A survey on achieving recombinant DNA vaccine (live delivery) against Streptococcusis in rainbow trout (*Oncorhynchus mykiss*) immunization

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Abstract

The main purpose of this study was to survey on achieving recombinant DNA vaccine (live delivery) against Streptococcusis for rainbow trout (*Oncorhynchus mykiss*) Immunization. Initially, a total of 515 samples were collected from the head kidney of diseased fish (weighing 50-200g) in 72 farms in 8 provinces. Approximately, 40% (206 samples) of specimens were infected with *Streptococcus* species. Then isolated 172 DNA samples were and consequently, five pathogenic species have been identified, including *Streptococcus iniae*, *Streptococcus faecium*, *Streptococcus agalactiae*, *Streptococcus dysgalatiae*, and *Streptococcus uberis*. The enzyme phosphoglucomutase (PGM) has recently been discovered to play an important role in polysaccharide capsule production and virulence in *S. iniae*. Therefore, was initially isolated *S. iniae* and cloned the phosphoglucomutase gene. Then, the PGM gene was amplified successfully and cloned in pTZ57R cloning vector. The recombinant plasmid was subcloned into the pETD uet-l expression vector by restriction enzymes and confirmed by PCR. Meanwhile, for amplifying *simA* and *cpsD* genes were used universal primers *pNZ814*8 and special for *simA* and *cpsD* genes. The recombinant bacteria *Lactococcus lactis* (NZ9000) was used to transform the plasmid into *L. lactis*. Vaccination was performed by bath and injection (peritoneal) methods. The efficiency of g2 was better than g1in these two methods and in all of the groups. The detection of anti *S. iniae* antibody and determination of IgM level was carried out by using ELISA. The results revealed that there was a significant (p<0.05) difference between the level of IgM in both two methods and experiment groups compared to the control group. The results of the challenge of vaccinated fish with *S. iniae* showed that fish RPS in all groups were more than 50 percent while in the control group was 21.43 percent. The highest fish RPS belonged to group 5(61.25 percent) and statistical analyses revealed that significant (p<0.05) difference between fish vaccinated RPS, compare to the control group.

Keywords: Recombinant DNA vaccine, Rainbow trout, Streptococcusis, Phosphoglucomutase

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Introduction

Streptococcus has been one of the most important bacterial diseases in rainbow trout farms in Iran for the past few years. The most important types of streptococcus can be mentioned (Buller, 2004; Shewmaker *et al*., 2007 , Garima et al., 2024; Romalde *et al*., 2008): *S. iniae*, *S. agalactiae*, *Streptococcus parauberis*, *S. dysgalactiae S. faecium*, *Streptococcus milleri*, *S. uberis*, *Streptococcus ictaluri*, *Streptococcus phocae*, *Streptococcus faecalis. Lactococcus piscium*, *L. garvieae*, *Vagococcus salmoninarum*, According to other researchers, *S.iniae* is the main cause of *Streptococcus* in farmed and wild fish in the world and the disease has been reported in the aquaculture industry in freshwater and saltwater fish (Klesius *et al*., 2006; Russo *et al*., 2006; Shoemaker *et al*., 2006; Yang and Li., 2009, Yee *et al*., 2024, Xiuzhen *et al*., 2023). The first pathogenic molecule in the capsule structure is the phosphoglucomu, tase gene, which converts glucose-6-phosphate and glucose-1-phosphate by producing the enzyme phosphoglucomutase, which plays a major role in the glucose structures of the capsule. Research has shown that loss of the pgm gene(phosphoglucomutase) in *S. iniae* causes changes in phenotypic characteristics, morphological changes in the cell wall, capsule production, and degradation of the innate immune defense of fish (Buchanan, 2023), (Nan *et al.*, 2021). The aim was to identify and express the *S. iniae* pathogenic gene in *L.lactis* and to investigate the possibility of producing a recombinant live vaccine against Streptococcusis in rainbow trout.

Material and methods

Material

Restrictive enzyme NcoI, KpnI, SacI , XbaI and related buffers (Litvani fermentase and takara), chloramphenicol antibiotic with a concentration of 30 g/mL, ampicillin antibiotic with a concentration of 100 mg/ml, T_4DNA ligase enzyme and related buffer, dNTP Mix (Fermentase) (100 mM), Taq DNA polymerase enzyme (with a concentration of 5 units/microliter) and related buffer (manufactured by Sinagen Company), PEG (Poly Ethylene Glycol), *E. coli* TOP10 and BL21 strains (Sinagen), plasmid pNZ8148 (MoBiTec), gel extraction kit (BioNeer), gel extraction kit (Fermentas), plasmid extraction kit (BioNeer), Blue bromophenol (Merck), EDTA (Ethylene diamine tetraacetic acid) (Fermentase), protein marker (Vivantis), protein marker (Fermentas).

Sampling

A sampling of sick and dying fish as well as seemingly healthy fish from 27 breeding farms in 8 provinces of Mazandaran, Gilan, Kermanshah, Lorestan, Chaharmahal Bakhtiari, Kohgiluyeh and Boyer Ahmad, Tehran and Fars provinces in the group of 50 - 200 g and was done during the summer. Primary culture of bacteria at the site of sampling, purification, and initial and complementary identification of bacteria (Astin and Astin, 2007-1999).

Molecular experiments

Molecular experiments were performed after some modification according to a study by Hanahan (1983), Smith (1984), Sambrook and Russell (2006), and Millard *et al.* (2012):

- DNA extraction was performed by phenol-chloroform method.
- Determining the quantity and quality of extracted DNA: Spectrophotometry and electrophoresis are used to determine the quantity and quality of extracted DNA and to determine the concentration and purity of extracted DNA.

Primer designing

Specific primers of each species are required to perform the PCR reaction. Sequences of 16.S.RNA and glucokinase genes of different strains of *Streptococcus* were used to design the primer. For this purpose, 5 pairs of primers were designed and made. Since the design of some primers was completely specific to each species, enzymatic digestion with restrictive enzymes was used (Table 1).

Table 1: Length of PCR product fragments using each of the primers.

Primer	Fragments length (base pair)					
	S. parauberis	***S. faecium***	***S. uberis***	***S. agalactiae***	***S. dysgalactiae***	***S. iniae***
Bac RNA	675	675	675	-	675	-
ENR	540	540	-	-	-	-
STRA	-	-	-	430	-	-
STRP	-	-	260	-	260	-
STRP1	-	-	-	-	-	554

PCR reaction

In this study, PCR reaction was performed using specific primers based on the sequence of ions 18s ribosomal RNA and Glocus kinase. Quantity and quality of PCR product were observed and evaluated using DNA 50 marker pb, 6% polyacrylamide gel electrophoresis and silver nitrate staining. Electrophoresis of PCR product, using 8% polyacrylamide gel: The PCR product was evaluated by 2% agarose gel electrophoresis and the size of the PCR fragments was compared with DNA marker (pBR322 DNA / AluI Marker, 20, MBI Fermentas) on 8% polyacrylamide gel and nitrate staining. Silver was obtained. Since the sensitivity of polyacrylamide gel is 200 to 300 times that of agarose gel. In the present study, this gel was used to evaluate the PCR results.

Isolation of gene causing streptococcusis and cloning

After identifying different strains of *Streptococcus*, *S. iniae* was used to isolate the phosphoglucomutase gene as a *Streptococcus* agent. After the PCR reaction confirmed that the isolated bacteria were *S.iniae*, another PCR was performed to amplify the *Streptococcus* gene. For this purpose, the desired gene sequence was identified using databases

and then a primer was designed. Thus, two pairs of primers were constructed to amplify the gene fragments encoding simA and cpsD proteins. To be surer of the test results and to avoid wasting time, simultaneous experiments were performed with two gene fragments of different sizes called g1 (simA) and g2 (cpsD).

Extraction and purification of SimA and CpsD fragments

After PCR, the amplified fragments, including Dimer primer and non-specific bands, were purified.

Cloning

The T. vector used in this study was PTZ57R, which was prepared by Fermentase Company.

To perform the ligation reaction, in addition to the T. vector, an insert is needed, which is the same as the purified PCR product. In this reaction, the PCR product is attached to the T. vector by the enzyme T4 DNA Ligase.

Transformation

Preparation of DNA- competent cells: A colony of the *E. coli* bacterium was cultured in 1 mL of LB liquid culture medium and incubated overnight in 37°C sheikering incubator (Hanahan, 1983). Stages of DNA transfer to living bacterial cells (Smith, 1984).

Plasmid extraction: Plasmid extraction using BioNeer kit Night cultures were prepared from the screened colonies.

Preparation of vector and gene fragment encoding simA and cpsD proteins

At this stage, the restriction enzymes are used to prepare the desired gene. According to the plasmid map of pGH and the amplified sequences of the simA and cpsD genes, there are sites in the amplified region restricted by restriction enzymes NcoI and KpnI, SacI and XbaI. The following materials have been used for enzymatic reactions (Tables 2 and 3).

Table 2: Enzymatic reaction to restrict pGH carrying simA and pNZ8148 gene.

Quantity	Materials
1µL (5 units)	(1) *NcoI* or (2) *KpnI*
2µL (1X)	Tango Buffe 10X
5 µg (1 µg)	*pNZ8148*, *pGH*
12µL	ddH_2O

Table 3: Enzymatic reaction to restrict pGH carrying cpsD and pNZ8148 gene.

Quantity	Materials
1µL (5 units)	(1) XbaI , (2) SacI
µL (1X) 20020 + 2µL (1X)	BSA+M Buffer 10X (XbaI) L Buffer 10X (SacI)
3µg (1 µg) Bring the volume to 20 µL	pNZ8148, pGH ddH2O

DNA recycling from agarose gel using BioNeer kit

Perform binding reaction

At this stage, plasmid pNZ8148 was used as a vector and the extracted gene fragments simA and cpsD were used as an insert.

Screening of colonies with recombinant plasmids

After transformation, the ligation product is spread on agar plates containing chloramphenicol and finally, several clones are observed on the plate. Monoclonals are transferred to LB

medium for overnight culture and then plasmid extraction is performed by alkaline lysis method

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Colony PCR

Approved colonies were selected from the sectioned plate and the plasmid was extracted after 24 hours.

Polymerase chine reaction (PCR) of simA and cpsD genes to confirm cloning

In this study, PCR was used to confirm the binding and presentation of the recombinant plasmid. In this step, to amplify the simA and cpsD genes, the universal primers pNZ8148 and specific for the simA and cpsD genes were used.

Transfer of plasmid to L. lactis

- Engineered *L. lactis* (NZ9000) was used to perform this reaction.
- Preparation of *Lactococcus* receptor cell
- Transfer stage; after the preparation of *Lactococcus* receptor cells, transfer was performed.
- Westren blotting polyacrylamide gel (Shewry and Fido, 1998).

Vaccination

Immersion method

One gene (g2) and two doses of $CfumL^{-1}$ 10^6 and $CfumL^{-1}$ 10^7 (g2d1 and g2d2) were used

In this method, one gene and two doses (2 treatments) and each treatment with three replications along with the control group were used for a total of 270 fish in 9 tanks, of which a total of 180 were vaccinated. The fish were immersed in the vaccine for 30 seconds for vaccination.

Injection method (intraperitoneal)

0.1 mL of two genes (g1, g2) and two doses of CfumL-1 107 and CfumL-1 108 (g1d1, g1d2 and g2d1, g2d2) were injected into the fish peritoneum.

In this method, two genes and two doses (4 treatments) and three replicate each with the control group, a total of 450 fish in 15 tanks were used, of which a total of 360 were vaccinated.

Blood sampling to measure IgM

Immersion method

In this method, blood sampling was performed in three stages and each at a distance of ten days. A total of 71 fish were collected during three stages.

Injection method (intraperitoneal)

Blood sampling was performed in three stages and each at a distance of ten days.

A total of 165 fish were collected during three stages.

Measurement of IgM

After fish vaccination, the ELISA method was used to detect antibodies against *S. iniae* and IgM levels.

Bacteria Challenge

Bacteria isolated from the kidney of all fish suspected of streptococcus were identified by microbial culture and biochemical tests (Buller, 2004; Austin and Austin, 2016). Then, in the molecular genetics' laboratory of the Caspian Sea Ecology Research center, the final diagnosis was confirmed using PCR reaction and specific primers designed from the srRNA16 gene. After preparing a dilution of 0.1 from the logarithm of 6 bacteria of *S. iniae*, 0.1 mL was injected intraperitoneally into 260 fish (12 treatments and a control group).

These fish were monitored for a maximum of 14 days (Rodas *et al*., 2002). After the onset of symptoms, from various organs such as liver, kidney and heart, sampling and culture in specific media (such as Blood agar and broth Hipporate) using Austin and Austin's method 2016 are also performed and the presence or absence of suspected colonies *S. iniae* was evaluated and the number of deaths was recorded in different treatments and the Relative Survival Percentage (RPS) was determined using the following formula (Ellis, 1988):

$$RPS = 1 - \left[\frac{\text{percent mortality in treatd group}}{\text{precent mortality in control group}}\right] \times 100$$

Statistical analysis

Data and descriptive statistics were analyzed and processed by the SPSS program. Excel 2007 software was used to draw the charts. One-way ANOVA test was used to determine the significance of the mean of variables and the Duncan test was used for grouping. The presence or absence of significant differences was determined with a 95% confidence interval and a P-value in the range of 0.05.

Results

After electrophoresis of the PCR product and enzymatic digestion of some samples, the molecular weight of DNA fragments was measured and species were identified based on it (Fig. 1).

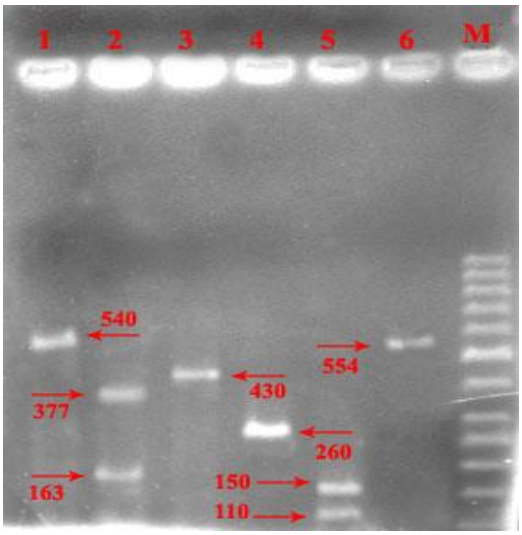

Figure 1: Band pattern of PCR product of different species of Streptococcus on agarose gel. (1) *S. faecium* (2): *S. parauberis,* (3) *S. agalactiae,* (4) *S. uberis,* (5) *S. dysgalactiae,* (6) *S. iniae,* (M) *Marker.*

Making of simA and cpsD sequences separately in pGH plasmid

As mentioned, the gene sequences encoding simA and cpsD proteins were made by Nedaye Fan in pGH vector.

Transformation of synthetic pGH plasmid containing simA and cpsD coding gene into TOP10 bacteria

To increase the number of recombinant plasmids containing the simA and cpsD protein-coding fragment, the recombinant plasmids were transferred into *E. coli* TOP10 strain cells using the mentioned method. After the incubation period, they were cultured in an ampicillin-containing medium, and after 16 hours of incubation at 37°C, the presence of colonies containing recombinant plasmid, which also has the ampicillin resistance gene, indicates the presence of and the accuracy of plasmid transformation in TOP10 cells (Fig. 2).

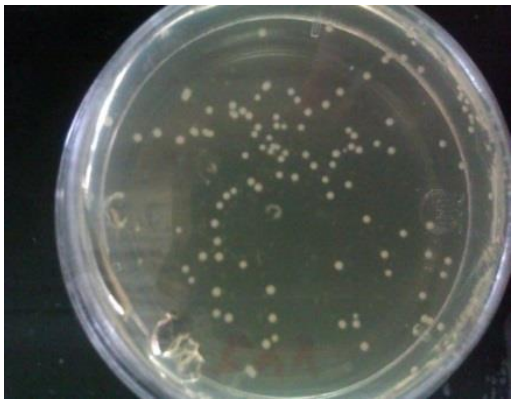

Figure 2: Growth of colonies containing recombinant plasmids.

Extraction of pNZ8148 and pGH plasmids containing simA and cpsD protein-coding fragments

Plasmid pNZ8148 and plasmids containing simA and cpsD gene fragments have 3208, 4495 and 3665 bp, respectively (Fig. 3).

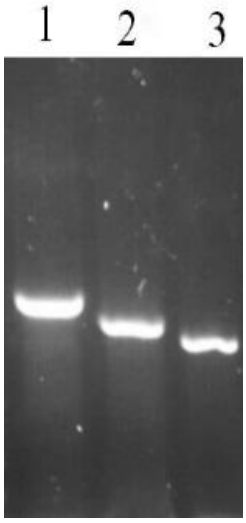

Figure 3: Extraction of plasmid pNZ8148 and pGH containing insert fragment from TOP10 cells. 1) The plasmid containing simA; 2) Plasmid containing cpsD; 3) Plasmid pNZ8148.

Extraction of genes using enzymatic digestion

The 1578 bp fragments belong to the simA gene and the 748 bp fragment to the cpsD gene. According to the results, the presence of 1578 and 748 bp gene fragments in the primary and synthetic pGH plasmid was confirmed (Figs. 4 and 5).

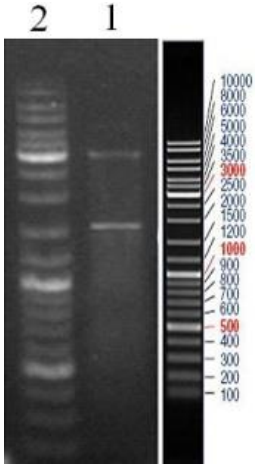

Figure 4: Sim A fragment restricted by NcoI and KpnI restrictive enzymes. 1) Restricted simA fragment; 2) 100 bp fermentase marker.

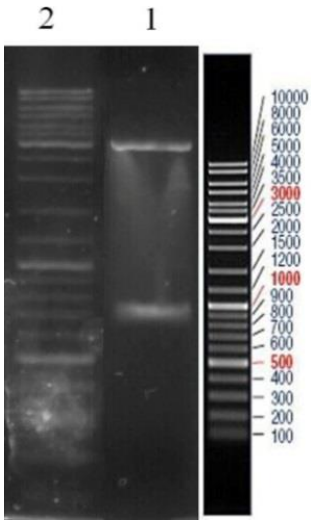

Figure 5: cpsD fragment restricted by SacI and XbaI restrictive enzymes. 1) Restricted cpsD fragment; 2) 100 bp fermentate marker.

Extraction of insert fragment from agarose gel

After removing the inserted enzyme piece on 1.5% agarose gel and extracting it, the resulting band was electrophoresed on 1.5% agarose gel to confirm the extraction (Fig. 6).

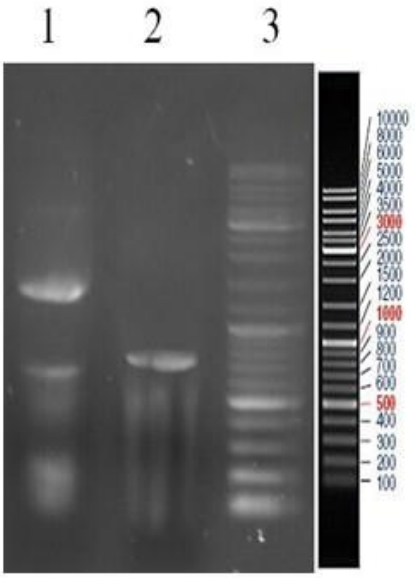

Figure 6: Band for insert fragment extracted from agarose gel. 1) Fragment simA; 2) Fragments cpsDc; 3) fermentate marker bp 100.

Subclones of simA and cpsD genes in pNZ8148 expression vector

Confirmation of plasmids containing the desired fragments

The PCR fragment in the plasmid carrying the simA and cpsD gene fragment should have 1778 and 948 bp, respectively. After PCR and electrophoresis of the products on 1.5% gel, the presence of the simA and cpsD gene fragments in the plasmids was confirmed (Figs. 7 and 8).

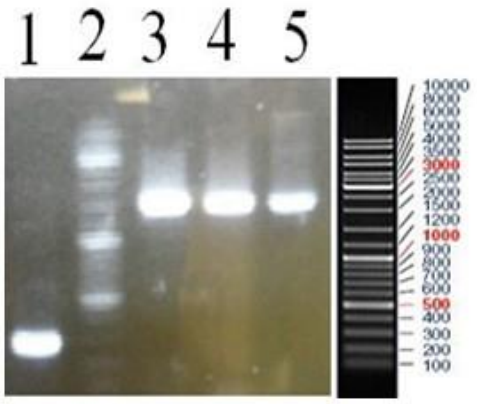

Figure 7: Confirmation of the presence of simA protein-encoding gene in pNZ8148 vector using universal primer: 1) Negative control (non-recombinant plasmid); 2) marker (3-5) Amplified simA gene.

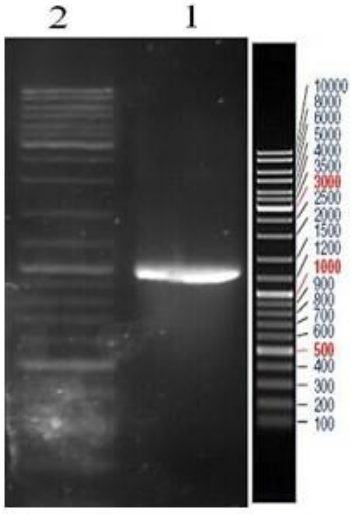

Figure 8: Confirmation of the presence of cpsD protein-encoding gene in vector pNZ8148 using universal primer: 1) cpsD gene amplified; 2) 100 bp marker.

Results of confirmation of the presence of the fragment by specific primers of simA and cpsD genes

Plasmid pNZ8148 showed no band in the PCR reaction, but in the presence of simA and cpsD gene fragments, 1578 and 748 bp bands were observed on 1.5% gel, respectively (Figs. 9 and 10).

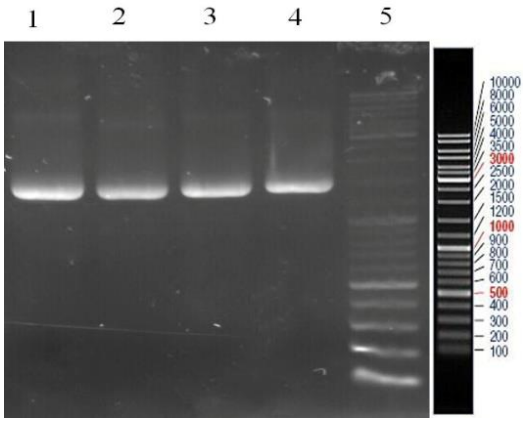

Figure 9: Confirmation of the presence of simA protein-encoding gene in vector pNZ8148 using specific primer: 1-4) simA gena amplified; 5) 100bp marker.

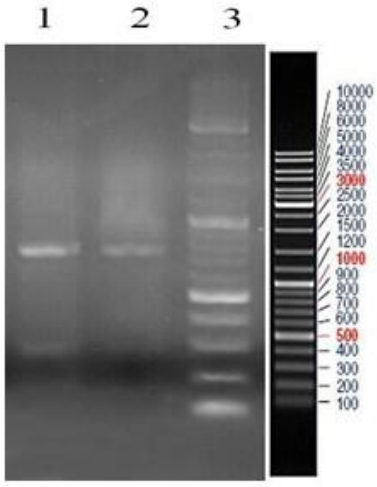

Figure 10: Confirmation of the presence of cpsD protein-encoding gene in vector pNZ8148 using specific primer: 1-2) amplified cpsD gene; 3) 100 bp fermentase marker.

Vaccination

Immersion method

Only the g2 gene was used in this method. The level of IgM in g2d1 (1.298±0.044) in the third stage is slightly higher than g2d2 (1.215±0.018) and its difference with the control is significant (p<0.05) (Table 4).

Table 4: Comparison of IgM levels in d1, d2 and control in the gene (g2) by immersion method.

	The first stage	The second stage	The third stage
g2d1	0.300±0.002	0.520±0.015	1.298±0.044
g2d2	0.276±0.012	0.483±0.010	1.215±0.018
C	0.258±0.013	0.365±0.008	0.446±0.007

Injection method (intraperitoneal)

In this method, in the g1 gene), the amount of IgM in g1d1 (1.291±0.065) in the third stage is more than g1d2 (1.243±0.082) and in comparison, with the control has a significant difference (p<0.05) (Table 5). In the gene (g2), the level of IgM in g2d1 (1.342±0.050) in the third stage was more than g2d2 (1.223±0.055) and compared with the control had a significant difference (p<0.05) (Table 6)

Exposed to bacteria

The results of exposure of fish vaccinated with *S. iniae* showed that out of 20 fish studied for each treatment, the relative survival rate of fish in all

treatments was over 50% and for the control group was 21.43±4.25 (Table 7). The highest percentage of relative survival of fish was related to treatment 5 (61.25±3.25) and statically analysis showed a significant difference between the percentage of relative survival of vaccinated fish with the control group (p<0.05).

Table 5: Comparison of IgM levels in d1, d2 and control in the gene (g1) by injection.

	The first stage	The second stage	The third stage
g1d1	0.238±0.031	0.580±0.012	1.291±0.065
g1d2	0.238±0.029	0.500±0.014	1.243±0.082
c	0.227±0.008	0.365±0.006	0.445±0.006

Table 6: Comparison of IgM levels in d1, d2 and control in the gene (g2) by injection.

	The first stage	The second stage	The third stage
g2d1	0.245±0.021	0.579±0.004	1.342±0.050
g2d2	0.246±0.004	0.512±0.005	1.223±0.055
c	0.245±0.008	0.365±0.006	0.445±0.006

Table 7: Mean relative survival percentage of vaccinated fish in different treatments and control groups after exposure to *S. iniae.*

MRSP (Mean ±SD)	Normal fish	number of deaths	Treatments number
22.80±7.54	11	9	1
55.00±7.51	10	10	2
42.80±7.54	11	9	3
12.40±7.54	11	9	4
50.25±3.61	12	8	5
75.30∘5± 5.51	10	10	6
25.43 ±4.21.	4	16	7

(1-2) Immersion method, (3-6) injection method, (7) controls; (MRSP) Mean relative survival percentage

Discussion

In this study, the results showed that the relative survival rate in all treatments was above 50% and also in both immersion and injection methods and all treatments, the efficiency of the vaccine resulting from the gene (g2) was better compared to gene (gl).

In this study, the pgm gene was amplified by PCR by using primers designed in the NCBI site gene bank with accession no: AY846302 from *S. inia* used by Akshaya *et al.* (2023), Huiliang *et al., 2024* with the difference that at the end of 5" primer, the restrictive BamH I and EcoR I enzymes was installed and in this study the T/A cloning steps were performed in Ptz57R vector. The PTZ57R has 2886 bp, with its cloning site (MCS) embedded in the lacz gene, making it easy to identify colonies containing the recombinant plasmid in blue and white. Also, this vector has a coding region of 654 bp for BamHI enzyme and 615 bp for EcoRI enzyme.

In this study, for cloning accuracy, in addition to PCR, the plasmid enzymatic digestion method with the above enzymes was used and by plasmid enzyme cleavage of Ptz57R/pgm a fragment of 1716 bp and a band of about 2800 bp of plasmid were observed. Finally, the desired fragment was

subcloned into pETDuet-1 vector and confirmed by PCR reaction. The pETDuet-1 is an expression vector that has 5420 bp and has two cloning sites (MCSI, MCS2) and can express two genes at the same time. The present study was similar to the study by Akshaya *et al.* (2023).

They generated the pgm gene using Forward 5-GAACTAGCTAGTTACTTTTGTAACTG-3 and Reverse 5-CTAATTCACAAAAGTGTTGATTTCAG-3 primers embedded in the primers of Bam HI and XbaI in the 5-primer primer. Cloning was performed on PCR2.1Topo vector and T/A cloning was confirmed by PCR reaction with specific pgm primers as well as enzymatic digestion reaction with two-fragments enzymes (Xbal, BamH1) and finally, pgm genes were subcloned into Pdc123 (7) shuttle expression vector and confirmed by PCR and enzymatic digestion.

Akshaya *et al.* (2023) showed that pgm mutants are initially distributed in the blood, brain and spleen, but are eliminated within 24 hours without harm to any organism, and 90 to 100% of fish that pgm mutants, were injected, survived by exposure to wild-type bacteria, and were shown to be able to stimulate the immune defense response and possibly as a recombinant vaccine, a good candidate for aquatic animals, confirming the purpose of this study.

Many studies have been performed with the NICE system, including the research of Wisselink *et al.* (2005), Naderi *et al.* (2020) for high production of mannitol by *L. lactis*; *Eimeria tenella* mannitol-1-phosphatase gene and *Lactobacillus plantarum* mannitol-1-phosphate dehydrogenase gene were cloned into the NICE expression system in *L.lactis.*

Much research has been done on streptococcus genes, including the following:

Millard *et al.* (2012) concluded in a study that capsule biosynthesis in *S. iniae* under the control of 21 kb operon contains about 20 genes. The five genes (cpsY, cpsD, cpsE, cpsG and cpsH) of the cps operon are highly variable and there is a direct relationship between changes in the cps genes and vaccine failure. Surprisingly, in some isolates, no capsules formed, yet the pathogen was still able to infect the host, however with completely different pathology. The results show that multivalent vaccines consisting of different types of cpsD sequences are somewhat effective. Mutations encoded in the capsule operon are associated with the failure of autogenous vaccines. The reason for selecting the cpsD gene in this study is the diversity of this gene among the genes synthesizing *S. iniae* capsule. Locke *et al.* (2010) investigated the effective potential of non-injectable vaccination using attenuated live vaccines. Three attenuated strains of *S. iniae* with genetic mutations removed by pathogens- polysaccharide capsules ($\Delta cpsD$), M-like protein ($\Delta simA$), and phosphoglucomutase ($\Delta pgmA$), in parallel with an adjuvant, lethal formalin, all *S. iniae* cells were examined. Vaccines $\Delta cpsD$،$\Delta pgmA$ and bacteria, showed a higher level of

vaccine immunity (0% mortality), while $\Delta simA$ despite causing 12 to 16% of vaccination-related deaths, one vaccine candidate for 100% protection in methods, intraperitoneal injection and immersion. Previously, through intraperitoneal (ip) HSB vaccination, it was found that the level of 100% protection against wild-type *S. iniae* with a mutant strain of M-like protein ($\Delta simA$) and there was 90 to 100% protection with phosphoglucomutase by translocation mutation ($\Delta pgmA$). Immersion vaccination has more benefits than injection-based methods, including avoiding fish damage and reducing costs, reducing manipulation and thus reducing stress in fish, and more effectively stimulating the fish's immune response. Through immersion, previous research has shown that $\Delta simA$ mutants have a reduced ability to attack epithelial cells. It can provide complete protection of the immune system against the challenge of the wild type. While the injectable vaccine is accurate and renewable, it is difficult and costly to work with, so it is used in a limited aquaculture program with valuable species such as salmon. Immersion vaccines are more cost-effective, and because of their proximity to natural infection routes used by aquatic pathogens, the outcome of the immune response is potentially stronger (Locke *et al.*, 2010).

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References

Akshaya R., Baskaralingam V., Pasiyappazham R., Sivakamavalli J, 2023. Oral vaccination for sustainable disease prevention in aquaculture-an encapsulation approach 31(2):867-891. DOI: 10.1007/s10499-022-01004-4

Austin, B. and Austin, D.A. 2016, Characteristics of the diseases. In Bacterial Pathogens: Diseases of Farmed and Wild Fish. Springer-Praxis, Praxis Publishing, Ltd. Chichester, cUK. pp 13-15.

Buller, N.B., 2004. Bacteria from Fish and other Aquatic Animals: A Practical Identification Manual, pp. 37-162. CABI Publishing, Cambridge.

Ellis, AE., 1988. General principles of fish vaccination. In: Ellis AE, editor. Fish vaccination. London: Academic Press. pp. 1-19.

Eyngor, M., 2008. Mergency of novel *Streptococcus iniae* exopoly saccharid - producing strains following vaccination with nonproducing strains. *Applied and Environmental Microbiology*, **DOI:** https://doi.org/10.1128/jcm.41.12. 5407-5413.2003. 74(**22**), 6892-689.

Garima S. R., Banikalyan S., 2024. Advancements in Fish Vaccination: Current Innovations and Future Horizons in Aquaculture Health Management. Methods Mol Biol , 14(13), 5672. doi: 10.1007/978-1-0716-1888-2_9.

Hanahan, D., 1983. Studies on transformation on E. coli with plasmids. *J. Mol. Biol*., 503-517. https://doi.org/10.1016/S0022-2836(83)80284-8

Huiliang Wu., Jiyuan Yin., Siming Li., Hao Wang., Peng Jiang ., Pengfei Li., Zhaoyang Ding., Han Yan ., Bo Chen ., Linchuan Wang ., Qing Wang, 2024. Oral immunization with recombinant L. lactis expressing GCRV-II VP4 produces protection against grass carp reovirus infection. Fish & *Shellfish Immunology*, p. 109439. https://doi.org/10.1016/j.fsi.2024.109439

Klesius, P.; Evans, J.; Shoemaker, C.; Yeha, H.; Goodwin, A.E. and Adams, A., 2006. Thompsond, K. Rapid detection and identification of *Streptococcus iniae* using a monoclonal antibody-based indirect fluorescent antibody technique *Aquaculture*, 258, 180– 186. DOI:10.1016/j.aquaculture.2005.06.040

Locke JB, Vicknair MR, Ostland VE, Nizet V, Buchanan JT 2010, Evaluation of *Streptococcus iniae* killed bacterin and live attenuated vaccines in hybrid striped bass through injection and bath immersion. *Diseases of Aquatic Organisms*, 89(**2**), 117. DOI:10.3354/dao02192.

Millard CM, Baiano JC, Chan C, Yuen B, Aviles F, Landos M, 2012, Evolution of the Capsular Operon of Streptococcus iniae in Response to Vaccination. Applied and environmental microbiology. ;78(23): 8219-26. https://doi.org/10.1128/AEM.02216-12.

Naderi-Samani M., Soltani M., Dadar M., Taheri-Mirghaed A., Zargar A., Ahmadivand S., Hassanzadeh R., Laleh Moazami Goudarzi. 2020, Oral immunization of trout fry with recombinant Lactococcus lactis NZ3900 expressing G gene of viral hemorrhagic septicaemia virus (VHSV). Applied and environmental microbiology. 12P. DOI: 10.1016/j.fsi.2020.07.007

Nan Q., Guangsheng D., Xiaofang Zh., Xun S., 2021 Recombinant lactic acid bacteria as promising vectors for mucosal vaccination. Fish Shellfish Immunol. ; 105:62-70. DOI: https://doi.org/10.1002/EXP.20210026

Rodas, B.A., Angulo, J.O., Cruz, J. and Garcia, H.S., 2002, Proparatian of probiotic buttermilk with *Lactobacillus reuteri. Milchwissenschoff Milk Sclence International*, 57, pp. 26-28

Romalde, J.L.; Ravel, C.; Valdes, I; Magarin, B.; Fuente, E.; San Martın, C.; Avendan-Herrera, R. and Toranzo, A.E., 2008. *Streptococcus phocae*, an emerging pathogen for salmonid culture, *Veterinary Microbiology*, 130, 198–207.

Russo, R., Mitchell, H. and Yanong, R.P.E, 2006. Characterization of *Streptococcus iniae* isolated from ornamental cyprinid fishes and development of challenge models, *journal of Aquaculture*, 256, pp. 105-110. https://doi.org/10.1016/j.aquaculture.2006.02.046

Shewmaker, P.L.; Camus, A.C.; Bailiff, T.; Steigerwalt, A.G.; Morey, R.E. and Carvalho, M.G.S., 2007. *Streptococcus ictaluri* sp. nov., isolated from channel catfish *Ictalurus punctatus* broodstock. *International Journal of Systematic and Evolutionary Microbiology*, 57, 1603–1606. DOI:10.1099/ijs.0.64810-0

Shewry, P. and Fido, R., 1998. Protein blotting , principle and applications, pp. 435-444.

Shoemaker, C.A., Vandenberg, G.W., Désormeaux, A., Klesius, P.H. & Evans, J.J. 2006. Efficacy of a *Streptococcus iniae* modified bacterin delivered using Oralject™ technology in Nile tilapia, *Oreochromis niloticus. Aquaculture*, 255, 151–156. https://doi.org/ 10.1016/ j.aquaculture.2005.12.016

Smith, C., 1984. *SDS polyacrylamide gel electorophoresis of proteins, in Methods in molecular biology*,. edited by W. Jm. Saunders Co, New York, pp. 41-45.

Soltani, M., Jamshidi, S. and Sharifpour, I., 2005. Streptococcosis caused by *Streptococcus iniae* in farmed rainbow trout (*Oncorhynchus mykiss*) in Iran: Biochemical characteristics and pathogenesis. *Bulletin of the European Association of Fish Pathologists*, 25, 95-106.

Wisselink HW, Moers AP, Mars AE,

Hoefnagel MH, De Vos WM, Hugenholtz J. 2005. Overproduction of heterologous mannitol 1-phosphatase: a key factor for engineering mannitol production by Lactococcus lactis. *Applied and environmental microbiology*, 71(**3**), 1507-14. doi: 10.1128 /AEM. 71.3.1507 –1514.2005

Yang, W. and Li, A., 2009. Isolation and characterization of *Streptococcus dysgalactiae* from diseased *Acipenser schrenckii*, *Aquaculture*, 294, 14–17. https://doi.org/10.1016/j.aquaculture.2009.05.018

Yee W., Megat H., Megat M., Khairb, Adelene Ai Lian Songbf, Mas Jaffri Masarudin c g, Jiun Yan Loh d, Chou Min Chonge, John Beardallh, Michelle Yee Mun Teo, Lionel Lian Aun . 2024, Recombinant lactococcal-based oral vaccine for protection against Streptococcus agalactiae infections in tilapia (Oreochromis niloticus) Fish & Shellfish Immunology, Volume 149, June 2024, 109572. https://doi.org/10.1016/j.fsi.2024.109572

Xiuzhen Sh., ORCID, Honghua Zh., Min L., Xiaoqian T., Jing X.,Heng C. and Wenbin Zh .2023, Development and Evaluation of Recombinant B-Cell Multi-Epitopes of PDHA1 and GAPDH as Subunit Vaccines against Streptococcus iniae Infection in Flounder (Paralichthys olivaceus). Vaccines 2023, 11(3), 624; https://doi.org/10.3390/vaccines11030624