

Oral Immunization of Mice With Vaccine of Attenuated *Salmonella typhimurium* Expressing *Helicobacter pylori* Urease B Subunit¹

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Objective To prepare the live recombinant vaccine of attenuated *Salmonella typhimurium* SL3261 expressing *Helicobacter pylori* (*H. pylori*) B subunit (UreB) and to determine whether it could be used as an oral vaccine against *H. pylori* infection. **Methods** Using genomic DNA of *H. pylori* Sydney strain (SS1) as template, the *H. pylori* UreB gene fragment was amplified by PCR and subcloned into the expression vector pTC01. The recombinant plasmid pTC01-UreB was then transferred into LB5000 to obtain modified forms, and further converted into the attenuated *Salmonella typhimurium* SL3261 to obtain recombinant SL3261/pTC01-UreB as an oral immunization reagent, which was then used to orally immunize Balb/c mice twice at a three-week interval. Twelve weeks later, anti-UreB IgA antibodies in intestinal fluid and IgG antibodies in sera were determined by ELISA. The relating data in control groups (including body weight, gastric inflammation, etc.) were also collected. **Results** The sequencing analysis showed that the UreB gene fragment amplified by PCR was consistent with the sequence of the *H. pylori* UreB gene. The restriction enzyme digestion revealed that the correct pTC01-UreB was obtained. SDS-PAGE and Western blot showed that a 61KD protein was expressed in SL3261/pTC01-UreB, which could be recognized by anti-*H. pylori* UreB antiserum and was absent in the control containing only *Salmonella typhimurium* SL3261 strain. The multiple oral immunization with SL3261/pTC01-UreB could significantly induce *H. pylori* specific mucosal IgA response as well as serum IgG responses. IFN- γ and IL-10 levels were significantly increased in SL3261/pTC01-UreB group, and no obvious side effect and change in gastric inflammation were observed. **Conclusion** The attenuated vaccine of *Salmonella typhimurium* expressing *H. pylori* UreB can be used as an oral vaccine against *H. pylori* infection.

Key words: *Helicobacter pylori*; Urease B subunit; Recombination; Attenuated *Salmonella typhimurium*; Oral vaccine

INTRODUCTION

H. pylori is one of the most pandemic bacteria in human beings and has been identified as the chief pathogen for chronic gastritis, peptic gastric ulcer and some kinds of carcinoma of epithelial tissue in gastric mucosa. Fortunately, related studies for the time being unanimously suggest that the vaccine of *H. pylori* might become the most effective method in the treatment and prevention of *H. pylori* infection worldwide^[1]. Most *H. pylori* vaccine studies have been focused on the protein vaccine, including studies on the extracts of whole *H. pylori* ultrasonic grinding and screening of *H. pylori* structural antigen. Since the preparation and purification of protein antigen are costly and time consumed, it is extremely

necessary to develop novel vaccines against *H. pylori* infection. Because protein can be easily degraded in gastrointestinal tract, adjuvants such as *Vibrio comma* CT and *Bacillus coli* Lt are usually added to induce effective and protective immunoreactions^[2]. While the body is immunized with protein vaccine, it suffers from various side effects caused by adjuvants. Urease is considered as the best candidate antigen for the vaccine preparation of *H. pylori* so far since urease is an inactive structural protein and indispensable in the implantation process of *H. pylori* into gastric mucosa^[3]. A lot of studies showed that oral immunization in both animals and human beings with either native or recombinant *H. pylori* urease B subunit (UreB) can successfully induce protective immunoreactions against *H. pylori* infection^[4-6].

Attenuated *Salmonella typhimurium* SL3261 is

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an engineered bacterium with mutational gene of aromatic amino acid-related gene (*aroA* gene). It could be used as an ideal vector due to its advantages of having invasiveness but without pathogenicity of *Salmonella*. It also has ability to induce effective and protective immunoreactions in gastric mucosa without degradation and damage in the gastrointestinal tract when orally administrated. In the study, we immunized Balb/c mice with the live recombinant vaccine of attenuated *Salmonella typhimurium* SL3261 expressing *H. pylori* *UreB*, tested anti-*UreB* antibodies, cytokines of *INF-γ* and *IL-10*, and index of gastric mucosal inflammation, and then determined whether attenuated SL3261/pTC01-*UreB* could be used as an oral vaccine against *H. pylori* infection.

MATERIALS AND METHODS

Materials

Experimental animals Four weeks old female Balb/c mice, weighing 15 g to 20 g were provided by Experimental Animal Resource Center of Zhongshan University, Guangzhou.

Plasmid, bacterial strains and reagents Pro-nucleus expression vector pTC01 (containing gene of chloramphenicol antibody) was from Invitrogen Company. *H. pylori* Sydney strain (SS1) was a generous gift from Dr. Hua CHEN, Department of

Gastroenterology of the First Military Medical University, Guangzhou. Attenuated *Salmonella typhimurium* LB5000 and SL3261 were from Dr. Da-Ming REN, Institute of Genetics, Fudan University, Shanghai. Restriction endonucleases *Sal* I and *Xba* I were from Sino-American Biotechnology Company. *INF-γ* and *IL-10* ELISA reagent box were from Gene Company.

Methods

Construction of Recombinant Expression Vector pTC01-*UreB* Expressing *H. pylori* *Urease B* Subunit

Culture and identification of *H. pylori* Sydney strain SS1, extraction of bacterial strain genomic DNA and amplification of *UreB* by PCR The culture of *H. pylori* SS1 followed the principles of Basic and Clinical Study on *H. pylori* Infection. *H. pylori* was incubated on agar made from sheep blood for three to five days at 37 °C under micro-oxygen condition. *H. pylori* SS1 genomic DNA was extracted and used as the template. Segment 16SrRNA and two segments in *VacA* (mid-region and signal sequences) of *H. pylori* SS1 were amplified by PCR. Final products of PCR were identified on 2% agarose gel. *H. pylori* strain 60190 (sl/mL) was used as the control group. Primer sequences of the gene *VacA* and the amplified 16SrRNA are listed in Table 1.

TABLE 1

Primer Sequences			
Amplified Gene	Genotype	Primer Sequences	Size of the Products (bp)
16SrRNA		5'TGGCAATCAGCGTCAGGTAATG3'	522
		5'GCTAAGAGATGAGCCTATGTCC3'	
<i>VacA</i> Mid-region	m1/m2	5'CAATGTGTCCAATCAAGCGAG3'	567/642
		5'GCGTCAAAATAATCCAAGG3'	
<i>VacA</i> Signal Region	s1/s2	5'ATGGAAATACAACAAACACA3'	259/286
		5'CTGCTTGAATGCGCAAAC3'	

Double restriction enzyme digestions of *EcoRI* and *PstI* were conducted using DNA extraction from *H. pylori* strains. Using the products of restriction enzyme digestions, *H. pylori* SS1 *UreB* was amplified by PCR. The sequences of primers were: 5'GCGCGAGCTCATGAAAAAGATTAGCAGAA AAGA3' (containing *Sac* I digestion site) and 5'TGCTCTAGACCTAGAAAATGCTAAAGAGT TGC3' (containing *Xba* I digestion site). PCR amplification was subjected to 1% agarose gel for identification. The related experiments of molecular biology mentioned in this paper were

performed based upon the Guideline to Molecular Clone.

Construction of recombinant expression vector pTC01-*UreB* Double restriction enzyme digestions with *Sac* I and *Xba* I were conducted for PUC18 vector and *UreB* PCR amplification. After ligation for 16 to 18 hours at 16°C, *Bacillus Coli* DH5α was transformed with the ligation and incubated on the plates of Amp⁺, IPTG⁺ and X-gal⁺ resistance. Plasmid inserted with *UreB* gene (pUC18-*UreB*) was extracted and sequenced. Then *UreB* in pUC8-*UreB* was subcloned to expression

vector pTC01.

Expression of H. pylori UreB in Attenuated Salmonella typhimurium

Expression of pTC01-UreB in Salmonella typhimurium SL3261 and E. coli DH5 α

LB5000 *Salmonella typhimurium* (*R* M^+) was attenuated by CaCl_2 method with obtained pTC01-UreB. The attenuated *Salmonella typhimurium* was transferred once again after the restriction enzyme digestion. Recombinant attenuated *Salmonella typhimurium* SL3261/pTC01-UreB was then obtained. The protein expression of SL3261/pTC01-UreB was conducted with or without IPTG for four hours in SL3261 and *Bacillus Coli* DH5 α (OD₆₀₀ about 0.5) respectively. The expression was determined with routine SDS-PAGE, stained with rabbit serum of anti-HP multi-clone antibody and HRP-conjugated sheep anti-rabbit IgG, and then colorated with DBA.

Expression stability of pTC01-UreB in SL3261
A single clone of SL3261/pTC01-UreB was cultured for 80 generations in succession in LB solid medium with chloramphenicol resistance. Plasmids extracted from six bacterial clones were analyzed every 20 generations with restriction enzyme digestion and electrophoresis. UreB expression in the last generation was determined by Western blot.

Efficiency of Oral Immunization of Balb/c Mice With the Attenuated Vaccine of Salmonella typhimurium Expressing H. pylori UreB

Grouping and immunization Balb/c mice were divided into three groups. Five mice in group, one was fed orally with 100 μL PBS. Five mice in group, two were fed orally with 100 μL 5.0×10^6 c.f.u *Salmonella typhimurium* SL3261. Fifteen mice in group, three were immunized with 100 μL 5.0×10^6 c.f.u SL3261/pTC01-UreB expressing *H. pylori* UreB. Before oral immunization, Balb/c mice were fasted overnight and 3% NaHCO_3 100 μL was inoculated orally to neutralize the gastric acid. Mice were inoculated as above once every three weeks. At the end of the 12th week, immunological detection was conducted.

Immunological determination Intestinal fluid and serum were collected from the mice of all three groups. Anti-UreB antibodies IgA in intestinal fluid and IgG in serum were determined by ELISA. The final values were read at A_{450} wavelength. Anti-UreB antibody IgG in serum was determined once again, i.e. the quantitative protein of *H. pylori* SS1 and SL3261 was subjected to SDS-PAGE, and stained with the

mice serum of group three and sheep anti-mouse IgG. Cytokines of IFN- HI and IL-10 were determined by ELISA.

Immunological Protection for H. pylori attacked Balb/c mice Balb/c mice in the study were divided into three groups. Immunological and bacterial detection were performed. Balb/c mice were attacked by *H. pylori* SS1 strain. Inoculation size of *H. pylori* was 1.0×10^9 c.f.u. Balb/c mice were inoculated three times every two weeks. The assay was based upon the detection of gastric tissue culture and the pathological study (Giese and HE stain) on gastric mucosa.

RESULTS

Identification of H. pylori SS1 Strain

A 522 bp segment of 16SrRNA amplified by PCR from *H. pylori* SS1 genome showed that the strain was the *H. pylori* strain and suggested that the vacA gene was positive (Fig. 1).

Construction of pUC18-UreB and pTC01-UreB

A 1700 bp genetic segment was amplified from *H. pylori* SS1 genome using recombinant UreB PCR products. The size of the amplified segment was similar with the UreB genetic segment (Fig. 2). It was cloned into sequencing vector pUC18 and then subcloned into expression vector pTC01 (Fig. 3). The pTC01-UreB was transferred to *Salmonella typhimurium* SL3261 and ready for inducing expression.

Expression of pTC01-UreB in SL3261

The 61 kD protein expressed by SL3261/pTC01-UreB was determined by SDS-PAGE in accordance with *H. pylori* UreB and there was no such protein in SL3261 in the control group. The expressed protein reacted to anti-*H. pylori* rabbit serum, showing that the protein had immunogenicity (Fig. 6). We also expressed UreB in *Bacillus coli* DH5 α and found its expression efficiency in DH5 α (Fig. 7).

Expression Stability of pTC01-UreB in SL3261

Bacterial clones were observed on LB plate containing chloramphenicol resistance after culturing for 80 generations in succession. Restriction enzyme digestion and PCR determination showed similar results in the plasmids extracted randomly from the clones each time. The last generation of plasmid still expressed UreB protein after having been transferred to SL3261.

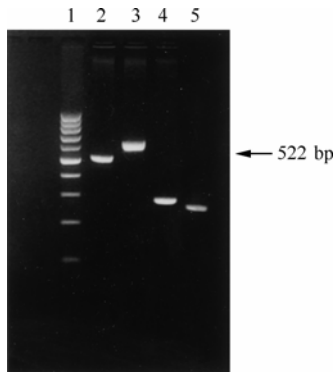


FIG. 1. *Hp* SS1 16SrRNA and VacA gene PCR amplification: 1. 100 bp DNA ladder, 2. 16SrRNA, 3. VacA m genotype, 4. VacA s Genotype, 5. *Hp*60190 strain s genotype.

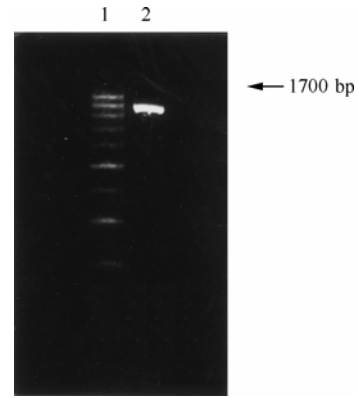


FIG. 2. *Hp* UreB gene fragment amplified by PCR: 1. 200 bp DNA ladder, 2. UreB gene fragment.

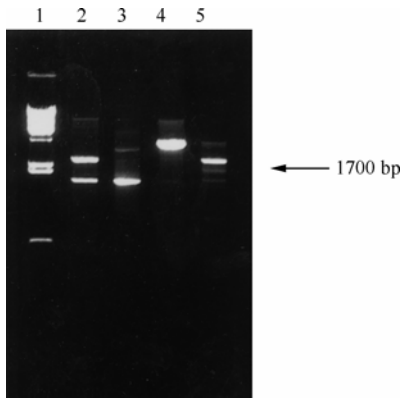


FIG. 3. Restriction enzyme digestion analysis of pUC18-UreB plasmid: 1. λ DNA/*Hind*III marker, 2. pUC18-UreB/*Sal* I+*Xba* I, 3. pUC18-UreB PCR production, 4. pUC18-UreB plasmid, 5. pUC18-UreB plasmid.

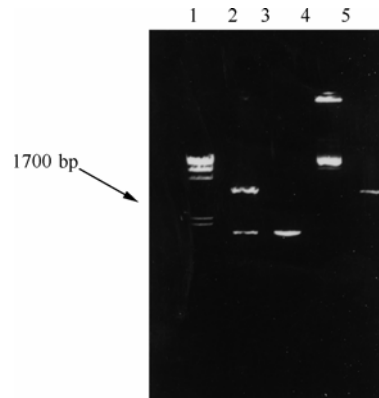


FIG. 4. Restriction enzyme digestion of recombinant pTC01-UreB plasmid: 1. λ DNA/*Hind*III marker, 2. pTC01-UreB/*Sal* I+*Xba* I, 3. pTC01-UreB PCR production, 4. pTC01-UreB plasmid, 5. pTC01 plasmid.

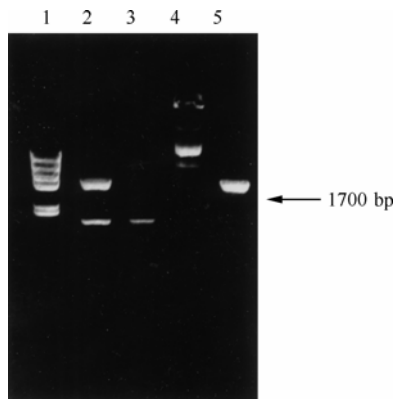


FIG. 5. Restriction enzyme digestion of pTC01-UreB plasmid in SL3261: 1. λ DNA/*Hind*III marker, 2. pTC01-UreB/*Sal* I+*Xba* I, 3. pTC01-UreB PCR production, 4. pTC01-UreB plasmid, 5. pTC01 plasmid.

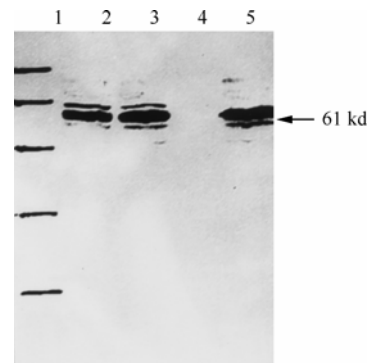


FIG. 6. Expression of UreB by SL3261 (Western blot): 1. Protein marker, 2. SL3261/pTC01-UreB ($A_{600} \approx 0.6$), 3. SL3261/pTC01-UreB ($A_{600} > 2.0$), 4. blank control, 5. SL3261 control.

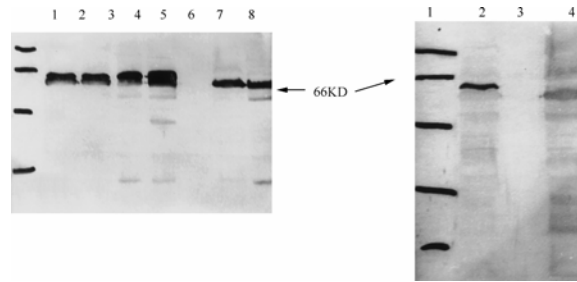


FIG. 7. Expression of UreB by SL3261 and DH5 α :1. Protein marker, 2. SL3261/pTC01-UreB, 3. SL3261/pTC01-UreB(IPTG), 4. DH5 α /pTC01-UreB, 5. DH5 α /pTC01-UreB(IPIG) 6. blank control, 7. SL3261 control. 8. DH5 α control.

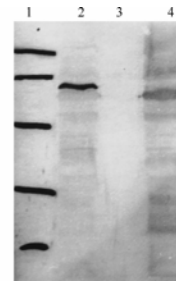


FIG. 8. Detection of anti-urease antibodies in sera from mice immunized with UreB-expressing *Salmonella*: 1. Protein marker, 2. *Hp*, 3. blank control, 4. SL3261 control.

ELISA of the Specific Antibody in Intestinal Fluid and Serum

Compared with PBS and SL3261 control groups, anti-UreB antibody levels of IgA and IgG were significantly increased in intestinal fluid and serum in mice immunized with SL3261/pTC01-UreB. The difference was statistically significant ($P < 0.01$, Table 2).

TABLE 2

ELISA Assay for Anti- *H. pylori* IgA Antibody in Mouse Intestinal Fluids and IgG in Mouse Serum (OD 450, $\bar{x} \pm s$)

	PBS Group	SL3261 Group	Experimental Group
IgA	0.056 \pm 0.022	0.108 \pm 0.025 ^a	0.522 \pm 0.159 ^a
IgG	0.053 \pm 0.022	0.142 \pm 0.039	0.373 \pm 0.100 ^b

Note. ^a $P < 0.01$ (IgA in the experimental group vs IgA in the control groups), ^b $P < 0.01$ (IgG in the experimental group vs IgG in the control group).

Determination of Anti-UreB Antibody IgG in Serum

The 61 kD UreB protein was observed in *H. pylori* somatic protein but not in the SL3261 control group. There were many nonspecific bands in *H. pylori* somatic protein samples, indicating that *H. pylori* and attenuated *Salmonella typhimurium* had many somatic proteins of cross reaction (Fig. 8).

Determination of Cytokines IFN- γ and IL-10

Cytokines IFN- γ and IL-10 in the supernatant of splenocyte culture are listed in Table 3. Cytokine IFN- γ contents were increased in both SL3261 and SL3261/pTC01-UreB groups compared with the PBS control group. But there was no significant difference between the SL3261 control and the SL3261/pTC01-UreB group ($P > 0.01$). While the level of IL-10 in the SL3261/pTC01-UreB group

was higher than that in the SL3261 and PBS control groups ($P < 0.01$).

Changes of Weight in Balb/c Mice

In early stages after the oral immunization with SL3261/pTC01-UreB, mice had hypocathexis and anorexia, but no diarrhea and death were found. There was a decline in weight compared with that before immunization, but the difference was not significant ($P > 0.05$, Table 4).

TABLE 3

Determination of Cytokines IFN- γ and IL-10 Secreted by Mice Splenocytes (pg/mL, $\bar{x} \pm s$)

	PBS Control Group	SL3261 Control Group	Experimental Group
IFN- γ	35.69 \pm 3.55	118.74 \pm 16.84 ^a	167.53 \pm 2993 ^a
IL-10	56.00 \pm 7.15	68.02 \pm 15.03 ^b	275.13 \pm 27.65 ^b

Note. ^a $P < 0.01$ (IFN- γ in the experimental group and the SL3261 control group vs the PBS control group). ^b $P < 0.01$ (IL-10 in the experimental group vs the SL3261 control group and the PBS control group).

TABLE 4

Changes of Mice Weight in the Experimental Group ($\bar{x} \pm s$)

	Immunization Before	Immunization After
Weight (g)	16.59 \pm 1.226	15.81 \pm 1.230

Pathological Detection in Mice Gastric Tissue

Giemsa stain on mice gastric tissue from all groups showed that no implantation of *H. pylori*. Gastric mucosa of mice from the PBS and SL3261 control had a little infiltration of inflammatory cells. Compared with the untreated mice, gastric tissue of mice in the SL3261/pTC01-UreB group had no

significant difference in inflammation (Figs. 9-11).

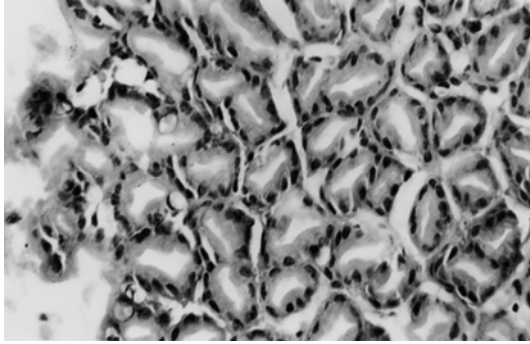


FIG. 9. Histological evaluation of gastric tissue from native mouse (HE stain, 160 \times).

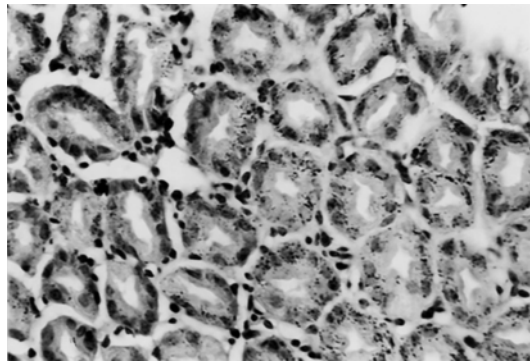


FIG. 10. Histological evaluation of gastric tissue from control mouse.

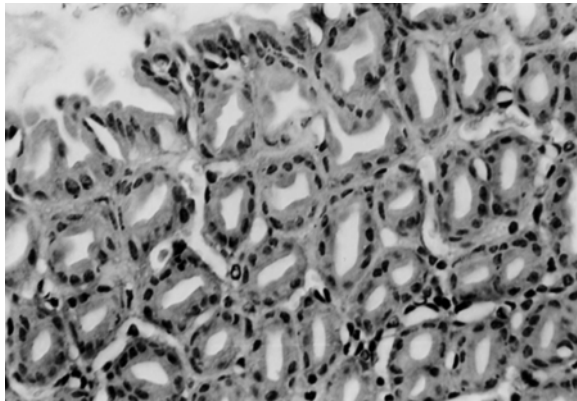


FIG. 11. Histological evaluation of gastric tissue from SL3261/pTC01-UreB immunized mouse (HE stain, 160 \times).

H. pylori Cultivation of Gastric Tissue

A few clones (40 to 60 c.f.u) on selective culture media were observed and identified as *H. pylori* by both urease test and Giemsa stain in the PBS and SL3261 control groups, which were 60% (3/5) and 40% (2/5), respectively. There was no *H. pylori* colony in gastric mucosa tissue of Balb/c mice in the experimental group.

DISCUSSION

H. pylori is the chief pathogen for chronic gastritis and peptic gastric ulcer, and is closely related to carcinoma of stomach and lymphoma of MALT^[1]. Incidence of *H. pylori* infection and carcinoma of stomach are relatively higher in China. UreB is now widely regarded as a promising candidate antigen for vaccine production. Moreover, UreB has a strong immunogenicity^[2-3]. Urease is composed of nine subunits, while UreB and UreA are two vital subunits of urease. UreB and UreA are located in cytoplasm and on the surface of membrane. Immunogenicity of UreB is much stronger than that of UreA^[5]. Experiments on mice and primate animals showed that both native and recombinant protein with adjuvant stimulated immunological protection against *H. pylori* infection^[6]. The immunization lasted for a long time and had no side effect. Attenuated vaccines of UreB protein have entered the second stage of clinical trial^[9-10].

H. pylori SS1 used in this study fit well to the pathogenic studies due to its positive CagA and VacA, strong pathogenicity and easy implantation. According to the sequence of Urease gene^[9], we developed a couple of PCR primers of UreB and a gene segment was amplified from *H. pylori* SS1 genome. Sequencing showed that the segment was identical with *H. pylori* UreB gene. The segment was cloned into expression vector pTC01, laying a foundation for the development of attenuated oral vaccines of *Salmonella typhimurium* expressing *H. pylori* UreB.

Compared with conventional vaccine types, attenuated *Salmonella typhimurium* as a vaccine vector has the following advantages. Firstly, it is unnecessary to be purified and adjuvanted to the antigen. Oral immunization is safe and easy to conduct without inducing immunological endurance. Secondly, it can avoid the degradation and degeneration of antigen in gastrointestinal tract. Thirdly, it can induce long-lasting immunologic reaction in mucosa for its strong pathogenicity to the gastrointestinal tract^[10-15]. Attenuated *Salmonella typhimurium* SL3261 is an engineered bacterium with mutational gene of aromatic amino acid-related gene (aroA gene). SL3261/pTC01-UreB, an attenuated vaccine of *Salmonella typhimurium* expressing *H. pylori* UreB, was successfully developed. Stable expression of gene of protective antigen in attenuated *Salmonella typhimurium* for a long-term period of time plays a key role in successful development of the vaccine. No pTC01-UreB plasmid was found in SL3261/pTC01-UreB cultured for 80 generations in succession on LB plates with chloramphenicol resistance. UreB protein expression was observed at

different growth stages, suggesting that pTC01-UreB expression vector is stably hosted in SL3261 without toxicity. Expressed UreB protein was proved to have immunogenicity by Western blot using anti-*H. pylori* rabbit serum, suggesting that attenuated SL3261/pTC01-UreB can be used as an oral vaccine against infection.

Successful vaccine against gastrointestinal tract pathogens mainly relies on the induction of mucosal and systematic immunizations. Experimental Balb/c mice in the study were immunized with SL3261/pTC01-UreB. Twelve weeks later, specific anti-UreB antibodies IgA and IgG were detected in mouse intestinal fluid and serum. The levels of IFN- γ and IL-10 in the supernatant of splenocyte culture increased, suggesting that immunological reactions in mucosa, body fluid and cells can be induced. The present study showed that the vaccine was effective. It is now widely accepted that the goal of developing vaccines is to adjust immunological reactions from Th1 to Th2^[19-20]. It was reported that the protection of vaccine against *H. pylori* infection is closely related to the increase of Th2 reaction and decrease of Th1 reaction^[20]. Our result showed that the secretion of IL-10 in immunized animals was elevated to a much higher level compared with the control, indicating that Th2 reaction was increased. The increased level of IFN- γ showed that there was also an increase of reaction Th1. Since IFN- γ was also raised in the SL3261 control, the increase of reaction Th1 might be related to the adoption of attenuated *Salmonella typhimurium* as a vaccine vector because attenuated *Salmonella typhimurium* could induce strong Th1 reaction^[5]. People are much concerned with the safety of vaccine. In our study, although most mice lost their weight to some extent during immunization, there was no significant difference compared with the control groups. In addition, no death or side effect as diarrhea were observed, and there was no inflammatory change in mice stomach. Therefore, it may be concluded that the vaccine is safe to immunize animals.

Whether the development of vaccine is successful should be confirmed by experimental animal models. We used Balb/c mice as the animal model infected with SS1 repeatedly and found that it was an ideal one. No *H. pylori* growth was observed on the gastric tissue slices in all three groups. Although *H. pylori* on some mice gastric tissues of the control groups was incubated, the relatively small amount could not prove that the absence of *H. pylori* in the experimental group was caused by the immunization of SL3261/pTC01-UreB. Infiltration of inflammatory cells in gastric mucosa of experimental mice was fewer than that in the two control groups, showing no significant difference. There might be

two reasons. Firstly, SS1 is human originated so that it cannot be easily reproduced in mice and its pathogenicity declines after generations of incubation. Secondly, the experimental conditions at present cannot meet the requirements of *H. pylori* incubation and the construction of animal model requires *H. pylori* with high activity. As for the absence of positive *H. pylori* in pathological examinations, it might be due to the small amount of *H. pylori* and their low sensitivity to staining. Giemsa stain is not the best and most sensitive method for *H. pylori* detection compared with silver staining. To set up better animal models as cat spirillum infected mice for the observation of SL3261/pTC01-UreB immunization is the goal of our further study.

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