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# Immunogenicity characterization of the multi-epitope vaccine CTB-UE with chitosan-CpG as combination adjuvants against *Helicobacter pylori*

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ABSTRACT

Urease is considered as an excellent vaccine candidate antigen against *Helicobacter pylori* (*H. pylori*) infection. Our previous study reported a novel multi-epitope vaccine CTB-UE which was composed of the mucosal adjuvant cholera toxin B subunit (CTB) and five cell epitopes from urease subunits. Murine experiments indicated that it could induce cellular and humoral immune responses intensively and attenuate *H. pylori* infection effectively in mice model. However, the body expression and lack of suitable adjuvant of this epitope vaccine restricted its application. In this study, new recombinant *Escherichia coli* strains was established to increase the solubility by fusing thioredoxin (Trx) and the combination adjuvants which composed of the chitosan and CpG were adopted to enhance the immunogenicity of CTB-UE for oral immunization. The experimental results indicated that the levels of IgG2a, IgG1 and IgA in the serum and the levels of sIgA in stomach, intestine and feces were significantly higher in the vaccinated group compared with the model control group. Additionally, chitosan-CpG combination adjuvants changed the ratio of IgG2a/IgG1 and conferred Th1/Th17-mediated protective immune responses. These results demonstrate that the oral vaccine with chitosan-CpG as combination adjuvants may be a promising vaccine candidate against *H. pylori* infection.



## 1. Introduction

*Helicobacter pylori* (*H. pylori*) was first discovered as a human pathogen in 1983 by Warren and Marshall and it is one of the main causes of the chronic gastritis, peptic ulcers, gastric adenocarcinoma, and gastric lymphoma worldwide [1]. Unfortunately, current therapy that are based on a combination of at least two antibiotics and a proton pump inhibitor, are not only costly but also associated with various problems such as poor patient compliance, increased antibiotic resistance, and reinfection [2,3].Consequently, vaccination should be a potential way to control the *H. pylori* infection [4].Urease which plays an important role in *H. pylori* gastral colonization is recognized as an excellent vaccine candidate antigen [5,6]In previous study, we designed a novel multi-epitope vaccine CTB-UE based on the mucosal adjuvant cholera toxin (CTB) and five cell epitopes from urease subunits. Pharmacodynamics results showed that oral immunization with this multi-epitope vaccine CTB-UE could induce a high level of urease-specific humoral, cellular and mucosal immune responses, and protect BALB/c mice from *H. pylori* infection [7].

In order to increase the solubility of the CTB-UE, some preferable strategies were applied such as fusion tags and fed-batch technology. Fusion tag thioredoxin (Trx) was selected to add into the N-terminal of the CTB-UE protein [8].In addition, the fed-batch cultivation mode was applied in the fermentation in order to control the environmental conditions and growth rate precisely [9,10].

The adjuvant was indispensable in the mucosal vaccines and some adjuvants were usually used in the protective immunity against *H. pylori* infection such as the bacterial enterotoxins cholera toxin [11,12] and aluminium-based adjuvants [13,14]. In this study, chitosan-CpG was selected to enhance the immunogenicity of this epitope vaccine. Chitosan is a cationic polysaccharide with many excellent properties such as mucosa adhesion, biodegradation and biocompatibility. Previous studies [15,16] showed that chitosan could protect antigen from the degradation by the gastric acid and

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pepsase, especially epitope antigen. Therefore, Chitosan-based vaccine delivery system could be used for mucosal adjuvant [17]. In addition, CpG oligonucleotides (ODNs) could activate APCs to express toll-like receptor 9 and it was considered to be a potent low-toxic and Th1-polarizing mucosal adjuvant [18]. Hu's study [19] reported that CpG could greatly enhance the efficacy of adjuvant when combination with chitosan adjuvant.

In this study, pET32a-Trx-CTB-UE vector containing the thrombin cleavage site was constructed and fed-batch culture technology was applied for high-yield soluble expression of CTB-UE to optimize CTB-UE expression and the chitosan-CpG was selected as combination adjuvants to increase immunogenicity of the multi-epitope vaccine CTB-UE.

## 2. Materials and methods

### 2.1. Materials and growth conditions

The empty vector pET32a was purchased from Novagen, Inc. (USA). The pET22b-CTB-UE plasmid was designed, constructed and conserved in our laboratory. The primers used for amplifying CTB-UE gene included P1-F (5′-TGG CCA TCT GGT GCC ACG CGG TTC TAC ACC TCA AAA TAT TAC TGA TTT GTG TGC -3′) and P2-R (5′-GTG GTG GTG GTG GTG GTG CTC GAG-3′). *Escherichia coli* BL21 (DE3) were grown in aerated Luria–Bertani (LB) medium or Terrific Broth (TB) medium at 37 °C with shaking. The enzyme EnZ I'm and the EnBase flo medium was purchased from BioSilta (Oulu, Finland). When required, ampicillin was added into all above culture medium at a concentration of 100 μg/mL for *E. coli*.

### 2.2. Expression of the multiple-epitope antigen CTB-UE in LB, TB and the EnBase flo medium and SDS-PAGE analysis

The *Msc I/Xho I* enzymes digested CTB-UE genes were inserted into *Msc I/Xho I* site of the expression vector pET32a, resulting in pET32a-Trx-CTB-UE. Fusion tag thioredoxin (Trx-) was added to the N-terminal of the CTB-UE. The plasmid construction were first established in *E. coli* DH5α and then introduced into *E. coli* BL21.

The LB medium, TB medium and the EnBase flo medium (BioSilta, Oulu, Finland) was adopted to enhance the expression level of CTB-UE protein. The EnBase flo medium, based on amylase-driven substrate releasing system, is a mineral salt medium supplemented with some complex nutrients, trace elements and the soluble glucose polysaccharide. Before inducing expression, the enzyme EnZ I'm (BioSilta) was added to control the speed of releasing glucose [20]. The culture was supplemented with a high concentration of complex nutrients (such as peptone and yeast extract) by adding the EnPresso Booster tablets. The whole process of cultivation could refer to the manufacturer's instructions (BioSilta, Oulu, Finland). Briefly, pre-culture *E. coli* was inoculated into the initial EnBase flo medium (1:200) including two tablets and 0.3U/L EnZ I'm enzyme. After overnight culture with shaking at 30 °C , two EnPresso Booster tablets (BioSilta), 0.6U/L EnZ I'm enzyme and the inducer 1 mM/L IPTG were added to induce expression in the final EnBase flo medium [21].

For the visualization and quantification of the total, soluble and insoluble proteins, the *E. coli* cell samples were harvested by centrifugation. The cell pellets were lysed by the addition of a 0.2-fold volume of lysis solution at 37 °C for 30 min. Then the bacterial lysates were disrupted by mild sonication and the protein samples were separated into soluble (S) and insoluble (In) parts by centrifugation and then SDS-PAGE analysis was performed to detect the solubility.

### 2.3. Multi-epitope vaccine CTB-UE preparation

The CTB-UE protein was expressed initially in the form of fusion protein Trx-CTB-UE and then digest to remove thioredoxin (Trx). The purification processes were carried out according to the protocol performed as previous described with some modifications. The supernatant obtained from above was heated for 5 min in the 70 °C water bath to remove the majority of thermolabile proteins and proceeded to hyperfiltrate by hollow-fiber ultrafiltration membrane with molecular weight cut-off (MWCO) of 30 kDa at 5000 rpm for 30 min at 4 °C. Thrombin reaction mother buffer was added to the ultrafiltrate (1:10) and 120U thrombin (Sigma, USA) was added into the solution to digest the fusion protein at room temperature. Then the enzymolysis proteins were analyzed by SDS-PAGE. The recombinant protein CTB-UE was purified by $Ni^{2+}$-charged column chromatography (Bio Basic Inc, Markham, canada) according to the recommendation of the manufacturer. The purity of CTB-UE was hyperfiltrated as above and then all the samples were dialyzed in PBS buffer, then concentrated and stored at −80 °C finally.

### 2.4. Adjuvants, immunization and sample collection

Ultrapure chitosan was purchased from Golden-shell biochemical Inc. (Yuhuan China). CpG ODN1826 (5′-TCCAT-GACGTTCCTGACGTT-3′) was synthesized by Shanghai Sangon Company. Aluminium adjuvant was obtained from Thermo Scientific. Five to six-week-old specific pathogen-free (SPF) male BALB/c mice were purchased from Comparative Medicine Center of Yangzhou University. All animal experiments were approved by the Animal Ethical and Experimental Committee of China Pharmaceutical University. The mice were randomly divided into six groups (six animals per group) and were orally immunized with PBS, CTB-UE, CTB-UE plus chitosan, CTB-UE plus CpG, CTB-UE plus combination adjuvants (including chitosan and CpG) or CTB-UE plus aluminium adjuvant, respectively.The vaccine formulation was listed in Table 1, and groups of mice each were administered in 500 μl final volume. Two weeks after the final immunization, mice were sacrificed. Blood, stomach, intestine and feces samples were collected for antibody assay and spleens removed for stimulation *in vitro*.

**Table 1**
Animal groups and antigens dosage.

| Groups | Antigens preparation | CTB-UE (μg) | Chitosan (mg) | CpG (μg) | Aluminium adjuvant (μl) |
|---|---|---|---|---|---|
| 1 | PBS | – | – | – | – |
| 2 | CTB-UE | 150 | – | – | – |
| 3 | CTB-UE + CpG | 150 | – | 50 | – |
| 4 | CTB-UE + chitosan | 150 | 50 | – | – |
| 5 | CTB-UE + chitosan + CpG | 150 | 50 | 50 | – |
| 6 | CTB-UE + aluminium | 150 | – | – | 250 |

All the mice were randomized into six groups (six mice in each group) and were inoculated orally with PBS, CTB-UE, CTB-UE plus chitosan, CTB-UE plus CpG, CTB-UE plus combination adjuvants (including chitosan and CpG), and CTB-UE plus aluminium adjuvant (alum). The antigen and adjuvant dosage were shown above.

### 2.5. Assessment of antigen-specific antibody responses

Urease specific serum antibodies IgG, IgG1 and IgG2a were measured by ELISA as previously described [22].To assess urease-specific mucosal secretory IgA (sIgA) production, the stomach, intestine, or feces samples were homogenized in 1 ml PBS containing 2 mM phenylmethylsulfonyl fluoride, 0.05 M ethylenediaminetetraacetic acid, and 0.1 mg/ml of soybean trypsin inhibitor. Suspensions were centrifuged and the supernatants was collected and diluted 1:5 in PBS for the analysis of mucosal sIgA antibodies. Briefly, ELISA plates were coated with 0.5 μg per well of natural *H. pylori* urease (Linc-Bio, Shanghai, China) at 4 °C overnight. The plates were washed with PBST and blocked with 5% (w/v) BSA in PBS. The plates were then washed with PBST and incubated with 100 μl of mouse sera or the supernatant samples, serially diluted in PBS at 37 °C for 1 h. After washing, HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a, and IgA (General Bioscience Corporation, USA) was added and the plates incubated again for 1 h. The color reaction based on TMB was terminated after incubation for 10 min at room temperature by the addition of 50 μl $H_2SO_4$ (2 M), and the absorbance at 450 nm was measured by a microplate reader. Serum samples from mice were assayed in triplicate.

### 2.6. Splenocytes proliferation assay

The spleens were obtained from immunized BALB/c mice according to the methods described by Li and Hu [19]. Cell concentrations were adjusted to $5 \times 10^6$ cells/mL in RPMI1640 supplemented with 10% FCS. 100 ul of the cell suspension was added to 96-well culture plate and incubated with 150 ug/ml CTB-UE or natural urease (Linc-Bio Inc, Shanghai, China) for 48 h at 37 °C in 5% $CO_2$ humid incubator. The cell proliferation was measured by CCK-8 assay and expressed as stimulation index.

### 2.7. Cytokine production determination

Lymphocytes were isolated from spleens with lymphocyte separation medium (Dakewe, shenzhen, China) and cultured ($2 \times 10^5$ cells/well) with CTB-UE (0.5 ug/ml) in RPMI-1640 in 96-well flat-bottom plates at 37 °C in 5% $CO_2$ for 72 h. Splenic lymphocyte culture supernatants were harvested to assay for interleukin (IL)-4, interferon-gamma (IFN-γ), and IL-17 using ELISA kits (R&D System, USA) according to the manufacturer's instructions.

## 3. Statistical analyses

All the statistical analyses were performed with the GraphPad Prism 5 software. Data were expressed as mean ± standard deviation (S.D). Statistical significance was tested using Student's paired *t* test. $p < 0.05$ was considered as statistically significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns: not significant).

## 4. Results

### 4.1. Expressing the fusion protein Trx-CTB-UE in three different culture medium

Three different culture medium (LB, TB and EnBase flo medium) was selected to cultivate the new recombinant strains *E. coli* under above conditions. The protein samples (from LB, TB and EnBase flo medium groups) were analyzed by SDS-PAGE gel and the results showed that the expression level of the protein Trx-CTB-UE from EnBase flo medium group were the highest (~37% of total bacterial protein as shown in Fig. 1A), increasing 14.7% than before. Additionally, the solubility rate of the CTB-UE protein from EnBase flo medium group could reach ~90% (as shown in Fig. 1A). However, the proteins Trx-CTB-UE from the LB and TB medium groups were mainly expressed in insoluble form (Fig. 1B–C).

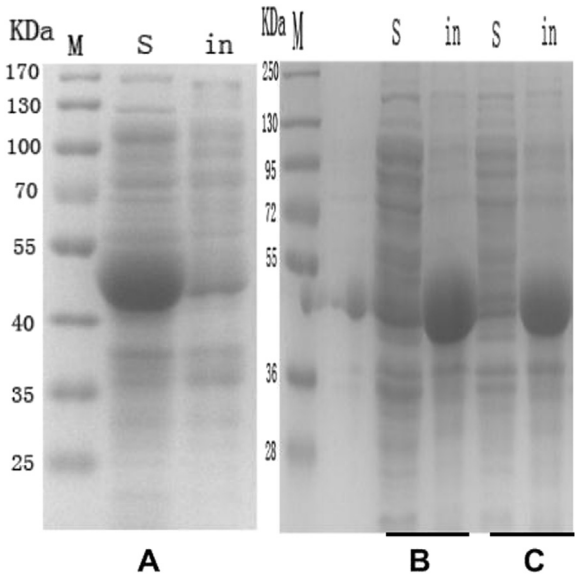


**Fig. 1.** SDS-PAGE analysis of total, soluble and insoluble cellular protein fractions after cultivation in EnBase flo medium, LB medium, and TB medium. M: protein molecular weight maker; A: the soluble (S) and insoluble (In) proteins of *E. coli* BL21 (DE3) expressing CTB-UE (42 KDa) after cultivation in EnBase flo medium; B: the soluble (S) and insoluble (In) proteins of *E. coli* BL21 (DE3) expressing CTB-UE (42 KDa) after cultivation in LB medium ;C: the soluble (S) and insoluble (In) proteins of *E. coli* BL21 (DE3) expressing CTB-UE (42 KDa) after cultivation in TB medium.

### 4.2. Purification of protein CTB-UE

The SDS-PAGE gel analysis showed that the fusion protein tag Trx-had been removed (precursor protein Trx-CTB-UE, ~44 kDa) and a 32 kDa protein band of mature protein CTB-UE could be detected (Fig. 2A). The cleavage efficiency was about 71% analyzed by the Quantity One software (Bio-Rad, US). The CTB-UE was purified by Ni2+-charged column chromatography (Bio Basic Inc, Markham, Canada) and anion-exchange chromatography using DEAE Sepharose FF (AmershamPharmacia Biotech AB, Sweden) and the purity of CTB-UE was about 98% (Fig. 2B).

### 4.3. The production of specific antibodies in serum

The animal immunization schedule and detection program were shown in Fig. 3. Groups of mice were immunized orally with PBS, CTB-UE, CTB-UE plus chitosan, CTB-UE plus CpG, CTB-UE plus combination adjuvants (including chitosan and CpG) or CTB-UE plus aluminium adjuvant [23].Two weeks after the final oral immunization, total IgG, IgG1, IgG2a and IgA antibodies were examined by ELISA assay. Compared with the PBS group, the levels of specific IgG, IgG1, IgG2a and IgA antibodies significantly increased in all immunized groups except for the CTB-UE group (without adjuvants) (Fig. 3A–B). Moreover, the combination adjuvants (chitosan-CpG) group could elicit higher level urease-specific IgG and IgG2a antibodies than the alum adjuvant group, but no statistically significant differences of IgA antibodies could be observed. Interestingly, combined of the chitosan and CpG adjuvants also changed the ratio of IgG2a/IgG1 (Fig. 3C).

To assess the effect of combination adjuvants chitosan-CpG on the mucosal immune responses, urease-specific secretory IgA (sIgA) antibodies from the gastric tissue, intestine mucus and feces

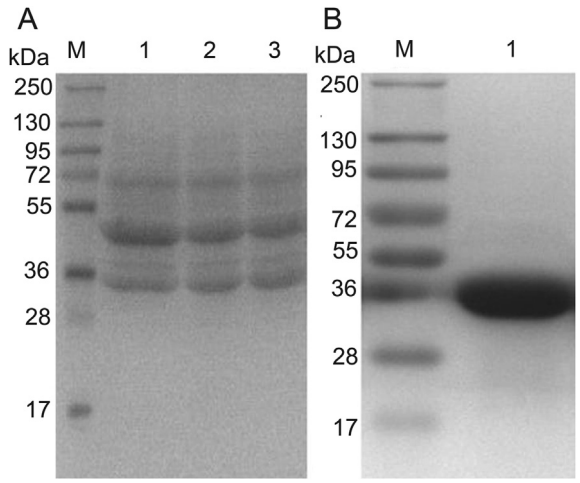


**Fig. 2.** A: SDS-PAGE analysis of the cleavage products of fusion protein Trx-CTB-UE digested by thrombin. M, protein molecular weight maker. Lanes 1–3, Trx-CTB-UE digestion products by thrombin. B: The products of CTB-UE purified by $Ni^{2+}$-charged column chromatography. M, protein molecular weight maker; lane 1, the purified CTB-UE products.

was detected (Fig. 3D). Oral immunization with the CTB-UE plus chitosan-CpG, as well as CTB-UE plus alum adjuvant could significantly elicit high level of specific sIgA antibodies compared with the control group.

### 4.4. Determination of the production of Th1 and Th17 cytokines

Chitosan had been demonstrated to be a mucosal delivery vehicles for various antigens to promote moderate dendritic cell maturation and was also effective in augmenting TLR agonist induced pro-inflammatory cytokines, including some Th1 and Th17 responses cytokines [31]. The production of Th1 and Th17 cytokines were determined to investigate whether the combined use of chitosan and CpG further enhanced the efficacy of adjuvants. The results showed that the chitosan-CpG adjuvants group induced more extensive production of IFN-γ and IL-17 than other groups (Fig. 4A–B). Moreover, the combination adjuvants chitosan-CpG group showed a significantly higher level of the IFN-γ and IL-17 responses cytokines than alum adjuvant. However, the induced IL-4 level in the chitosan-CpG group markedly reduced (Fig. 4C). Therefore, the dominant level of IFN-γ and IL-17 revealed that combined use of chitosan and CpG induced mixed Th1/Th17 T-cell responses.

### 4.5. Splenocytes proliferation assay

In order to evaluate the adjuvanticity of the chitosan, CpG and the combination adjuvants, the splenocytes from the mice of the above groups were stimulated with natural urease (2 μg/culture) or CTB-UE (2 μg/culture) *in vitro*. Results indicated that the splenocytes from the mice immunized orally with CTB-UE alone showed no significant responses compared with the PBS group ($P > 0.05$). However, the splenocytes amounts from the mice immunized with CTB-UE plus chitosan or CpG increased obviously than PBS group ($P < 0.05$). More importantly, the splenocytes from mice immunized with CTB-UE plus chitosan-CpG adjuvants showed strongly proliferative responses to CTB-UE or urease compared with the control groups ($P < 0.001$) (Fig. 4D).

## 5. Discussion

*H. pylori* is a Gram-negative bacterium that colonizes gastric epithelium and is responsible for chronic gastritis, peptic ulceration and even gastric cancer. Vaccination is an effective strategy against *H. pylori* injection. Previous reviews [24,25] have summarized various candidate vaccines that showed prophylactic or therapeutic effect against *H. pylori* infection, such as whole bacteria vaccine, recombinant subunit vaccine and DNA vaccine. Recent studies reported that epitope vaccines was considered an attractive strategy against *H. pylori* infection due to its more security, specificity and

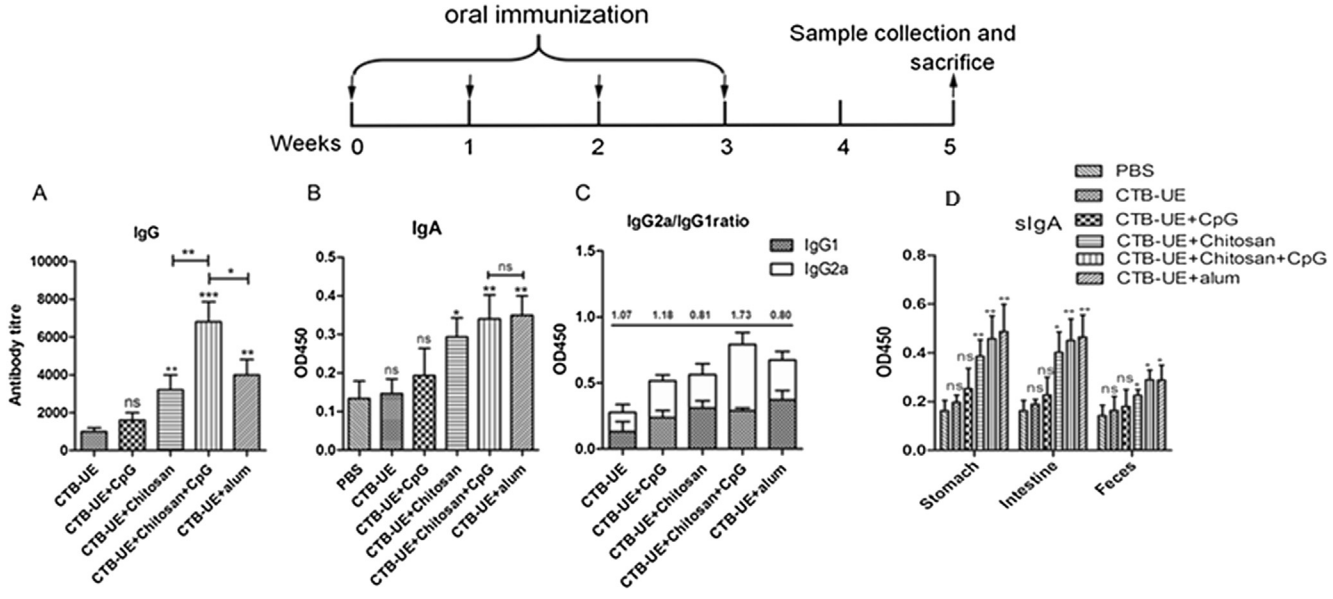


**Fig. 3.** Assessment of antigen-specific antibody responses. Groups mice were orally immunized with PBS, CTB-UE, CTB-UE plus chitosan, CTB-UE plus CpG, CTB-UE plus combination adjuvants (including chitosan and CpG) and CTB-UE plus aluminium adjuvant (alum). Two weeks after the final oral immunization, total IgG (A), IgA (B), IgG2a and IgG1 (C) antibodies were examined by ELISA. Data are the mean ± SD. A value of $p < 0.05$ was considered statistically significant: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns: not significant. Measurement of mucosal sIgA(D) antibodies against *H. pylori* urease after oral immunization. Data are the mean ± SD. A value of $p < 0.05$ was considered statistically significant: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns: not significant. Supernatants of homogenized stomach, intestine, and feces were collected for detecting the levels of mucosal sIgA against *H. pylori* urease.

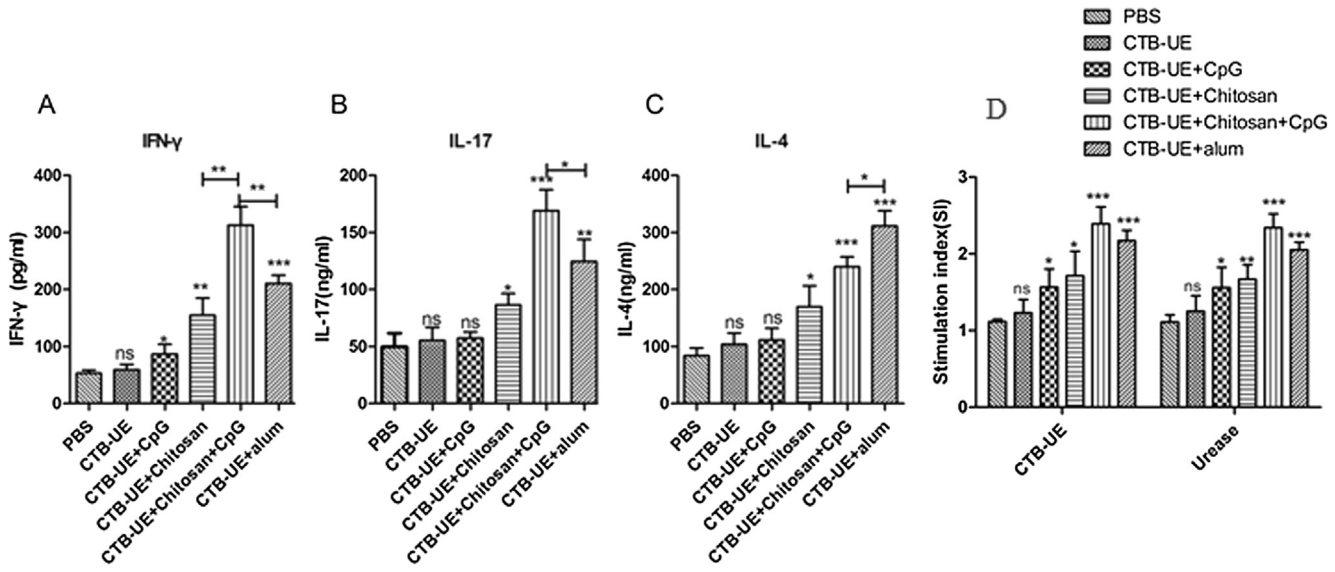


**Fig. 4.** Determination of the production of Th1 and Th17 cytokines. The splenic lymphocytes were stimulated with CTB-UE for 72 h, and the supernatants were collected and cytokine production IFN-γ (A), IL-17 (B) and IL-4 (C) were determined by ELISA. Data are the mean ± SD. A value of $p < 0.05$ was considered statistically significant: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns: not significant. *H. pylori* urease-specific splenocyte proliferation measured by the stimulation index (D). Splenocyte lymphocytes were stimulated with 20 μg/mL CTB-UE or 20 μg/mL urease and proliferation was measured by CCK-8 incorporation. Data are shown as means ± S.D. A value of $p < 0.05$ was considered statistically significant: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns: not significant.

potent immunogenicity by removing other unfavorable epitopes in the complete antigen.

In our previous study, the multi-epitope vaccine CTB-UE was designed and mainly expressed in insoluble form [7]. After Trx-tag was fused into the N-terminal of the CTB-UE, the target protein's solubility was increased enormously (~65%) in EnBase flo medium. Additionally, the expression of the CTB-UE antigen increased almost 14% in the EnBase group. There were two reasons for the above results. Firstly, the Trx-tag could help the heterologous protein folding correctly. In addition, the controlled-release system reduced incorrect fold of the target proteins when express quickly, resulting in reducing the toxicity of foreign proteins.

Our previous data showed mice immunized with the multi-epitope vaccine CTB-UE induced high levels of urease-specific serum antibodies, systemic and mucosal IgG and IgA, but the vaccine's immunogenicity is relatively low because of the small molecular weight [26]. Combined inoculation of the antigen and chitosan has been confirmed to be an effective way to enhance the efficacy of vaccine. Splenocytes proliferation is usually due to B cells proliferation in spleen and the protective antibody titer is associated with the number of B cells [27].Oral administration with multi-epitope antigen CTB-UE with chitosan-CpG as combination adjuvants could induce strong serum IgG antibodies, mucosal sIgA antibodies responses and the prominent splenocytes proliferation. In addition, chitosan-CpG adjuvants group obviously increase the ratio of IgG2a/IgG1, which was directly related to Th1-polarizing immune response. The Th1 and Th17 cell-mediated immune responses is considered to mediate the protect immunity against *H. pylori* infection [28–30]. The capacity of the chitosan-CpG adjuvants to induce significantly high levels of antigen-specific IFN-γ and IL-17 cytokines but not IL-4 is a special finding about the adjuvant properties of the chitosan-CpG [31]. The secretion of Th1 and Th17-cell polarizing cytokines demonstrated that chitosan-CpG adjuvant was a potent stimulus for inducing Th1/Th17 responses.

*H. pylori* infected the host via the mucosal route and mucosal surfaces were the first line to defense *H. pylori* infection. Therefore, the mucosal route was considered favorable to develop the mucosal vaccination. Mucosal vaccination could trigger immune responses not only mucosally but also systemically. The induction of antigen-specific secretory immunoglobulin A (sIgA) antibodies production played an important role in inhibition of *H. pylori* adhesion at mucosal epithelial cells. Our results showed mucosal immunization with the multi-epitope antigen CTB-UE plus chitosan-CpG also surpassed parenteral immunization with CTB-UE plus other adjuvants in the level of antigen specific mucosal sIgA antibodies in stomachs, small intestines, and feces. Briefly, the combination adjuvants chitosan-CpG can induce effective mucosal immune response and improve the antigen's adhesion to the gastrointestinal mucous membrane to elicit mucosal immunity preferably.

In summary, we optimized the expression of multi-epitope vaccine CTB-UE in the solubility and the expression level of the target protein. Additionally, we demonstrated the combination adjuvant chitosan-CpG have the potential capacity to enhance potent humoral, cellular and mucosal immune responses. However, we have to say that the flaw of the study was not adopting *H. Pylori* challenge test and lacking the corresponding data about the immune preventive effect of this vaccine.

## Conflict of interest

The authors declare that they have no conflict of interests.

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## Transparency document

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