Original Article

The design of the constructs of cpsD and simA genes of Streptococcus iniae

Mohsen Rahmanian Kooshkaki[1], Mojgan Bandehpour[2, 3*], Bahram Kazemi[2, 3]

[1] Department of Biology, Colledge of Basic Sciences, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran
[2] Cellular & Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3] 3Department of Biotechnology, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Abstract

Background & Objective: *Streptococcosis* is one of the bacterial infections in fish, especially rainbow trout which infects brain and nervous systems of fish and is caused by *S. iniae*. Estimation of the impact of disease prevalence by *S. iniae* in fish farming in some countries is reported about 100 million dollars per year. Some of the most effective proteins in pathogenicity of these bacteria are *SimA* and *CpsD*. In order to design new and effective vaccine, in this study cloning of two genes of Streptococcus was performed into *pNZ8148* vector and expressed in *Escherichia coli*.

Materials and Methods: *simA* and *cpsD* genes were subcloned into *pNZ8148* vector. Obtained constructs were transformed to expressing *E. coli BL21* strain. After induction with nisin, SDS PAGE electrophoresis and Western blotting were used to confirm the procedures.

Results: Using PCR with specific and universal primers, the accuracy of cloning was confirmed. Final verification of expressed protein was carried out by SDS-PAGE and western blotting.

Conclusion: With regard to the obtained results, it seems that the generated gene construct in this study can be used as a vaccine against *Streptococcosis* in future researches.

Key Words: Streptococcus iniae, cpsD gene, simA gene, pNZ8148 vector, cloning, Expression

***Corresponding Author:** Mojgan Bandehpour, Department of Biology, Colledge of Basic Sciences, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran. Email: Bandehpour@gmail.com.

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Introduction

Streptococcosis is one of the common bacterial diseases in human and fish, especially rainbow trout which infects brain and nervous systems of fish and is caused by *S. iniae* bacteria[1]. This disease maybe either chronic or acute and the mortality rate has been reported to be between 20 to 50% [2].

This bacterium causes more than 100 million dollars damage for fish farming annually, with more than 30 species of fresh and salt water fish demonstrating susceptibility to the disease and many of which are economically important such as rainbow trout, tilapia, salmon, barramundi, flounder, yellowtail, ayu, red drum, and hybrid striped bass (HSB) [3]. Fishes in dense and crowded farming as well as stressful environments (for example suboptimal temperatures, poor water quality, overcrowding, displacement, etc.) are more vulnerable to *S. iniae* infection [4]. Common clinical symptoms of *S. iniae* infection in fish include ulcers,

loss of orientation, exophthalmia, lethargy, and erratic swimming [3, 5]. Vaccination of farm fishes, for prevention of infectious disease is used as one of the main methods of prevention [6].

Capsular polysaccharides play an important role in protecting several pathogenic microbes against the host defenses during infection [3]. Operons of *Streptococcus* capsule of one region are about 21kb with 700 nucleotides and contain a conserved group of genes (cpsA to –E) that are collectivity responsible for capsule chain length determination and export [7].

Another protective protein of these bacteria is *SimA*, which is coded with 1566bp of *simA* gene, is 57.5 kD and has 521 amino acids [8, 9].

Materials and Methods

Designing of *simA* and *cpsD* genes

The *simA* and *cpsD* genes were synthesized into *pGH* vector separately, based on the sequences submitted in the genbank (Nedaye Fan, Iran).

Plasmid and Strain of Bacteria

pNZ8148 plasmid (MoBiTec, USA) was chosen as an expression vector, while and *E. coli TOP10* and *E. coli BL21* strains (CinnaGen, Iran) were selected as prokaryotic hosts. This vector has chloramphenicol resistance gene, which is used for screening of bacteria.

Preparation of genes and vector

After transformation, proliferation and extraction of synthesized plasmids from *E. coli TOP10* strain using the alkaline lysis method [10], in order to obtain the expression of *simA* and *cpsD* genes, the recombinant vectors, *pGH/simA* and *pGH/cpsD*, were digested using *NcoI-KpnI* (Fermentas, Lithuania) and *SacI-XbaI* (Takara, Japan) enzymes, respectively. *simA* and *cpsD* genes were purified from agarose gel with a gel extraction kit (Bioneer, Korea), and were separately cloned into *pNZ8148* expression vector. The resultant of aforementioned reactions was transformed to *E. coli TOP10* strain and cultured on chloramphenicol plates.

Confirmation of bacteria carrying the recombinant plasmid using PCR

For final approval of the plasmids extracted, colony PCR was performed using specific and universal primers (Table 1). Reaction products were evaluated on 1.5% agarose gel.

TABLE 1. Sequence of specific and universal primers.

Primers name	Sequence	Tm temperature
Forward (*pNZ8148*)	AACGGCTCTGATTAAATTCTG	50.4 ºC
Reverse (*pNZ8148*)	TGCTTTATCAACTGCTGC	46.5 ºC
Forward (*simA*)	ATGGCTAAACAAATCAAAGC	48.7 ºC
Reverse (*simA*)	TTATTCTTCCTCTTTGCGTTTAC	51.6 ºC
Forward (*cpsD*)	ATAAAGGAGGTAAATAATGTCAC	45.9 ºC
Reverse (*cpsD*)	TCACTTTCTGGAATGTTTTTTAC	49.7 ºC

Expression of *SimA* and *CpsD* proteins using SDS-PAGE and Western blotting

For this purpose, confirmed constructs were extracted and transformed to *E. coli BL21* strain competent cell on LB medium containing chloramphenicol. One milliliter of the culture was assigned as the zero sample, then the rest of it was induced with the nisin (5 ng/ml) for 3 and 5 hours. Samples were lysed and evaluated for the protein marker on 10% polyacrylamide gels and then transferred onto nitrocellulose membrane and examined using specific antibodies [10].

Results

After enzymatic digestion, purified plasmids were cloned in *pNZ8148* vector. In order to verify its accuracy, verification tests including PCR with specific and universal primers caused amplification of pieces with length 1578bp and 1778bp for *simA* gene and 748bp and 948bp for *cpsD* gene (Figures1&2).

For expression of recombinant proteins, gene construct was induced by the addition of nisin, and the results of SDS-PAGE and Western blotting at 0, 3 and 5 hours after induction showed that the recombinant proteins, *SimA* and *CpsD*, were expressed with approximated sizes of 57.5 and 27 kDa, respectively (Figures 3, 4, 5, 6).

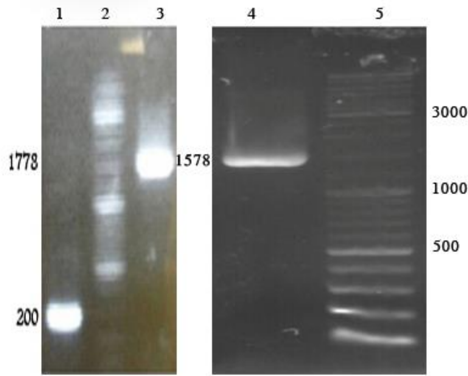

FIG. 1. Confirmation of the presence of *simA* gene in *pNZ8148* vector by PCR on 1.5% Agarose gel. Negative control (1). 100 bp DNA Ladder (2,5). The PCR product of the recombinant vector by using universal primer of *pNZ8148* (3). The *simA* gene PCR product of the recombinant plasmid (4)

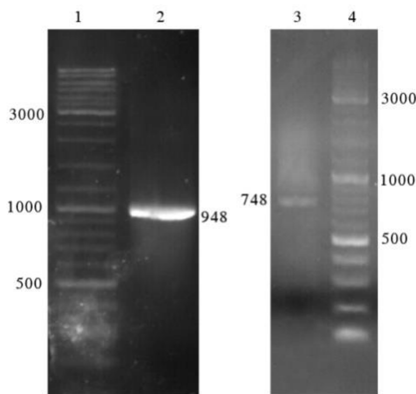

FIG. 2. Confirmation of the presence of *cpsD* genes in *pNZ8148* vector by PCR on 1.5% Agarros gel. 100 bp DNA Ladder(1,4). The PCR product of the recombinant vector by universal primer of *pNZ8148* (2). The *cpsD* gene PCR product using specific primer (3)

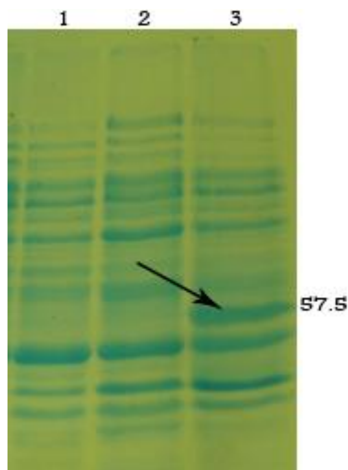

FIG. 3. Comparison of lysates on 10%SDS PAGE for *SimA* protein
Before induction (1). 3 h after induction (2). 5 h after induction by nisin (3)

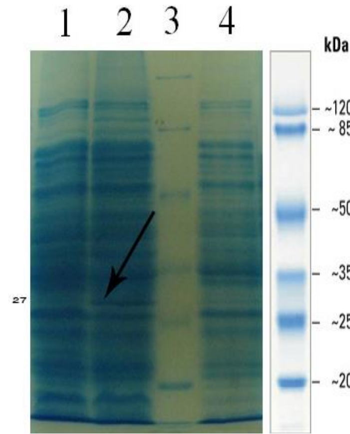

FIG. 4. Comparison of lysates on 12%SDS PAGE for *CpsD* protein
BL21 Cells (1). 5 h after induction (2).Stained Protein Marker (3). 3 h after induction by nisin (4)

FIG. 5. Western blotting of SimA Protein expression, using specific antibodies
5 h after induction by nisin (1).Vivantis prestained protein marker (2)

FIG. 6. Western blotting CpsD Protein expression, using specific antibodies
5 h after induction by nisin (1).Vivantis prestained protein marker (2)

Discussion

Bacterial pathogens are important causes of infectious diseases and mortality in wild and farmed fishes reared in confined conditions. The use of antibiotics to control fish diseases has faced limited success and has the potential danger of antibiotic resistance development in aquatic bacteria. As aquaculture is one of the fastest-growing food production industries in the world, the demand for sustainable ways combating fish disease is gaining significance. There is tremendous scope for the development of new vaccines and therapeutic drugs against bacterial fish pathogens [11].
Streptococcosis disease caused by *S. iniae*, provokes significant economic losses in fish farms in several countries, including farmed trout in Iran [12]. In this study two surface antigens of these bacteria which are effective in its pathogenesis were chosen. *simA* and *cpsD* genes were cloned in *pNZ8148* expression vector.

This vector was designed for gene transfer to *Lactococcus lactis* genome. In the next step, the resultant recombinant bacteria would be applied as a vaccine for fish and relief for human infections caused by *S. iniae* which have been neglected.

pNZ8148 vector was used due to better expression membrane proteins, secretion of proteins into the medium, over-expression of heterologous and homologous genes for functional studies, as well as to obtain large quantities of specific gene products. In addition, it could be used for controlling the production of toxic proteins in metabolic engineering and soluble protein expression [13]. In a study by *H.Wisselink Wisselink* et al. the mannitol 1-phosphatase gene of *Eimeria tenella* and the mannitol 1-phosphate dehydrogenase gene mtlD of *Lactobacillus plantarum* were cloned in the nisin-dependent *L. lactis* NICE overexpression system in order to induce high production of mannitol by *Lactococcus lactis*,and reached the aforementioned conclusion [14].

Lots of researches have been done to control *Streptococcosis* disease. *Candice M. Millard* and colleagues in their research reached the conclusion that capsular biosynthesis in *S. iniae* is under the control of a 21kb operon which includes about 20 genes. Five genes (*cpsE*, *cpsD*, *cpsG*, *cpsH* and *cpsY*) of cps operon are highly variable and there is a direct relationship between changes in cps genes and failure in vaccination. Surprisingly, no capsule was formed in some of our isolates, yet the pathogen was still able to infect the host, albeit with a completely different pathology. The results show that polyvalent vaccines consisting of a variety of cps sequences are somewhat effective. Mutations in the capsular operon coincided with infection in previously vaccinated stocks [15].

Due to the diversity of this gene among capsule synthesis genes in *S. iniae*, it was chosen as the antigen in this study.

A potential target for serologically cross-protective vaccines is the M-like protein SiMA, an essential virulence factor in *S. iniae* that is highly conserved amongst virulent strains [16].

High conservation, surface location and key role in virulence has made SiMA a potential target for serologically cross-protective vaccines against *S. iniae* in fish[9] and it is a good candidate for aquaculture, which in this study was the second antigen.

The first confirmed streptococcal infection in cultured fishes in Iran was reported in 2002 in rainbow trouts. Since then the infection has spread rapidly throughout the country causing considerable mortality in rainbow trout stocks along significant economic losses. It also should be considered that *S. iniae* emerged as a threat to public health due to its zoonotic agent, being isolated from humans infected due to injuries during handling of fresh fish. Despite the fact that there are many reports indicating occurrence of the disease in Iran and other countries, *Streptococcosis* is less studied in ornamental fish [17].

Attempts to identify more factors of this pathogenic bacterium in order to find therapeutic targets and vaccine developments would continue. Some problems in this search include the diversity of strains and serotypes of *S. iniae* and the fact that different environments may have a stronger impact on virulence than what was previously thought to be.

Vaccination covering a number of isotypes is one of the most practical ways to prevent the losses due to this zoonotic bacterial disease in aquaculture industry. Previous work has shown that it was possible to isolate the bacterium from different parts of Iran leading to production of a local commercial vaccine inside the country. However, because of the existence of heterogeneous strains of *S. iniae*, there is a lot of genetic diversity. Vaccines that have been manufactured for this purpose were single valence and inactive, which are faced with limited success [18].

This study opens doors for future research which would considerably increase our knowledge of bacteria and treatment options for fish pathogens.

Conclusion

In this study, two key genes of *Streptococcus iniae*, *simA* and *cpsD*, were cloned in the standardized aquatic vector, called *pNZ8148*. Then their expression in *E. coli* was evaluated using SDS-PAGE electrophoresis and Western blotting.

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