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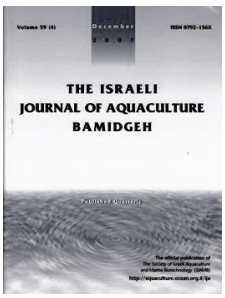

Immune Response in Tilapia, *Oreochromis niloticus*, Induced by the Surface Immunogenic Protein (Sip) of *Streptococcus agalactiae*

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Abstract

The main aim of this study was to demonstrate that the surface immunogenic protein (Sip) of the aquatic pathogen *Streptococcus agalactiae* is a potential candidate for vaccine development for *Oreochromis niloticus*. In this study, a Sip was identified. Alignment analysis indicated that the Sip protein was highly homologous with Sip proteins from other *S. agalactiae* isolated from humans (99%). SDS-PAGE procedure indicated that the Sip protein was successfully expressed in *Escherichia coli* BL21 (DE3). The recombinant Sip protein was purified by affinity chromatography, and the mouse anti-Sip serum was produced. Tilapia *Oreochromis niloticus* were vaccinated with Sip, and immunogenicity was confirmed by subsequent western blotting. Enzyme-linked immunosorbent assay (ELISA) analysis demonstrated that Sip produced an observable antibody response in all sera of the vaccinated fish. The relative percentage survival (RPS) value for the Sip vaccine was 90.62. Tilapia vaccinated with Sip produced specific antibodies, and were highly resistant to infection by the virulent *S. agalactiae*. These results indicate that Sip is an effective vaccine candidate against *S. agalactiae* for tilapia, *Oreochromisniloticus*.

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Introduction

Streptococcus spp. is considered to be a diverse group, ranging from commensal organisms which occupy various niches of the human body to pathogens that infect a wide range of hosts. *Streptococcus agalactiae*is is considered a pathogen responsible for causing infections in different animals, poultry, fish, rodents, mammals, and humans (Elliott *et al*., 1990; Evans *et al.*, 2002; Evans *et al*., 2000; Evans *et al*., 2006; Hetzel *et al*., 2003; Yildifim *et al.*, 2002a; Yildifim *et al*., 2002b; Zappulli *et al*., 2005). *S. agalactiae* infections can cause severe economic losses in wild and cultured hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) worldwide (Wu *et al*., 2013). Control of streptococcosis through antibiotic treatment is not always successful and may harm the environment since resistant bacteria may be produced and transferred to aquatic animals or humans. Therefore, the development of vaccines to protect aquatic animals against streptococcosis is important (Kasper *et al*., 1996; Wessels *et al*., 1995).

Based on the composition of the capsular polysaccharide, *S. agalactiae* can be divided into ten serotypes (Ia, Ib and II to IX) (Chaffin *et al*., 2000; Persson *et al*., 2004; Slotved *et al*.,2007). Only serotype Ib has been detected and reported in fish (Vandamme *et al*., 1997). An alternative strategy for protecting fish would be to develop a vaccine based on a ubiquitous protein. Bacterial surface proteins have numerous advantages for vaccine development. Indeed, such bacterial proteins have been shown to be present in most pathogenic strains where they induce cross-protective immunity (Fikrig *et al*., 1990; Martin *et al*., 1997). Furthermore, these proteins do not need to be conjugated to other molecules, since they elicit an effective T-cell dependent antibody response resulting in long-term immunity (Brodeur*et al*., 2000). *S. agalactiae* surface proteins, already being investigated as potential vaccine candidates, are the Rib protein, the α and β subunits of the c protein, and the Rib protein (Flores & Ferrieri., 1989; Gravekamp *et al*., 1999; Stålhammar-Carlemalm *et al*., 1993). All these proteins are capable of eliciting antibodies in mice and to some extent prolong life and protect against lethal bacterial infections.

Bernard *et al*. (2000) identified a surface immunogenic protein (Sip) by immunological screening of a genomic library. Sip was identified in *S. agalactiae* strains representing all nine serotypes. Importantly, immunization of mice with purified recombinant Sip protein elicited protective immunity against lethal infection with *S. agalactiae* strains of several serotypes (Brodeur *et al*., 2000). This data indicates that Sip is an interesting candidate for the development of a protein-based vaccine against *S. agalactiae* disease.

Oreochromis niloticus is an important species in commercial fisheries in southern China, particularly in the Guangdong province. It is also a species commonly found in brackish water in estuaries around the world (Vijayan *et al*., 1996). With the expansion and intensification of fish farming, disease outbreaks have increased and are recognized as a significant limitation on sustainable aquaculture (Bondad-Reantaso *et al*. 2005). One of the primary causes of disease in many aquaculture systems is bacterial infection, streptococcosis being the most common in cultured *O.niloticus.* Therefore, protecting cultured fish from disease is essential for the expansion and sustainability of the aquaculture industry. We investigated the potential of a 53-kDa protein, Sip to protect *O.niloticus* against experimental infection with *S. agalactiae* ZQ0910.

Materials and Methods

*Bacteria and culture conditions***.** *S. agalactiae* strain ZQ0910 was isolated from diseased tilapia, *O. niloticus* in Zhaoqing City of Guangdong Province in China. The isolate was cultured overnight in Brain Heart Infusion (BHI, Huankai) at 37 ^{0}C in the presence of 8% CO_2. *Escherichia coli* strains DH5α and BL21 (DE3) were cultured on Luria-Bertani (LB, Oxford) medium with 1.5% (w/v) agar at 37 ^{0}C. When required, the medium was supplemented with 100 μg/ml ampicillin (LB medium). Plasmid pMD-19T vector (Takara, Japan) was used for sequencing. The pET-32a(+) plasmid which contains a T7 promoter, Trx coding sequence, His-Tag sequence, and an ampicillin resistance gene was used for recombinant expression of Sip.

Extraction of genomic DNA. S. agalactiae strain ZQ0910 was preserved in Brain Heart Infusion (BHI, Huankai) incubated at 37 ^{0}C in the presence of 8% CO_2. Genomic DNA was extracted by MiniBEST Bacterial Genomic DNA Extraction Kit (Takara).

Gene cloning and plasmid construction. The *SIP gene*s from the strain ZQ0910 were amplified from purified chromosomal DNA by PCR with recombinant Taq DNA polymerase (Takara, Japan) as described by the manufacturer. The primers (PA1:5'-ATGAAAATGAATAAAAAGGTACT-3') and (PA2: 5'-TTATTTGTTAAATG ATACGTGAACG -3') were designed according to the published sequence of *SIP gene* of *S. agalactiae* ZQ0910 (GenBank accession no. AKAP01000043.1). Polymerase chain reaction (PCR) was performed for 4 min at 95 ^{0}C, 30 cycles for 60s at 94 ^{0}C, 30s at 57 ^{0}C and 60s at 72 ^{0}C, and a final elongation period of 10 min at 72 ^{0}C. The products obtained after amplification were sequenced for the *SIP gene* from strain ZQ0910, then ligated into pMD-19T vector. The recombinant plasmid containing the *SIP gene* was named pMD-sip and transformed into E. coli DH5a. The sequence of the insert was also verified. To obtain a thioredoxin-Sip fusion protein with an N-terminal polyhistidine tag; the *SIP gene* was also amplified by PCR using primers (PB1:5'-CGCGGATCCATGAAAATGAATAAAAAGGTACT-3') and (PB2:5'-ACGCGTCGACTTATTTGTTAAATGATACGTGAACG-3'), which contained *BamH I* and *Sal I* restriction sites, respectively, and were used to perform the amplifications. The amplification product was ligated into plasmid pET-32a(+), and after sequencing, the recombinant plasmid named pET-Sip was transformed into *E. coli* BL21(DE3).

Purification of the recombinant Sip protein. The *E. coli* BL21(DE3) cells containing recombinant plasmids were inoculated at a ratio of 1:100 (v/v) into Luria-Bertani broth (LB, Huankai) with antibiotics, were vigorously shaken, and incubated at 37^{0}C until the OD_{600} value reached 0.6. Then isopropylthio-β-D-galactoside (IPTG) was added to the culture at 1 mM concentration and incubated at 37 ^{0}C for 4 h. The bacteria were then collected after centrifugation at 4000g for 10 min. The pellets were re-suspended in 15 ml of pH 7.4 lysis buffer (20 mM PBS, 0.5 M NaCl and 8 M urea). The cell suspension was then sonicated, and the lysed mixture was centrifuged at 12000 g for 10 min at 4 ^{0}C. The supernatant was then collected and filtered through a 0.22-μm spin filter (Ultrafree-MC, Millipore). The fusion protein was purified by affinity chromatography using a nickel-charged resin (Ni-nitrilotriacetic acid Superflow: Qiagen Inc., Mississauga, Canada) following the manufacturer's instructions. The purity of the recombinant Sip protein was evaluated by SDS-PAGE, and the amount of protein was determined by bicinchoninic acid (BCA) assay according to manufacturer's instructions (Pierce Chemical Company, Rockford, III).

Vaccine preparation and vaccination. The purified recombinant Sip (1 mg/ml) and sterile PBS (10 mM, pH7.2) were emulsified with an equal volume of Freund's incomplete adjuvant (Sigma, USA). *O.niloticus* weighing approximately 30g were randomly divided into two groups, 120 fish per group. The vaccination group was immunized by intramuscular injection with the emulsified recombinant Sip, and the control group with emulsified sterile PBS. After vaccination, fish were maintained at 25 ^{0}C and fed commercial dry pellets daily.

Generation of a Sip-polyclonal antibody (Sip-PcAb). BALB/c mice (Guangdong medical college, Zhanjiang, China) were injected subcutaneously (s.c) three times at 3-week intervals with 50μg of purified recombinant thioredoxin-Sip protein in 0.1ml of PBS mixed with 50μg of Freund's adjuvant (Sigma, USA). Freund's complete adjuvant was used in the first injection and Freund's incomplete adjuvant was used in all subsequent injections. The mouse anti-Sip serum was collected on the seventh day after the fourth immunization. Simultaneously, the negative serum was produced as control by immunizing mice with PBS and Freund's adjuvant (Sigma, USA) using the same procedures.

Western blot analysis. Cultures of recombinant plasmid pET-Sip (transformed into *E. coli* BL21 cells) were centrifuged at 10,000 rpm for 1 min. The pellets were washed twice with phosphate buffered solution (10 mM, pH7.2) and re-suspended in PBS (10 mM, pH7.2). The cell suspension was boiled at 100 ^{0}C for 5 min. The crude protein extracts

were electrophoresed by SDS-PAGE containing 5% stacking gel and 12% separating gel. The expression of recombinant Sip protein was examined with Western blot analysis using mouse anti-Sip serum (1:10,000) as the primary antibody and HRP- conjugated goat anti-mouse IgG (1:500, Sigma Co) as the secondary antibody. Simultaneously, the normal mouse serum was used as negative control. Western blot analysis was also carried out using His-tag monoclonal antibody (1:2000, Sigma Co) as primary and HRP-conjugated goat anti-mouse IgG (1:500, Sigma Co) as secondary antibody to detect whether the fusion protein with His-tag was successfully expressed. The antibody-bound proteins were then visualized by 3, 3'-di-aminobenzidine (DAB).

Analysis of antibody levels. Nine fish from each treatment group (vaccinated and control) were examined weekly for 7 weeks post-vaccination for antibody response against Sip by enzyme-linked immunosorbent assay (ELISA). 96-well plates were coated with the purified recombinant Sip (8μg/ml diluted in the preliminary assay). Two-fold serial dilutions of the fish serum were added to duplicate wells of the plates. Antibody binding to the antigen was detected using mouse anti-Tilapia (*O. niloticus)* IgM monoclonal antibody (Aquatic Diagnostics Ltd, Scotland), followed by horse radish peroxidase (HRP)-conjugated Goat-anti-mouse IgG at 1:4000 dilution. The reaction was developed with the 3,3,5,5-tetramethylbenzidine (TMB) substrate in H2O2 and stopped with 2.0M H_2SO_4. Optical density was measured at 450 nm using a microplate reader (μQuart, BioTek). Results were considered positive if the absorbance was at least double the control serum, and antibody titers were scored as the highest positive dilution.

Bacterial challenge experiments. Virulent *S. agalactiae* strain ZQ0910 was cultured at 37 ^{0}C in BHI medium for 18h. Four weeks post-vaccination, fish in each group (100 per group) were used for the challenge test. Bacterial suspension of *S. agalactiae* strain ZQ0910 was prepared and adjusted to a cell density of approximately 1×10^8 cfu/ml. The fish were anesthetized and challenged by intraperitoneal inoculation with 0.1 ml of 1×10^8cfu/ml (1×10^7cfu/ml per fish) cell suspension according to a preliminary experiment. Each sub-group of fish was then maintained in a separate tank with fresh running water at 25 ^{0}C for 14 d. Dead fish were autopsied to determine cause of death and to detect the presence of *S. agalactiae* in the tissues by bacterial culture in Brain Heart Infusion broth (BHI) agar. Relative percentage survival (RPS) was calculated from the cumulative mortalities using:

$$RPS = (1 - \frac{\text{mortality of vaccinated group}}{\text{mortality of control group}}) \times 100\%$$

Data are expressed as mean±SD. Statistical analysis was performed to assess serum antibody levels and mortality for significance (P<0.05) using student's t test. Probabilities of 0.05 or less were considered statistically significant.

Results

Cloning and sequence analysis. The open reading frame (ORF) sequence of Sip containing 1305 nucleotides was obtained from the genome DNA of *S. agalactiae* strain ZQ0910 using PCR and sequencing. The deduced amino acid sequences of *SIP gene* consisted of 433 amino acid residues with predicted molecular weight of 45.5kDa. GenBank accession no. is AKAP01000043.1.

BLAST analysis showed that the Sip sequence of *S. agalactiae* was highly similar to the homologous genes of other *Streptococcus spp*. in the GenBank database (ranging from 99-100% identity at DNA level, 98-100% identity at deduced amino acid level). The result of multiple amino acid sequence alignment analysis also indicated that they were highly conserved (Fig.1).

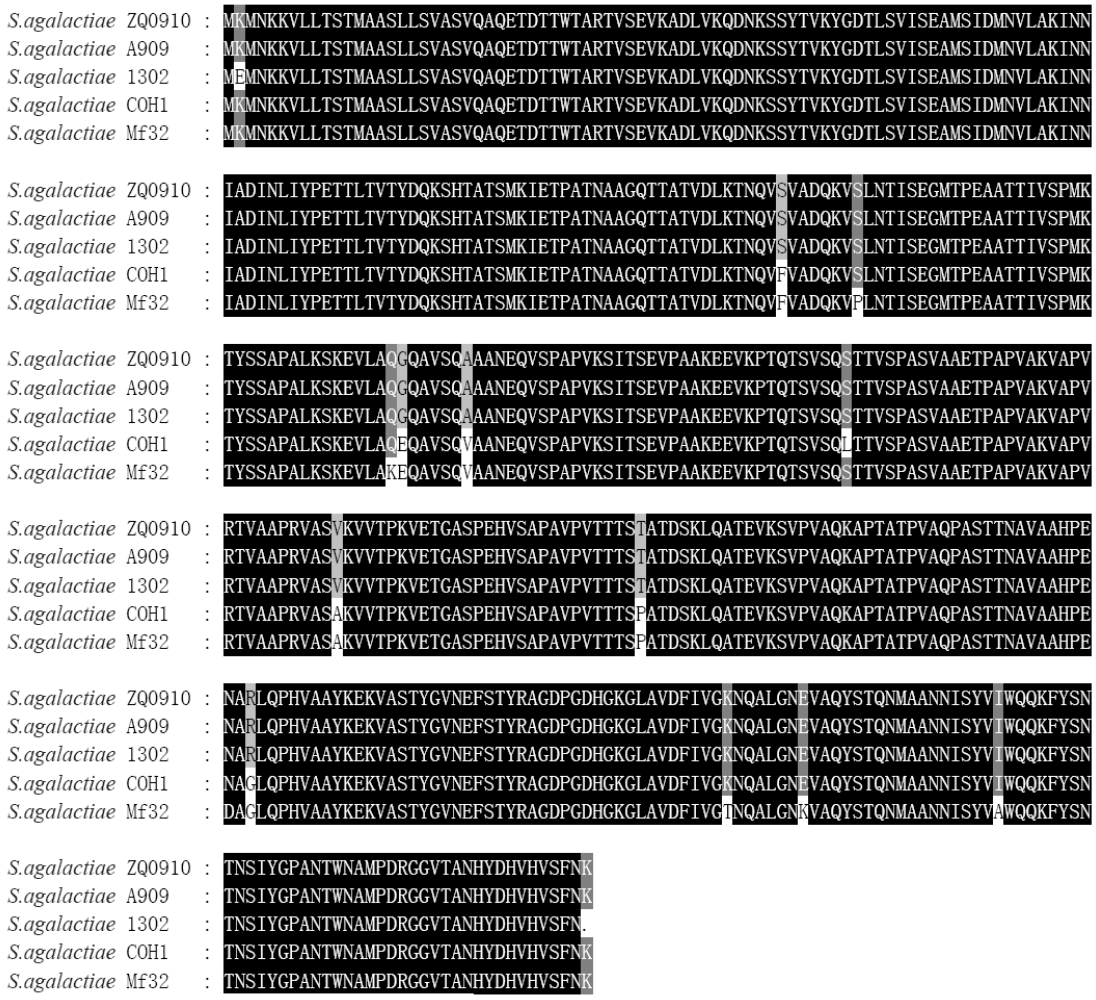

Figure 1. Multiple amino acid sequence alignment of Sip from *Streptococcus agalactiae* ZQ0910 and other strains. Identical amino acid residues presented were boxed in black, and boxes in grey indicated the location of similar residues.

Expression and purification of the recombinant Sip. The predicted molecular mass of the Sip protein is 45.5 kDa, and the actual molecular mass of recombinant Sip protein seen in SDS-PAGE is 65.9 kDa because of the fusion protein which consists of 20.4kDa TRX-His-Stags. Western bolt analysis showed that His-Tag monoclonal antibody specifically recognized the Sip fusion protein expressed in *E. coli.* BL21. SDS-PAGE analysis showed that the His-tagged Sip protein was homogeneously purified because only a single band was detected in the electrophorogram (Fig. 2).

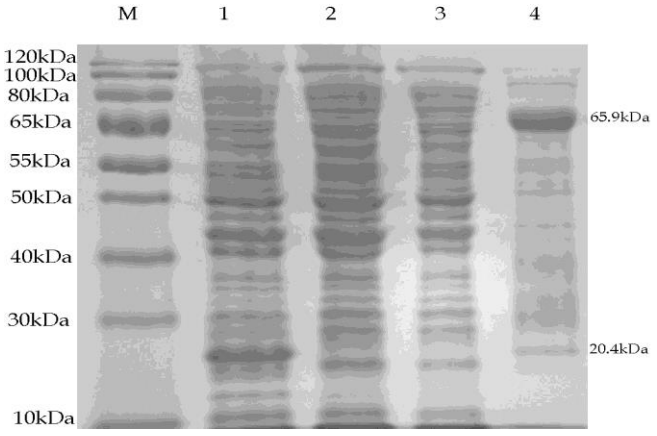

Figure 2 *Streptococcus agalactiae* SDS-PAGE profile of Sip. Lanes: M, molecular mass markers; 1, pET-32a (+) with IPTG induction; 2, pET-32a (+) without IPTG induction; 3, pET-sip without IPTG induction; 4, pET-sip with IPTG induction

Western blot analysis. Western blot analysis revealed that the prepared mouse anti-Sip serum did not react specifically to the Sip fusion protein, but also to natural Sip protein of *S. agalactiae*, while the normal mouse serum as negative control did not react to the Sip fusion protein (Fig 3).

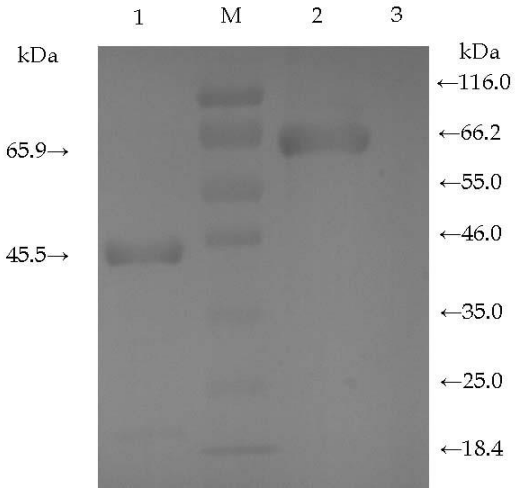

Figure 3 Western blot analysis of pET-Sip expression in *Escherichia coli* BL21(DE3). Lanes M: prestained protein marker; Lane 1: the whole cell lysates of *Streptococcus agalactiae*ZQ0910 (using mouse anti-Sip serum as the primary antibody); Lane 2: pET-Sip with IPTG induction (using mouse anti-Sip serum as the primary antibody); Lane 3: pET-Sip with IPTG induction (using normal mouse serum as the primary antibody).

The ELISA results indicated that the titer of antisera was 1:512, 000.

Analysis of serum antibody levels. The antibody production against the Sip of *S. agalactiae* in Tilapia is shown in Fig. 4.

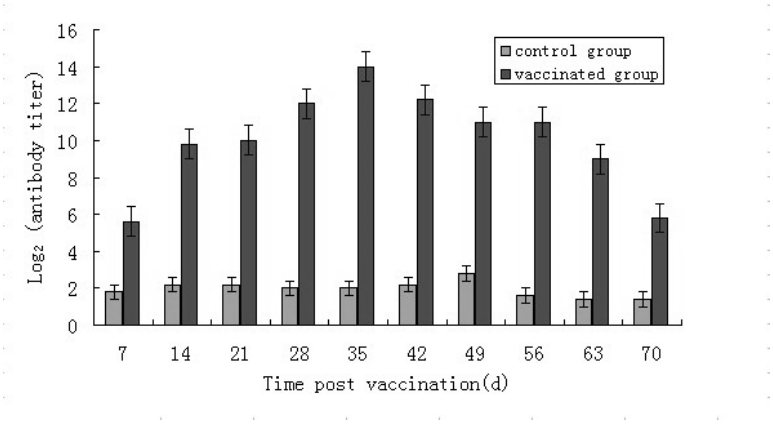

Figure 4 Analysis of serum antibody levels against Sip in *Streptococcus agalactiae*. Each bar represents mean $\log_2$ value ± SD of the highest dilution of the serum when P/N ≥2.7 (P/N= OD_{450} of the inspected sera/ OD_{450} of the negative serum). Significant differences from the control were *P<0.05*

The results show that a specific antibody titer against Sip was detected in all sera of the vaccinated fish. During weeks 1 to 10 post-vaccination, $\log_2$ (antibody titers) in the sera of the vaccinated groups reached levels above 6.0, (the maximum reached was 14.0), while that of the control group was only 1.0-3.0 at all times. There was a significant difference ($p < 0.01$) in antibody titers between the vaccinated and control group

Efficacy of the Sip vaccine. The cumulative mortality of Tilapia is shown in Table 1.

Table 1. Cumulative mortality rate for Tilapia (*Oreochromis niloticus*) after challenge with *S. agalactiae*.

Days after challenge	*Vaccinated group*		*Control group*	
	No. of dead fish	*Mortality rate,%*	*No. of dead fish*	*Mortality rate, %*
1	0	0	0	0
2	0	0	0	0
3	0	0	10	10
4	0	0	20	20
5	0	0	26	26
6	2	2	34	34
7	2	2	38	38
8	4	4	42	42
9	7	7	71	71
10	8	8	78	78
11	8	8	90	90
12	9	9	90	90
13	9	9	93	93
14	9	9	96	96

The fish immunized with the Sip vaccine demonstrated a high level of protection. Results showed that after challenge with *S. agalactiae* fish mortality rate in the vaccinated groups was significantly lower ($P < 0.01$) than in the control group. The dead fish displayed disease symptoms typical of streptococcal infection. Abnormal behaviors included erratic swimming, swimming and whirling on the surface; ocular abnormalities included opacity, periorbital and intraocular hemorrhage, purulence and exophthalmia; reddening and hemorrhaging in the integumental and muscoskeletal system was common. In addition, typical of streptococcal infection, opercula were clear creating a 'window to the gill' and no pathogen other than *S. agalactiae* was isolated. The RPS value for the Sip vaccine was 90.62.

Discussion

Some GBS surface proteins, such as the R protein, the α and β subunits of the C protein, and the Rib protein (Flores *et al*.,1989; Michel *et al*.,1991; Stålhammar-Carlemalm *et al*., 1993), have been investigated as potential vaccine candidates. Unfortunately, these proteins were not found to be present in all clinical isolates (Ferrieri & Flores., 1997; Stålhammar-Carlemalm *et al*., 1993). A surface immunogenic protein (Sip), has been identified (Bernard R *et al*. 2000). Since Sip-specific antibodies efficiently recognized surface exposed epitopes on GBS cells, antibodies against these particular epitopes could play a role in antibody-mediated protective immunity (Rioux *et al*., 2001). This immunity appears to be important in preventing GBS infection. In this study, *SIP gene* was cloned and identified from *S. agalactiae* ZQ0910. The BLAST result showed that the putative amino acid sequence of *SIP gene* was conserved among different Streptococci strains. The multiple amino acid sequence alignment analysis indicated there were discernible similarities at the amino- and carboxy termini, while the central region was more diverse, reflecting the functional importance of the terminal amino acids in forming the tertiary structure of the Sip protein monomers. In additional, this could be one of the important reasons that Sip are particularly attractive and used for the development of vaccines.

The Sip protein of *S. agalactiade* was successfully expressed and purified. When mouse polyclonal antibodies against the recombinant Sip were tested in Western blot with crude Sip, and the isolated Sip, a strong reaction was observed. Thus the recombinant Sip of *S. agalactiae* retained natural antigenicity. Therefore, we presume the recombinant Sip could be used as potential vaccine.

There is evidence that indicates that antibodies directed against GBS surface proteins conferring protective immunity are sufficient to prevent GBS infection (Larsson et al., 1999; Michel et al., 1991; Stålhammar-Carlemalm *et al*., 1993). The present study suggests that a strong antibody response was induced and significant protection was achieved by vaccination with the Sip of *S. agalactiae* in mice. The immune response induced after immunization with recombinant Sip efficiently protected mice against experimental infection with different GBS strains, which included representatives of all nine serotypes (Riou*et al*., 2001). It has been proven that recombinant Sip of *S. agalactiae* isolated from humans could provide protection for mice, and its RPS was 89 (Brodeur*et al*., 2000). In our study, tilapia (*Oreochromis niloticus*) vaccinated with the isolated Sip of *S. agalactiae* were protected with a RPS of 94.

The development of a versatile vaccine that provides heterologous protection for *S. agalactiae* has been a major concern because the pathogen has different serotypes. Recently more attention has been focused on the possibility of developing vaccines based on genetically conserved antigens present mainly in *S. agalactiae,* and all their serotypes. Such proteins would not only induce more effective and durable immune responses but also could potentially protect against all clinically relevant pathogens and their serotypes (Cai *et al*., 2013). Sip is an interesting candidate for the development of a protein-based vaccine against *S. agalactiae* disease. In this study, we applied the Freund's incomplete adjuvant in Sip vaccine as previously reported (Li *et al*., 2010). However, Freund's adjuvant was not applied in fish vaccines because of the cost and induced inflammation in the injection site (Sobhana *et al*., 2002). We plan to test the oil adjuvant in the future.

In conclusion, the *SIP gene* from *S. agalactiae* was cloned and expressed in E. coli. This recombinant Sip is a protective antigen of *S. agalactiae* in *O.niloticus*. The present report confirms that Sip is exposed at the surface of intact *S. agalactiae* cells. Most importantly, the surface exposure of Sip is not hindered by other surface antigens. These observations promote further interest in Sip as a potential vaccine candidate against infection by *S. agalactiae* in *O.niloticus*.

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