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Safety and immunogenicity of an oral DNA vaccine encoding Sip of *Streptococcus agalactiae* from Nile tilapia *Oreochromis niloticus* delivered by live attenuated *Salmonella typhimurium*

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ABSTRACT

Attenuated *Salmonella typhimurium* SL7207 was used as a carrier for a reconstructed DNA vaccine against *Streptococcus agalactiae*. A 1.02 kb DNA fragment, encoding for a portion of the surface immunogenic protein (Sip) of *S. agalactiae* was inserted into pVAX1. The recombinant plasmid pVAX1-*sip* was transfected in EPC cells to detect the transient expression by an indirect immunofluorescence assay, together with Western blot analysis. The pVAX1-*sip* was transformed by electroporation into SL7207. The stability of pVAX1-*sip* into *Salmonella* was over 90% after 50 generations with antibiotic selection in vitro while remained stable over 80% during 35 generations under antibiotic-free conditions. The LD_{50} of SL/pVAX1-*sip* was 1.7×10^{11} CFU/fish by intragastric administration which indicated a quite low virulence. Tilapias were inoculated orally at 10^8 CFU/fish, the recombinant bacteria were found present in intestinal tract, spleens and livers and eventually eliminated from the tissues 4 weeks after immunization. Fish immunized at 10^7, 10^8 and 10^9 CFU/fish with different immunization times caused various levels of serum antibody and an effective protection against lethal challenge with the wild-type strain *S. agalactiae*. Integration studies showed that the pVAX1-*sip* did not integrate with tilapia chromosomes. The DNA vaccine SL/pVAX1-*sip* was proved to be safe and effective in protecting tilapias against *S. agalactiae* infection.

1. Introduction

Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae*, is a Gram-positive coccus that can act as either a commensal organism or a pathogen with a broad host range [1–3]. To date, *S. agalactiae* consists of ten serotypes: Ia, Ib, and II to IX [4], of which serotypes Ia, III and V cause the majority of human infections [5] and serotypes Ia, Ib and III have been previously reported in fish [6,7]. Piscine GBS has increased in prominence and numerous species of fish have been found susceptible to infection [8]. In July 2009, a severe streptococcal infection in tilapia outbroke in intensive tilapia cultures in Hainan Province, China. The pathogen had been isolated and characterized as *S. agalactiae* in our laboratory.

Vaccination is a very important alternative for disease prevention. As an encapsulated bacteria, the capsular polysaccharides of *S. agalactiae* have attracted the most attention with regard to vaccine development, however, it was observed that the protection conferred by capsular polysaccharides is type specific [9]. Cell surface proteins of GBS have also been considered as possible virulence factors and vaccine components. Though some surface proteins of *S. agalactiae* such as the alpha and beta C protein, the R protein and the Rib protein have already been suggested to be potential vaccine candidates, these proteins have not been found in all *S. agalactiae* isolates [10]. Surface immunogenic protein (Sip) was a GBS surface-exposed protein and found to be present in every serotype of GBS isolates [11] and highly conserved [10]. Sip as a potential vaccine

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candidate has been identified for subunit vaccines [9,12], however, the immunogenicity of prokaryotic expression antigen protein is not as well as that of eukaryotic expression due to the lack of glycosylation and post-translational modification [13]. Considering the advantages of long-term antigen expression and easy production, a DNA vaccine encoding Sip against *S. agalactiae* in fish has been an attractive choice. However, intramuscular injection of vaccine is limited in the actual application in aquaculture, because it is laborious and also stressful to fish as well. By contrast, oral delivery of vaccines which is more effective and practical has been described as the most desirable method for vaccinating fish [14].

In fish farming, a safe and effective vaccine by oral route is highly needed. Attenuated *Salmonella* with the ability of adhesion and invasion but not pathogenic [15], has been used as a delivery system for recombinant heterologous antigens to induce protective immunity against several infectious diseases and tumor sources in animal models [16]. Attenuated *Salmonella* as a DNA vaccine delivery can elicit both cellular and humoral responses against the pathogens by carrying the DNA vaccine plasmid to antigen presenting cells (APCs) [17]. By now, attenuated *Salmonella typhimurium* has rarely been reported as a DNA vaccine vector in fish and more studies are needed to prove its feasibility in fish farming.

In this research, we cloned the gene encoding a portion of Sip into pVAX1 and introduced the recombinant plasmid pVAX1-*sip* into attenuated *S. typhimurium* SL7207 by electrotransformation. We evaluated the safety and stability of the recombinant DNA vaccine delivered by SL7207 both in vivo and in vitro as well as the protective effects to tilapia by oral vaccination.

2. Methods

2.1. Bacterial isolates, cell lines, media, plasmids and culture conditions

The *S. agalactiae* (JF423941, Ia) was isolated from an outbreak epidemical diseased tilapias from Hainan, P.R. China in 2009. The attenuated *S. typhimurium* SL7207 aroA mutant was kindly provided by Professor Sanjie Cao of College of Veterinary Medicine, Sichuan Agricultural University, Sichuan, P.R. China. The eukaryotic expression plasmid pVAX1 was obtained from Invitrogen. Epithelioma papilloma cyprini (EPC) cells were kindly offered by Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences. *S. typhimurium* SL7207 and *S. agalactiae* were grown in Luria–Bertani (LB) medium and in brain heart infusion medium (BHI, BD Corporation, USA) at 37 °C, respectively. Kanamycin (Kan) was used at a final concentration of 50 μg/ml.

2.2. Fish

Healthy tilapias (100 ± 5.0 g) were purchased from a tilapia culture farm in Chongqing municipality, China. The fish were reared in 100-L tanks with aeration and fed a commercial diet twice a day throughout the study. Water was partly replaced daily and the temperature was maintained at 30 ± 1.0 °C. We did not observe any mortality due to infectious disease during this period.

2.3. Construction of expression plasmids

S. agalactiae DNA was extracted from pure cultures of the bacterial isolate using bacterial Genomic DNA kit (Tiangen, China). The *sip* segment was amplified from the DNA using the forward primers 5′-GGATCCACCATGGCTGCCACTTCAATGAAA-3′ and reverse primers 5′-AAGCTTTTATTTGTTAAATGATACGTG-3′ with *Bam*HI and *Hind*III restriction sites, respectively. The purified PCR product of *sip* gene was linked into pMD19-T (TaKaRa) and then sequenced. The *sip* gene fragment was cut by restriction enzyme and ligated into pVAX1. The recombinant plasmid was named pVAX1-*sip*.

2.4. Expression of pVAX1-sip in EPC cells

Epithelioma papulosum cyprinid (EPC) cells were propagated in MEM with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 25 °C on glass coverslips in 24-well plates [18]. EPC cells were transiently transfected with pVAX1-*sip* or pVAX1 by using Lipofectamine 2000 (Invitrogen) at a DNA/lipofectamine ratio of 1:2.5 (μg/μl) according to the manufacturer's protocol. Cells were washed with cold PBS at 24–36 h after transfection and then fixed in methanol-acetone, 1:1, for 30 min at 4 °C and washed with PBS once again followed by incubation with primary antibodies at a dilution of 1:100 in PBS containing 0.5% bovine serum albumin (BSA) and secondary antibodies (1:200 dilution in PBS containing 1% BSA) both at 37 °C for 1 h, respectively. After washing with PBS, the fluorescence signals were analyzed by fluorescence microscopy. Primary antibodies were self-prepared antiserum of rabbit to *S. agalactiae*. Rabbits were immunized with Formalin-killed whole bacterial cells (1×10^9 CFU) emulsified in complete Freund adjuvant by multipoint subcutaneous injections. After 2 weeks, the rabbits received additional weekly booster injections (usually twice) of the same dose of GBS in incomplete Freund adjuvant until the antibody titer reached 1:16 determined by double immunodiffusion test before each injection. Then the rabbit received a final intravenous injection a week later with GBS suspended in normal saline. Three days after injection, antiserum was collected and purified by ammonium sulfate precipitation method [19,20]. Secondary antibodies were FITC-conjugated goat anti-rabbit IgG (Abgent).

For Western blot analysis, 48 h after transfection, cells were separated on 12% SDS-PAGE and transferred onto a PVDF membrane which was probed with serum obtained from a *S. agalactiae*-infected rabbit. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Abgent) was used to detect the bound antibodies. HRP was detected using an enhanced HRP–DAB colour development kit (Tiangen, China).

2.5. Transformation into attenuated S. typhimurium

The purified plasmid pVAX1-*sip* or pVAX1 was electroporated into *S. typhimurium* SL7207 (2.5 kV, 200 Ω, 25 μF). The positive transformants selected for kanamycin resistance were identified by PCR amplification, restriction endonuclease analysis and sequence analysis with universal 16S rDNA primers. The recombinant *Salmonella* strains containing plasmid pVAX1-*sip* or pVAX1 were named SL/pVAX1-*sip* and SL/pVAX1, respectively.

2.6. In vitro and in vivo stability of plasmid

Bacterial cultures were passaged for 50 generations (10 days) at 37 °C with or without antibiotic selection, respectively. The ratio of the number of bacterial colonies on kanamycin media (cells containing the plasmid) to the viable counts on non-kanamycin media (the total number of cells) at each passage was calculated to evaluate the persistence of a plasmid in a population of attenuated *S. typhimurium* in vitro [21].

Tilapias were randomly divided into two groups, 25 fish per group. One was orally immunized with SL/pVAX1-*sip* in 10^8 CFU dosages per fish using gastric tubes and the other was PBS control. Five tilapias in each group were killed with an overdose of MS-222 at 3, 7, 14, 21, 28 days after immunization. The intestinal tract, livers and spleens were collected, respectively and stored at −20 °C. The collected tissues were homogenized in PBS. The recovery of recombinant *Salmonella* from the organs was estimated by counting of