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Full Article

Experimental vaccine against lactococcosis in cultured rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Lactococcus garvieae is the etiological agent of lactococcosis, an emerging disease which affects several fish species and causes important economic losses both in marine and freshwater aquaculture. Lactococcosis usually happens when water temperature increases over 15°C during the year. Normally, it causes a hyperacute and haemorrhagic septicemia in fish. This paper presents a procedure for producing experimental vaccine for rainbow trout (*Oncorhynchus mykiss*) lactococcosis including aspects such as pathogen characterization, pathogenicity, mass cultivation, safety, potency and field trial tests for immersion use. In the potency test, after challenging the vaccinated fish with live pathogenic bacteria (1×10^7 bacteria per milliliter of immersing solution) and observing for 72 hours thereafter, 10% of fish died while the control group showed 60% mortality within the observation time. In the field trial from vaccination time onward till marketing of the fish, those mortalities that occurred in groups of vaccinated and non-vaccinated fish were recorded. Total death occurred in the vaccinated group was 11%, while in non vaccinated group this number was approaching 23%. This observation indicates a 50% reduction in mortality in the vaccinated group. This is the first report on experimental vaccine against lactococcosis in fish that is produced and tested in Iran.

Keywords: Experimental vaccine, Lactococcosis, Rainbow trout

INTRODUCTION

During last decades there has been a continuous growth of aquaculture industries all over the world and such intensive production would experience disease problems. Infectious diseases that occur as sporadic events in wild fish populations may cause high mortalities when appearing in intensive fish farming (Gudding *et al* 1999). Lactococcosis is a bacterial disease caused by *Lactococcus garvieae* that is a Gram-positive, non-motile, ovoid cocci, occurs in pairs and short chains, produces a-haemolytic colonies on blood agar, and is oxidase and catalase negative, non-acid fast and non-sporulating (Ravelo *et al* 2001). Lactococcosis occurs in freshwater as well as seawater and has been a source of major economic losses since the early seventies for rainbow trout (*Oncorhynchus mykiss*) and marine fish industries in Asia (rainbow trout and yellowtail, *Seriola quingueradiata*) as well as in the United State of America and South Africa (Vendrell *et al* 2006). In a study in 2002 in Iran from 235 cases of streptococcosis among rainbow trout

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collected from farms of Fars province, streptococci were isolated from 199 cases. These isolates were biochemically allocated to *Streptococcus iniae* and *Lactococcus garvieae* (Akhlaghi & Keshavarzi 2002). Also *L. garvieae* outbreaks in farmed rainbow trout are described from different parts of Iran (Soltani *et al* 2008). Losses due to lactococcal septicemias in cultured fish, the expense involved in using antimicrobial compounds and the reported increasing drug resistance of the causative Gram-positive cocci (Kav & Erganis 2008) pointed out the need for developing immunoprophylactic measures to prevent these infections (Ravelo *et al* 2005). Although some chemotherapeutic agents have activity against *L. garvieae*, the improper use of antibacterials can cause antibiotic resistance, legal restrictions and difficulties due to anorexia. Therefore, a safe and efficacious vaccine against lactococcosis in rainbow trout is needed (Vendrell *et al* 2007).

Several attempts have been carried out in laboratory and field trials by anesthetizing fish and inoculating them intraperitoneally. Rainbow trout weighing 35 g was immunized with a killed vaccine and a control group was injected with PBS intraperitoneally. On day 29 post-vaccination, both groups were challenged by intraperitoneal injection with 0.1 ml of a virulent heterologous strain of *L. garvieae* at 3×10^6 cfu/ ml and fish were observed for a further 21 days. At the end of the experiment, the survivals of the vaccinated fish and control group were 94 and 4%, respectively (Vendrell *et al* 2007). Data on immersion vaccination in fish is scarce; Liaghat *et al* (2011) demonstrated that healthy non-exposed fish responded well to *Streptococcus iniae* bacterin used by the immersion route. In this study attempts were made to produce formalin killed lactococcosis experimental vaccine using immersion route that is a feasible vaccination route in fish farms for Iran and evaluate its efficiency in cultured rainbow trout.

MATERIALS AND METHODS

Bacteria. *Lactococcus garvieae* was isolated from diseased fish showing signs of lactococcosis which were collected from rainbow trout farms in Fars province. The disease is characterized by uni- or bilateral exophthalmia with haemorrhages in the periocular area, in the opercula, in the buccal area, at the base of the fins and on the surface, darkening of the skin and distended abdomen (Figure 1). To isolate the bacteria, specimens from internal organs of diseased fish were cultured using blood agar by streaking method and incubation at 30 °C overnight aerobically. On the next day colonies showing characteristics of streptococci were selected for further studies such as Gram staining, biochemical testing, and molecular characterization (Ravelo *et al* 2001, Austin & Austin 2007).

Figure 1. Rainbow trout suffering from lactococcosis (skin blackening and exophthalmia are evident).

PCR assay. Genomic from DNAs *L. garvieae* were extracted as described by Holmes and Quiqley (1981). The PCR assay used was previously developed for definitive identification of *L. garvieae* based on the 16S rDNA sequence of *L. garvieae* by Mata *et al* (2004). Oligonucleotide primers were as pLG-1 (5'-CATAACAATGAGAATCGC-3') and pLG-2 (5'-GCACCCTCGCGGGTTG-3'), to identify *L. garvieae*. The specificity of these primers was checked on all sequences available from the GenBank database using the Blast program. The primers were commercially synthesized by Cinnagen Company (Iran).

Samples were tested at least in duplicate and sterile water, used as the negative control. The amplification of 16S rDNA was confirmed by running the amplification product in 2% agarose gel electrophoresis (Gibco BRL, Karlsruhe, Germany) in

1× TAE buffer and visualized by staining with ethidium bromide (final concentration of 0.5 µl/ml) under UV transilluminator. Images were captured on a computer and printed.

The PCR products of 16S rDNA of the isolates were sequenced. Sequencing of each PCR product was performed using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) after sequencing reactions with a Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). Forward and reverse nucleic acid sequence data were used to construct a continuous sequence of inserted DNA. Further comparison of the continuous sequences was made with previously available sequences in NCBI (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool).

Estimation of pathogenicity index. The isolated *L. garvieae* was semi-mass cultivated in 100 ml triptose phosphate broth (TPB) and incubated at 30 ^{o}C over-night. Using immersing method in a solution containing 1×10^{7} bacteria per mililiter in 500 ml volume, ten fish (10 g weight) with two replicates were infected. The infected fish were observed for 72 h and the pathogenicity index was estimated (Amend 1981).

Mass cultivation of the isolate. The isolated bacterium was mass cultivated in TPB supplemented with 5% yeast extract under aerobic condition at 30 ^{o}C in 20 L glass flasks at production section of Razi Vaccine and Serum Research Institute of Iran-Shiraz branch. After 24 h incubation before addition of formalin, a sample was collected from mass cultivated bacteria for viable counting per ml using colony forming unit (c.f.u). Formalin (37%) was added to mass cultivated organisms up to 0.3% and the culture was further incubated at 30°C overnight (Organization Internationale des Epizooties 2008). On the next day a sample was collected from formalized culture for total counting as well as testing for sterility and purity.

Sterility testing. Samples from formalized culture were inoculated on blood and TPB agar and incubated at 30°C over night, on the next day the inoculated plates were inspected for any microbial growth by colony formation.

Purity testing. Smears were prepared from samples collected from formalized culture and Gram stains were performed. Stained smears were observed microscopically to see any unrelated organisms.

Formalin estimation. Residual formalin in experimentally prepared vaccine was estimated by the method described by Organization Internationale des Epizooties (2008) through colorimetric methods.

Safety testing. Ten rainbow trout of 10 g weight were immersed in a solution containing 2×10^{9} formalin killed bacteria/ ml (double recommended dose of the vaccine to be used)(Vendrell *et al* 2007) for one minute and the exposed fish were observed for 72 h thereafter for any signs of morbidity and/ or mortality in the Shiraz veterinary school, fish health unit.

Potency testing. Ten rainbow trout (mean weight of 10 g) with two replicates were vaccinated with 1×10^{9} /ml formalin killed bacteria for one minute by immersing method in the fish health unit. Ten fish with two replicates as control group were also immersed in normal saline for one minute. After that for 72 hours the vaccinated fish were watched for any abnormal signs. Thirty days later the vaccinated fish as well as the control group were challenged with the pathogenic bacteria (exposure of fish to 1×10^{7} live pathogenic bacteria/ ml for one minute by immersing method). Thereafter the exposed fish were inspected for 72 hours. Attempts were made to isolate *L. garvieae* from diseased fish by standard microbiological methods. Relative percent of survival (RPS) was calculated according the formula proposed by Amend (1981). RPS= (1- % mortality in vaccinated/ % mortality in control) × 100.

Field trial. The experimentally prepared lactococcosis vaccine was used to immunize 18,000 rainbow trout each having three grams weight in a farm raceways by exposing them to the vaccine for one minute by immersion method (first vaccination dose). Forty five days after first vaccination, the same fish when each had 10 grams weight were again vaccinated by the

same method (second vaccination dose) (Figure 2). In the same farm in separate raceways, 18,000 rainbow trout fish with the same age as the vaccinated group were kept as control group without vaccination. The rainbow trout farm has experienced a 2 year time challenging with enterococcosis according to its responsible veterinarian's report. The same conditions such as water quality and temperature, type and amount of feeding as well as environmental stresses were kept the same for both groups in the fish farm. Relative percent of survival was calculated as described above.

Figure 2. Immersion vaccination of the rainbow trout.

RESULTS

Results of biochemical tests on the *L. garvieae* isolated in this study were compared with the published results of biochemical tests of *L. garvieae* isolated by other researchers (Ravelo *et al* 2001, Austin and Austin 2007). In the specific PCR assay, DNAs extracted from all *L. garvieae* gave the expected 1107-bp PCR fragment of 16S rDNA sequenses, which is specific for *L. garvieae* (Mata *et al* 2004). The 1107 bp band was not observed with distilled water and DNA obtained from other bacteria (Figure 3).

In the pathogenicity index estimation using 1×10^7 bacteria per milliliter, our isolate showed an index of 60 percent mortality (mean of replicates). Mass cultivation of the isolate in TPB along with 5% yeast extract showed 10^9 bacteria/ ml by viable counting after 24 h incubation at 30 °C aerobically. While the bacteria cultivated in TSB without yeast extract showed 10^8bacteria / ml at the same incubation time and temperature using colony forming unit method.

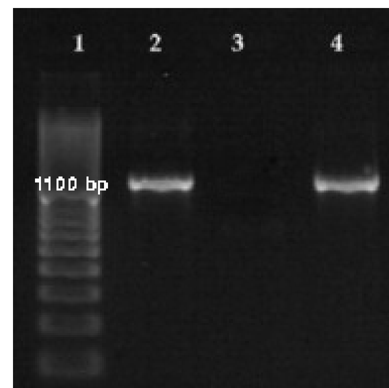

Figure 3. Electerophoretic analysis of DNA amplified fragments from *L. garvieae* isolated in this study. Lane 1, Marker: Lane2, Positive control; Lane 3, Negative control (distilled water); Lane 4, Our *L. garvieae*.

The sterility test showed no live bacteria in the prepared vaccine. The purity test did not reveal any organism other than the desired one (*L. garviaeae*) in the prepared vaccine. The formalin estimation test showed 0.03 % formalin in preparation which is in the range of recommended formalin concentration in a vaccine stock to be used in fish (Organization Internationale des Epizootics 2008). Among the fish exposed to double doses of vaccine no sign of disease or death was observed within 72 hours after exposure.

In the potency test after challenging the vaccinated fish with the live pathogenic bacteria and observing for 72 hours thereafter, 10% of fish died while the control group showed 60% mortality (mean of replicates) within the observation time (Table 1). In the field trial from vaccination time onward till fish marketing, any death occurred in each group of vaccinated and non-vaccinated was recorded. Total death occurred in vaccinated fish group were 1920 fish (11%), while in the non-vaccinated group this number was 4175 (23%). This observation indicates more than 50% reduction in the mortality rate in the vaccinated group. *Lactococcus garvieae* was isolated from moralities

occurred during the field trial with typical signs of the disease in the fish farm (Table 1).

Table 1. Mortality and relative percent survival after challenging of rainbow trout with *L. garvieae* isolate after immersion immunization with the prepared lactococcosis vaccine; a) potency testing, b) field trial testing.

	Fish groups (Number of fish)	Number of fish	Mortality Number (%)	percent survival
(a)		10	1	83
1	**Immunized fish (30)**	10		100
		10	2	67
		10	7	0
2	**Control fish (30)**	10	5	0
		10	6	0
(b)				
1	**Immunized fish**	18000	1920 (11%)	54
2	**Control fish (Non-immunized)**	18000	4175 (23%)	0

DISCUSSION

Lactococcosis is a bacterial disease, caused by *L. garvieae* that occurs in both freshwater and seawater and has been a source of major economic losses since the early seventies for the rainbow trout and marine fish industry (Vendrell *et al* 2006).

Lactococcal septicemia caused by a beta-hemolytic *L. garvieae* appears to be most severe when water temperature rises above 20 °C in tilapia, rainbow trout and ayu (Austin and Austin 2007). Environmental stresses such as sudden temperature changes, poor water quality parameters and poor nutrition may also influence severity of the disease. Species of fish may also play a role in the severity of enterococcal losses. Apart from the usual preventive measures such as the reduction of over crowding, overfeeding, unnecessary handling and prompt removal of diseased or died fish, control of lactococcosis has included vaccination, chemotherapy as well as the use of non-specific immunostimulants (Romalde *et al* 2004).

Vaccination may be the best form of prevention for lactococcal septicemia in cultured fish. Akhlaghi *et al* (1996) used immunization (i.e. by injection and immersion in formalin killed bacteria) to look at the possibility of vaccination of rainbow trout against streptococcus (enterococcus). After one month, a positive effect of vaccination (relative percent survival of 88 and 11 respectively) was observed in immunized fish. In their experiment, the percent of mortality in control group was 75%.

A common approach in vaccine production is to inactivate the pathogen and toxins by physical (e.g. UV and heat) or by chemical (e.g. killed by formol) means so that it is no longer capable to replicate in or damaging the host. It is critically important to maintain the structure of epitopes on antigens during inactivation. Administration of these bacterins is carried out by injection or immersion providing that the serotypes used for vaccine preparation cover the field of virulent strains and that the vaccines are used correctly, these vaccines are effective and give negligible side effects (Romalde *et al* 2004). Most vaccines used in aquaculture to date have been inactivated bacterial vaccine. The ability of fish vaccines to induce a protective immunity is based on experimental challenge studies and/ or field experiments (Gudding *et al* 1999). Autovaccines have also been developed with strains of *L. garvieae* (inactivated with formalin) isolated from the fish farm where the outbreak was occurring (Eldar *et al* 1997). According to Sommerset *et al* (1993) lactococcosis vaccine is tested successfully in Italy, France, U.K and Japan to immunize rainbow trout and amberjack/ yellowtail mortalities. The use of locally isolated pathogenic microorganism in vaccine preparation would help in increasing the vaccine efficiency, in this study the locally isolated *L. garviae* from diseased

rainbow trout, was used for experimental vaccine production. To mass cultivate the isolate, various culture media along with and without yeast extract and different incubation times and temperature were applied to get the highest growth yield. Triptose phosphate broth medium supplemented with 5% yeast extract and incubation at 30 °C for 24h were found to be the best cultivation conditions. By application of the mentioned cultivation conditions, 1×10^9 bacteria per milliliter were obtained, the mass cultivated bacteria were inactivated using 0.3% of 37% formaldehyde for 24 hours at 30 °C. When fish were exposed to double dose of prepared vaccine (i.e. 2×10^9) for one minute by immersion, no illness or dead were observed in the exposed fish for 72 hours after exposure which illustrates the safety of prepared vaccine. In the potency testing, prepared experimental vaccine was evaluated at laboratory as well as the field trial by exposing (vaccinating) 18000 rainbow trout in a fish farm. At our laboratory the vaccinated fish showed 98% relative percent survival, while for the field trial this percent was about 54. It has been stated that the relative percent survival of above 70 would be a good potency index for an aquaculture vaccine (European Pharmacopoeia 2002), which in this study we achieved above 70% at the laboratory level. At field, this percent is somehow lower than the laboratory one due to effects of uncontrolled stresses such as overcrowding, sudden water temperature changes, and poor water quality that may not be controlled easily at the field level and therefore may influence the potency of any vaccine. To our knowledge this study is the first aquaculture vaccine prepared from locally isolated pathogen in Iran for use in cultured rainbow trout fish, which its safety and potency has been evaluated at both laboratory and field trials. This vaccine is suitable to immunize non-exposed juvenile rainbow trout in fish farms that helps health measure strategies in fish farm to eradicate lactococcosis.

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