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Surface display of *Clonorchis sinensis* enolase on *Bacillus subtilis* spores potentializes an oral vaccine candidate

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ABSTRACT

Clonorchis sinensis (*C. sinensis*) infections remain the common public health problem in freshwater fish consumption areas. New effective prevention strategies are still the urgent challenges to control this kind of foodborne infectious disease. The biochemical importance and biological relevance render *C. sinensis* enolase (*Cs*enolase) as a potential vaccine candidate. In the present study, we constructed *Escherichia coli*/*Bacillus subtilis* shuttle genetic engineering system and investigated the potential of *Cs*enolase as an oral vaccine candidate for *C. sinensis* prevention in different immunization routes. Our results showed that, compared with control groups, both recombinant *Cs*enolase protein and nucleic acid could induce a mixed IgG1/IgG2a immune response when administrated subcutaneously ($P < 0.001$), intraperitoneally ($P < 0.01$) and intramuscularly ($P < 0.001$) with worm reduction rate of 56.29%, 15.38% and 37.42%, respectively. More importantly, *Cs*enolase could be successfully expressed as a fusion protein (55 kDa) on *B. subtilis* spore indicated by immunoblot and immunofluorescence assays. Killed spores triggered reactive Th1/Th2 immune response and exhibited protective efficacy against *C. sinensis* infection. *Cs*enolase derived oral vaccine conferred worm reduction rate and egg reduction rate at 60.07% ($P < 0.001$) and 80.67% ($P < 0.001$), respectively. The shuttle genetic engineering system facilitated the development of oral vaccine with *B. subtilis* stably overexpressing target protein. Comparably vaccinal trails with *Cs*enolase in different immunization routes potentialize *Cs*enolase an oral vaccine candidate in *C. sinensis* prevention.

1. Introduction

Human clonorchiasis is a key foodborne zoonosis which is caused by the consumption of raw or undercooked freshwater fish infected with *Clonorchis sinensis* (*C. sinensis*) metacercariae. According to recent reports, it is globally estimated that more than 200 million people are at risk of *C. sinensis* infection except that 15–20 million people have been infected in eastern Asian countries [1]. Accumulating evidence showed that the long-lived flukes inside human bodies could induce various pathological impairments, such as pyogenic cholangitis, cholelithiasis, cholecystitis and hepatic fibrosis, even cholangiocarcinoma and hepatoma [2]. However, molecular mechanisms by which *C. sinensis* causes pathological changes remain unclear to date despite valuable gene information of *C. sinensis* has been available in recent years [3]. New effective prevention strategies are still the challenges to control this kind of infectious disease even some vaccine trials have been carried out previously [4,5].

Enolase is a multifunctional enzyme that is involved in the reversible dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate in glycolysis and gluconeogenesis pathways, this enzyme was also identified as a key component of excretory–secretory products (ESP) of many parasites [6]. Besides, through binding to plasminogen and nucleic acid, enolase played an important role as a cell surface receptor in host-pathogen interactions and pathogenic diseases [7,8]. The biochemical importance and biological relevance of enolase enable its potential application as a vaccine candidate in various parasitic diseases, such as *Ascaris suum* [9] and *Candida albicans* [10]. More importantly, our previous investigations suggested that, as a key component of *C. sinensis* ESP, *Cs*enolase might play vital roles in controlling the parasite growth [11].

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Gram positive bacterium *B. subtilis* is a widely used oral vaccine delivery system since it has been classified as a novel food probiotic for both human and animal consumption [12]. Extensive investigations confirmed that *B. subtilis* is an attractive vehicle for delivery of heterologous antigens to gastrointestinal tract as bioactive molecule [13,14]. In our previous study, we successfully reported the use of cotC, a major component of the *B. subtilis* spore coat, as a fusion partner for the expression of *C. sinensis* protein on the spore coat [15]. Encouragingly, the surface displayed antigen on *B. subtilis* conferred considerable protective efficacy against *C. sinensis* [4]. In the present study, by employing oral vaccine engineering platform we constructed before, we constructed *Escherichia coli*/*B. subtilis* shuttle genetic engineering system and investigated the potential of *Cs*enolase as an oral vaccine candidate for *C. sinensis* prevention in different immunization routes. Our comparably vaccinal trails with *Cs*enolase in different immunization routes confirm *Cs*enolase could be a promising oral vaccine candidate in *C. sinensis* prevention.

2. Materials and methods

2.1. Ethics Statement

C. sinensis metacercariae were isolated from experimentally infected freshwater fish *Ctenopharyngodon idellus* as methods described before [5]. *C. sinensis* adult worms were recovered from infected livers of Sprague–Dawley rats. Animals were purchased from animal center of Sun Yat-sen University and raised carefully in accordance with National Institutes of Health on animal care and the ethical guidelines. All experimental procedures were approved by the animal care and use committee of Sun Yat-sen University (Permit Numbers: SCXK(Guangdong) 2009-0011).

2.2. Bioinformatics analysis of Csenolase

As we described before [11], the open reading frame of *Cs*enolase contains 1308 bp encoding 436 amino acids. To evaluate the possibility of *Cs*enolase as vaccine candidate in the context of B cell response, B cell linear epitope, epitope antigenicity, epitope hydrophilicity, and epitope surface accessibility of full length *Cs*enolase were predicted by B Cell Epitope Prediction Tools (http://tools.immuneepitope.org).

2.3. Vaccine trials with recombinant Csenolase and nucleic acid

First, full length of *Cs*enolase was cloned into pET28a(+) to obtain recombinant protein as described previously [11]. Eukaryotic expression plasmid pcDNA3.1B was used to construct recombinant pcDNA3.1-ENO with specific primers (F: CGCAAGCTTATGTCGATCCTCAAAATTAC, R: CGTCTCGAGCTAACAGAGTGTACTC, underlined are restriction sites).

Next, we carried out the vaccine experiments in different immunization routes. Recombinant protein rENO (200 μg) was mixed with equal volume of complete Freund's adjuvant and injected into six-week-aged Sprague–Dawley rats ($n = 8$) subcutaneously and intraperitoneally, respectively. The control group ($n = 8$) was immunized with equal volume of PBS emulsified with equal volume of complete Freund's adjuvant. 100 μg of protein emulsified with incomplete Freund's adjuvant was given for two booster immunizations at a 2-week interval. For nucleic acid immunization, 200 μg of pcDNA3.1-ENO plasmid was directly injected into rats ($n = 8$) intramuscularly in quadriceps, rats ($n = 8$) immunized with equal amount of pcDNA3.1 plasmid were included as control. Also, 100 μg of pcDNA3.1-ENO or pcDNA3.1 plasmid was given for two booster immunizations at a 2-week interval. After that, each rat was challenged with 100 living *C. sinensis* metacercariae through intragastric administration under anesthesia, and all rats were raised under the same conditions.

2.4. Construction of gene fusions for spore vaccine

For oral vaccine development, at this time, we optimized and improved our genetic engineering by using *E. coli*/*B. subtilis* shuttle vector PEB03 to facilitate the gene transformation, which was ever an issue to impede further study in the previous trials. *E. coli*/*B. subtilis* shuttle vector PEB03 was a kind gift from Zhang [16]. Firstly, we extracted genomic DNA from *B. subtilis* WB600 strain, the fragment of cotC gene (380 bp) containing the promoter sequence and coding sequence (198 bp) was amplified from *B. subtilis* genome with specific primers (F: CATGTCGACTGTAGGATAAATCGTT, R: CGGAAGCTTGTAGTGTTTTTTATGC, underlined are restriction sites), and ligased to the pBluescript II SK(+) using *Sal* I and *Hind* III as restriction sites. The cotC gene was followed by full-length coding sequence of *Cs*enolase (1308 bp) amplified with specific primers (F: CGCAAGCTTATGTCGATCCTCAAAATTAC, R: CGTGAGCTCCTAACAGAGTGTACTC, underlined are restriction sites), *Cs*enolase was ligased to pBluescript II SK(+) using *Hind* III and *Sac* I as restriction sites. All the constructs were confirmed by complete sequencing, the right fusion fragment of cotC-ENO was subcloned into *E. coli*/*B. subtilis* shuttle vector PEB03 using *Sal* I and *Sac* I as restriction sites. To prepare immunization control, we also constructed PEB03-cotC plasmid by cloning cotC gene directly into PEB03 using corresponding primers (forward: GGCGTCGACTGTAGGATAAATCGTT, reverse: CGCGAGCTCTTAGTAGTGTTTTTTATGC, underlined are restriction sites). All the genetic work was performed in *E. coli* DH5α strain, and the recombinant plasmid PEB03-cotC-ENO could be easily transformed into *B. subtilis* WB600 strain for protein expression by electrotransformation method [17].

2.5. SDS-PAGE and Western blotting analysis of fusion protein expression

Transformant containing PEB03-cotC-ENO was grown in LB medium with 100 μg/ml spectinomycin at 37 °C, sporulation was made in Difco Sporulation Medium (DSM) by the exhaustion method as previously described [18]. Spores at different time points (2 h, 6 h, 12 h, 18 h and 24 h) were harvested and routinely treated for SDS-PAGE to evaluate the presence of fusion expression of *Cs*enolase. Moreover, spore coat proteins were extracted from spores at 24 h using SDS–DTT extraction buffer (0.5% SDS, 0.1 M DTT, 0.1 M NaCl). To confirm the surface display of *Cs*enolase on the spore coat, extracted proteins were subjected to 12% SDS-PAGE and then transferred onto Polyvinylidene Fluoride (PVDF, Whatman). The immobilized membrane was blocked overnight at 4 °C using 5% non-fat milk in PBST. After five times of washing with PBST, *Cs*enolase antiserum (1:200 in PBST) was used to probe the membrane by incubating for 2 h at RT. Finally, the probed membrane was incubated with rabbit anti-rat HRP-conjugated secondary antibody (1:2000 in PBST) and visualized by ECL method.

2.6. Surface display by immunofluorescence microscopy

To confirm the fusion expression of *Cs*enolase in *B. subtilis*, 1 ml of sporulation cultures at 6 h and 24 h were harvested and fixed on slides as methods adapted from previous report [4]. Samples were blocked with normal goat serum overnight at 4 °C followed by incubation with *Cs*enolase antiserum (1:200 in PBST) for 2 h at RT. Naïve rat serum at the same dilution was also used to probe the corresponding samples as a control. Cy3-labeled goat anti-rat IgG (Invitrogen, 1:500 in PBST) was employed to visualize