosal immunization induced by oral administration of rCTB recombinant cholera toxin B-subunit suppressed proliferation of *H. pylori*. Oral vaccination may be a new strategy additively supportive of conventional antibiotic eradication therapy against *H. pylori* infection. But IgG levels antibody of Oral vaccination declined quicker than the IgG of injection whole-cell *H. pylori* vaccine.

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