

• *H pylori* •

Construction of a recombinant attenuated *Salmonella typhimurium* DNA vaccine carrying *Helicobacter pylori* hpaA

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Abstract

AIM: To construct a recombinant attenuated *Salmonella typhimurium* DNA vaccine carrying *Helicobacter pylori* hpaA gene and to detect its immunogenicity.

METHODS: Genomic DNA of the standard *H pylori* strain 17 874 was isolated as the template, hpaA gene fragment was amplified by polymerase chain reaction (PCR) and cloned into pUCmT vector. DNA sequence of the amplified hpaA gene was assayed, then cloned into the eukaryotic expression vector pIRES through enzyme digestion and ligation reactions. The recombinant plasmid was used to transform competent *Escherichia coli* DH5 α , and the positive clones were screened by PCR and restriction enzyme digestion. Then, the recombinant pIRES-hpaA was used to transform LB5000 and the recombinant plasmid isolated from LB5000 was finally used to transform SL7207. After that, the recombinant strain was grown *in vitro* repeatedly. In order to identify the immunogenicity of the vaccine *in vitro*, the recombinant pIRES-hpaA was transfected to COS-7 cells using Lipofectamine™2000, the immunogenicity of expressed HpaA protein was detected with SDS-PAGE and Western blot.

RESULTS: The 750-base pair hpaA gene fragment was amplified from the genomic DNA and was consistent with the sequence of *H pylori* hpaA by sequence analysis. It was confirmed by PCR and restriction enzyme digestion that *H pylori* hpaA gene was inserted into the eukaryotic expression vector pIRES and a stable recombinant live attenuated *Salmonella typhimurium* DNA vaccine carrying *H pylori* hpaA gene was successfully constructed and the specific strip of HpaA expressed by pIRES-hpaA was detected through Western blot.

CONCLUSION: The recombinant attenuated *Salmonella typhimurium* DNA vaccine strain expressing HpaA protein with immunogenicity can be constructed and it may be helpful for further investigating the immune action of DNA vaccine *in vivo*.

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Key words: *Helicobacter pylori*; hpaA Gene; DNA vaccine

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INTRODUCTION

Helicobacter pylori is a Gram-negative microaerophilic bacterium which clones human gastric epithelium. Infection of *H pylori* is strongly associated with chronic gastritis, peptic ulcer or gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma^[1-4]. More than 50% of the population worldwide is infected with *H pylori*. The current standard treatment for it consists of antibiotics in combination with proton pump inhibitors^[5-7]. Because of the emergence of antibiotic-resistant strains, vaccination of humans against *H pylori* infection is an effective and economical approach to the control of this pathogen.

Recently, DNA vaccine has been demonstrated to induce both humoral and cellular immunity and it is becoming a promising treatment for viral, bacterial and parasitic pathogens. Protective immunity against HIV, influenza virus, rabies virus, malaria and tuberculosis has been shown in animal models^[8-12].

In this study, we constructed a recombinant live attenuated *Salmonella typhimurium* DNA vaccine carrying *H pylori* hpaA gene, and identified its immunogenicity in COS-7 cells *in vitro*.

MATERIALS AND METHODS

Strains and plasmid

Attenuated *S typhimura* LB5000 and SL7207 were kindly provided by Professor Bruce Stocker of Stanford University, USA. They were cultured in Amp (-) LB medium. COS-7 cell line was provided by the Department of Immunology, Secondary Military Medical University of China. *E. coli* DH5 α used for cloning experiments was grown in LB medium containing 50 mg ampicillin per liter. Standard *H pylori* strain CCUG17874 (NCTC11638) was kindly provided by the Italian IRIS Research Center and cultured on *H pylori*-selective agar plates with 10% defibrillated sheep blood and antibiotics (Merck Company, Germany) at 37 °C under microaerophilic conditions with 50 ml/L O₂, 10 ml/L CO₂ and 85% N₂. Vector pIRES was purchased from Clontech, USA.

Amplification of hpaA gene fragment

H pylori strains were collected from the agar plates in PBS, then genomic DNA was extracted as previously described using CTAB. According to the complete DNA sequence of *H pylori* published and multiple clone sites of pIRES, the primers to amplify hpaA containing *EcoRI* site in P1 and *MluI* site in P2 were designed: P1: 5'GAATTCCACCATGAAAAAAGGTAGTTTGGC3', P2: 5'ACGCGTCTACTTTTCGTTTTTTTCATTTAC3'. Amplification was done in a total volume of 50 μ L under conditions: at 94 °C

for 5 min, then 30 cycles at 94 °C for 45 s, at 55 °C for 45 s and at 72 °C for 1 min, followed by 5 min at 72 °C. The PCR products were analyzed on 1.2% agarose gels stained with ethidium bromide.

Sequence analysis of hpaA

PCR products were separated using a QIAquick gel extraction kit (QIAGEN, CA, USA). Purified hpaA DNA fragments were subcloned into TA cloning vector pUCmT (Takara, Dalian, China), and then the sequence of hpaA was analyzed using an automatic sequencer.

Construction of recombinant pIRES-hpaA

Fragments of *Eco*RI and *Mlu* I-digested pUCmT-hpaA were inserted into the *Eco*RI/*Mlu* I site of eukaryotic expression vector pIRES, through a series of enzyme digestion and ligation reactions. Then the recombinant pIRES-hpaA was confirmed by PCR and restriction enzyme digestion.

Construction of recombinant attenuated salmonella typhimurium carrying H pylori hpaA gene

Recombinant pIRES-hpaA was used to transform attenuated *Salmonella typhimurium* LB5000 with calcium chloride, then the recombinant plasmid was extracted to transform the final host strain SL7207 using electroporation. The attenuated *Salmonella typhimurium* SL7207 carrying hpaA gene was cultured in LB medium to 80 generations. The recombinant plasmid in transformed SL7207 were isolated from every 10 generations and identified by restriction enzymes and PCR.

In vitro transfection

To detect the protein expressed by recombinant pIRES-hpaA, pIRES-hpaA was transfected into COS-7 cells. COS-7 cell line was cultured at 37 °C, 5 mL/L CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Gibco-BRL, UK), 100 U/mL penicillin and 100 µg/mL streptomycin, 15 mmol/L HEPES, and 2 mmol/L L-glutamine. Twenty four hours before transfection, 5 × 10⁵ COS7 cells were seeded into six-well plates, and the mixture of pIRES-hpaA and Lipofectamine™2000 (Invitrogen, USA) were added to the cells. Forty-eight hours after transfection, cells were washed with PBS, and protein extraction reagent (Pierce, USA) was added. After shaking for 5 min, the lysate was collected and centrifuged at 12 000 g for 5 min at 4 °C. Supernatant containing the proteins was maintained at -80 °C until later use.

Expression of hpaA protein detected by western blot

Supernatant containing the proteins was determined by electrophoretic analysis in a 12% polyacrylamide gel, subsequently electrotransferred onto nitrocellulose membranes (Bio-Rad, Germany), nonspecific binding sites were blocked with 2% bovine serum albumin (BSA), then rabbit anti-*H pylori* and peroxidase-labeled anti-rabbit immunoglobulin G (IgG) were added (DAKO, Denmark). The antigens were visualized by chemiluminescence (Bio-Rad, Germany) according to the manufacturer's instructions.

RESULTS

Sequence analysis of hpaA nucleotide

PCR products of hpaA were cloned into TA cloning vector pUCmT. The sequence of amplification fragment was consistent with that of *H pylori* hpaA published in the gene bank.

Construction of recombinant pIRES-hpaA, PCR and restriction enzyme confirmation

After pUCmT-hpaA and pIRES were digested by both *Eco*RI and *Mlu* I, a 750-bp fragment of hpaA was directly cloned into *Eco*RI/

Mlu I site of pIRES, resulting in a recombinant plasmid pIRES-hpaA. pIRES-hpaA was digested by both *Eco*RI and *Mlu* I. P1 and P2 were used as primers to amplify hpaA from pIRES-hpaA, and the products analyzed on agarose gel (Figure 1) showed that the recombinant plasmid contained the objective gene hpaA.

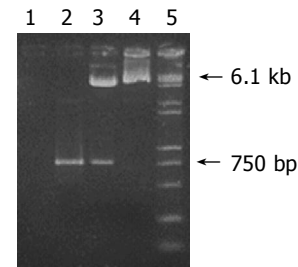


Figure 1 Agarose gel electrophoresis analysis of recombinant pIRES-hpaA. Lane 1: PCR product of pIRES as a negative control; lane 2: PCR product of pIRES-hpaA; lane 3: pIRES-hpaA after digestion with *Eco*RI and *Mlu* I; lane 4: pIRES after digestion with *Eco*RI and *Mlu* I; lane 5: DNA Marker (DL2 000+15 000).

Recombinant attenuated Salmonella typhimurium DNA vaccine and its stability

After transformed by pIRES-hpaA, the recombinant plasmid extracted from LB5000 was used to transform SL7207. Plasmid stability was essential to assure the stable expression of antigens encoded by genes which were cloned into the plasmid. Therefore, SL7207 carrying plasmid pIRES-hpaA was grown *in vitro* up to 80 generations to examine the plasmid stability. The 750-bp objective fragment could be seen on the map of agarose gel of the PCR products and the products of *Eco*RI and *Mlu* I-digested recombinant plasmid were isolated from transformed SL7207 (Figures 2, 3).

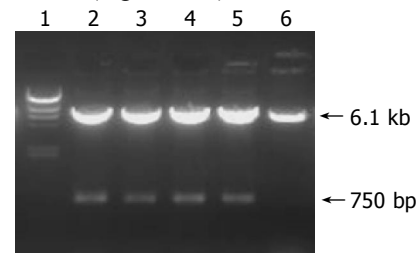


Figure 2 Agarose gel electrophoresis analysis of recombinant attenuated *Salmonella typhimurium* DNA vaccine strain with restriction enzyme digestion. Lane 1: DNA ladder (1 kb); lanes 2-5: Recombinant plasmid pIRES-hpaA from strains of different generations after digestion with *Eco*RI and *Mlu* I; lane 6: pIRES after digestion with *Eco*RI and *Mlu* I.

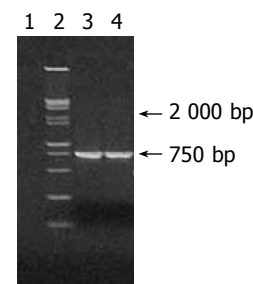


Figure 3 Identification of recombinant attenuated *Salmonella typhimurium* DNA vaccine strain carrying hpaA by PCR. Lane 1: Product amplified from pIRES as a negative control; lane 2: Marker (DL2 000+15 000); lanes 3-4: Products amplified from recombinant

plasmid from strains of different generations by PCR.

Immunoreactivity of expressed recombinant protein

Identification of pIRES-hpaA *in vitro* expression was carried out. The lysate of COS-7 cells transfected by pIRES-hpaA was analyzed by Western blotting. It revealed the immunoreactive band of 30-kD corresponded to HpaA protein, but the control transfected with pIRES had no specific band (Figure 4).

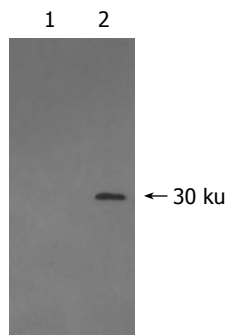


Figure 4 Western blotting of expressed pIRES-hpaA products Lane 1: COS-7 cells transfected by pIRES as a control; Lane 2: COS-7 cells transfected by pIRES-hpaA.

DISCUSSION

DNA vaccine is a novel vaccine. It has been widely used in laboratory animals and non-human primates to induce humoral and cellular immune responses. Clinical trials have shown that DNA vaccine is safe and well tolerated. Moreover, some reports have indicated that it could produce long-lasting immunity. The vaccine is a recombinant plasmid with heat stability. It can be used not only for protection but also for treatment in the presence of targeted infectious pathogens^[13-15].

However, at present researches of *H pylori* vaccine mostly focus on protein vaccine, including *H pylori* whole-cell sonicate or one of the recombinant proteins of *H pylori* as the antigen of the vaccine in combination with mucosal adjuvants such as cholera toxin or heat-labile toxin of enterotoxigenic *E. coli*^[16-18]. The manufacture of such vaccines is complicated, and some mucosal adjuvants have gastrointestinal toxicity. It was reported that mucosal immunization with *Helicobacter heilmannii* urease B or *H pylori* urease, given nasally with cholera toxin, could protect BALB/c mice against *Helicobacter heilmannii* infection and significantly reduce the pre-existing infection. However, immunization could aggravate gastric corpus atrophy^[19].

H pylori adhesin A (HpaA) belonging to a group of outer membrane proteins of *H pylori* has been described as an adherence factor for blood cells and plays an important role in adhesion of microbes^[20,21]. HpaA could mediate binding to sialic acid, a putative neuraminylactose-binding hemagglutinin. HpaA is a highly conserved protein among *H pylori* clinical isolates and immunogenic in humans^[22,23]. Therefore, HpaA is an ideal antigen candidate for *H pylori* vaccine.

It has been shown that live attenuated bacteria carrier including attenuated strains of *Salmonella* and *Shigella* *in vitro* could deliver DNA vaccines to human cells. Bacterial DNA vaccine delivery has also been demonstrated *in vivo* in several experimental animal models of infectious diseases and tumors. They allow vaccination via mucosal surfaces and specific targeting to professional antigen-presenting cells in mucosa-associated lymphoid tissue^[24-26].

In this study, we constructed a live recombinant attenuated *Salmonella typhimurium* DNA vaccine strain expressing HpaA protein. First, the complete hpaA gene fragment was amplified

from genomic DNA of *H pylori*; then sequence analysis was performed after it was cloned into the TA cloning vector pUCmT. Subsequently, purified hpaA was cloned to eukaryotic expression vector pIRES. Both the enzyme digestion and PCR confirmed the successful construction of recombinant plasmid pIRES-hpaA. Recombinant attenuated *Salmonella typhimurium* carrying *H pylori* hpaA gene was successfully constructed after pIRES-hpaA was first used to transform LB5000 and SL7207. The stability of the protective antigen is very important for a vaccine; we assessed the stability of the recombinant plasmid *in vitro*. It is confirmed by PCR and restriction enzyme that pIRES-hpaA is present in all the transformed strains of SL7207 up to the 80th generation, which reveals the stability of the recombinant plasmid in the bacterium.

It is also demonstrated *in vitro* in our present study that the COS-7 cells transfected by pIRES-hpaA could express the specific protein of 30 kD, but the COS-7 cells transfected by pIRES could not express the protein. The pIRES-hpaA DNA vaccine could express the specific HpaA protein which can react with anti-*H pylori*.

Recombinant attenuated *Salmonella typhimurium* DNA vaccine carrying *H pylori* hpaA gene can express HpaA protein with immunogenicity. Further study is needed to explore its protective and therapeutic effect on animal models *in vivo*.

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