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RESEARCH ARTICLE

Oral Vaccination Against Lactococcosis in Rainbow Trout *(Oncorhynchus mykiss)* Using Sodium Alginate and Poly (lactide-co-glycolide) Carrier [1]

Soner ALTUN * Ayşegül KUBİLAY ** Seçil EKİCİ **
Behire Işıl DİDİNEN ** Oznur DİLER **

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* Uludag Univercity, Faculty of Veterinary Medicine, Department of Microbiology, TR-16059 Gorukle, Bursa - TURKEY
** Suleyman Demirel Univercity, Faculty of Fisheries, Department of Aquaculture, TR-32500 Isparta - TURKEY

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Summary

As an alternative immunization procedure against lactococcosis in rainbow trout, encapsulated antigens immobilized in sodium alginate (SA) and poly lactide-co-glycolide (PLGA) polymers were administrated as a feed additive. Positive controls were intraperitoneally (IP) administered with aqueous-based bacterin (vaccine) while negative controls were not vaccinated. Positive control groups were immunized intraperitoneally by using the aqueous-based bacterin. Relative percentage survival (RPS) values of groups immunized orally with SA and (PLGA) encapsulated vaccines were not significantly different and was 53.48% and 62.79% respectively (on the 30th day). No statistically significant difference was determined between the SA and (PLGA) vaccine groups and relative percentage survival (RPS) values of the two groups determined to be 53.48% and 62.79%, respectively. To determine the effect of the booster immunization by using encapsulated immobilized vaccines, booster immunization was performed on the 61st day after oral administration of the same vaccines and RPS value was more than 60% on the 90th 120th days. After an aqueous-based vaccine followed by an immobilized vaccine for booster, the RPS value has increased over more than 80% indicating that booster application has increased the protection of rainbow trout against lactococcosis. It can be suggested that both SA and PLGA oral vaccines can be effectively used in rainbow trout against lactococcosis and there was no significant differences between the protection levels, however, since the preparation costs of SA oral vaccines are relatively lower compared to PLGA ones its usage for vaccination appears more appropriate.

Keywords: *Lactococcus garvieae, Rainbow trout, Oral immunization, Sodium alginate, Poly (lactide-co-glycolide)*

Taşıyıcı olarak poli (lactid-ko-glikolid) ve Sodium Alginate Kullanılarak Gökkuşağı Alabalıkları *(Oncorhyncus mykiss)*'nın Lactococcus garvieae'ye Karşı Oral İmmunizasyonu

Özet

Sodium aljinat (SA) ve poli laktid-ko-glikolid (PLGA) polimerleriyle immobilize edilmiş antijenler, gökkuşağı alabalıklarında lactococcozise karşı alternatif bir immunizasyon yöntemi olarak kullanıldı. Su bazlı bakterinin periton içi enjeksiyonla verildiği grup pozitif kontrol olarak, aşı uygulanmayan grup ise negatif kontrol olarak belirlendi. SA ve PLGA ile immobilize edilmiş oral aşılarla immunize edilen grupların korunma oranları arasında önemli bir fark bulunmadı ve RPS (30. gün) değerleri sırasıyla % 53.48 ile % 62.79 olarak tespit edildi. Balıklarda immobilize edilmiş oral aşılarla ilk aşılamadan 61 gün sonra booster yapıldığında ise RPS değerlerinin her iki grupta da 90 ve 120. günlerde % 60'ın üzerinde olduğu belirlendi. İlk olarak İP enjeksiyonla aşılanan balıklara daha sonra immobilize edilmiş oral aşılarla (61. gün) booster yapıldığında RPS değerlerinin %80'in üzerinde çıktığı ve gökkuşağı alabalıklarında laktokokkozise karşı korunma süresini artırdığı tespit edildi. Tüm eprüvasyon günlerinde SA ve PLGA oral aşı gruplarının koruyuculuğu arasında istastiki bir farklılık olmaması, SA oral aşıların PLGA'ya göre çok daha az maliyetle hazırlanabilmesi nedeniyle SA oral aşıların gökkuşağı alabalıklarında laktokokkozise karşı kullanımında tercih edilebileceği görüldü.

Anahtar sözcükler: *Lactococcus garvieae, Gökkuşağı alabalığı, Oral immunizasyon, Sodyum aljinat, Poli (laktid-ko-glikolit)*

İletişim (Correspondence)
☎ +90 224 2941264
✉ soneraltun2003@yahoo.com

INTRODUCTION

Lactococcus garvieae is a facultatively anaerobic, non-motile, non-spore forming, Gram positive ovoid coccus occurring in pairs and short chains and producing α-haemolysis on blood agar (BA). It is the etiological agent of lactococcosis, an emergent disease which affects many fish species and causes important economical losses both in marine and freshwater aquaculture when water temperature increases over 16°C in summer months [1]. The first outbreak of lactococcosis in rainbow trout from Spanish fish farms has occurred in 1988. The spread of *L. garvieae* throughout Mediterranean Europe has been rapid; *L. garvieae* infections of trout were recorded in Spain in 1991, the same pathogen was detected in Italy. The pathogen and accordingly the disease have stated to spread rapidly throughout the southern part of the European continent, including countries such as Portugal as well as the Balkans [1]. In Turkey, *L. garvieae* was has been firstly isolated in 2001 at an outbreak occurred in rainbow trout farms [2,3]. Since then, such infections have been reoccurred, especially during the warm summer months. Therefore, *L. garvieae* is now considered one of the most important pathogens at in the rainbow trout industry in Turkey [2,3]. The existence of two serological groups associated with the presence of capsular material has been demonstrated for *L. garvieae*. In addition, capsulated strains are more virulent to rainbow trout than non-capsulated strains [4].

Ravelo et al.[5], have reported that *L. garvieae* were separated into three genetic groups, composed of the Spanish, Portugues, English and Turkish strains (Group A), the Italian and French strains (Group B) and the Japanase strains (Group C). Altun et al.[3] have determined that Turkish strains are 99-100% identical to Spanish and English strains in the point of view sequence analysis of 16s rDNA of *L. garvieae*.

Mortality of lactococcosis has stated to be more than 50% in rainbow trout in summer season and the treatment of the disease is not successful with chemo-therapeutics because of development of the resistance and recurrent infection. Therefore, development of effective vaccines is needed to prevent lactococcosis outbreaks [6]. Attempts to develop effective vaccines against lactococcosis have been carried out [7-9]. Fish vaccines can be administered by immersion, injection or oral route. Injection is evaluated as an effective immunization method in inducing immunity, but is stated to be not suitable for extensive aquaculture because of handling stress and high labor costs. For Gram-positive fish pathogens [10,11], good protection levels are could have been achieved when vaccines are had been administered intraperitoneally (IP) [7]. However, the short duration of the immunity (2 to 3 months) constitutes the main inconvenience for the success of these vaccines, since this period is not enough to protect during warm seasons in which water temperature is higher than 16°C when and the majority of lactococcosis outbreaks occur [8]. To overcome such problems, several approaches including use of adjuvants in the vaccine formulation, the combination of selection of genetically resistant fish and vaccine strains, and use of booster immunization have been evaluated. Oral vaccines have been evaluated as good alternatives since there is no need to handling of the fish; it is not a stressful method and does not require extensive labor [12]. Activation of mucosal immunity has stated to be appeared an important factor since many pathogens can enter into the body of the fish through mucosal surface [3]. However, oral vaccination has stated to have some drawbacks; i) the applied antigen is often destroyed due to protease activity present in the intestinal tract, (ii) oral tolerance can be evoked and (iii) the antigen does not necessarily enter the gut mucosa and consequently an immune response is not initiated [13-15].

An oral vaccine should be in such form that the formulation may protect the antigen from inactivation and digestion during passage through the stomach and the anterior gut [16]. One option is acid-stable coating. Nevertheless, this method has stated to be relatively expensive. Piganelli et al.[17] have described the coating of antigen microspheres (ECAMs) with Eudragit LD-30 co-polymer for oral vaccine delivery. Vervarcke et al. [18] have examined the effect of lag time of the coated pellets on uptake and immune response to inactivated *V. anguillarum*. Joosten et al.[19] and Romalde et al.[8] have successfully used the encapsulation of inactivated bacteria in alginate microparticles for vaccination. The aim of this study is to test determine the efficacy and protection level of different oral vaccine formulations against lactococcosis as well as their usefulness as primary or secondary immunization method materials. Also, duration of protection was investigated in order to propose an appropriate vaccination program to prevent *L. garvieae*.

MATERIAL and METHODS

Selection of Bacterial Strains for Vaccine Formulation

The vaccine was prepared from *L. garvieae* M1 strain. This strain was has been previously isolated at a natural outbreak that occurred in a rainbow-trout farm which located in Fethiye - Mugla, Turkey [4]. The strain was selected among several lab strains on the basis of

previous studies in which we have determined its antigenic characteristics determined in a previous study [9,20]. The reference strain NCDO 2155 (ATCC-43921) was also used in that study to compare its property with the lab strain. The strains were inoculated on tripticase soy agar (TSA, Difco Laboratories, Detroit, MI) and incubated at 25°C for 24-48 h. Pure bacteria were transferred to triptic soy broth (Difco Laboratories, Detroit, MI). Then, pure culture stocks were stored at -80°C in triptic soy broth (TSB, Difco) including 15% glycerol.

Preparation of the Bacterin

The formalin killed bacterin was prepared as stated in the previous studies [9,20]. Bacterial cells were inactivated by adding formalin until final concentration of 0.7%. The solution suspension was incubated at 25°C for 3 h, and then at 4°C overnight. Thereafter Then, inactivated bacterial cells were washed three times with phosphate buffered saline (PBS pH 7.2) by centrifugation at 6000 rpm for 30 min at 4°C. The formalin killed vaccine was then resuspended in phosphate buffered saline (PBS) and optical density of final suspension was adjusted to OD_{600} of 1.2 to make its cell concentration about $1x10^{10}$ cfu/ml.

Preparation of SA (Sodium alginate) Microbeads and PLGA poly (lactide-co-glycolide) Microspheres

The previously prepared bacterial suspension was mixed separately with SA (Sigma aldrcih A2033, Darmstadt, Germany) and PLGA (Sigma-Aldrich P2191, Darmstadt, Germany) solutions at the concentration of (10^{10} cells/ml).

Sodium alginate microbeads were prepared by using orifice-ionic gelation method which was previously described by Gonzales-rodriguez et al.[23]. Sodium alginate solution in distilled water (4%-w/v) was prepared and mixed with the bacterial homogenate containing 10^{10} cells/ml. The resultant mixture was homogenized with Ultra Turrax T25 at 11.000 rpm. This suspension was added dropwise (30 drops/min) through a 18 G needle into the aqueous solution of $CaCl_2$ (0.3 %-w/v) stirred on a magnetic stirrer for 24 h for the crosslinking. The microbeads were then filtered (Whatman cellulose acetate filter) and washed with distilled water. Finally, the microbeads were left to dry at room temperature until they reach to a constant weight. The microbeads were kept at 4°C until use [21-23].

PLGA microsphers containing bacterial strains were prepared by using spray drying method. Briefly, PLGA (50:50) was dissolved in dichloromethane resulting in the final solution of the polymer at 2% (w/v). This solution was added on the bacterial homogenate to prepare 10^{10} cells/ml, and the resulting suspension was homogenized by using Ultra Turrax T25 at 11000 rpm for 2 min. This homogeneous suspension was then sprayed through a 0.5 mm nozzle with the apparatus Büchi B-190 spray dryer (Büchi Labortechnik, Postfach, Switzerland). The flow rate was set as 1 ml/min at 4 atm pressure. Inlet/outlet temperatures of the method were set as 65°C and 35°C, respectively. The microcapsules were stored at +4°C until use [24-27].

Preparation of Fish Feed Including Vaccine Materials

A commercially available fish feed (Ecobio, feed No: 3, containing 45% protein, 20% lipids, Ekobio Feed, İzmir-Turkey) was wetted by adding distilled water and the feed was ground in a blade mill to obtain a paste. This paste was subsequently mixed with microparticles or cell suspension with pestle and mortar in order to obtain a homogeneous mixture. The final feed paste was given to fish at a rate of 1% of body weight per day. So, daily feed portion of each fish were included $1x10^{10}$ formalin-killed cells. Since the paste has proper plastic properties, it is extruded through a 20-ml syringe, dried during 24 h at room temperature and cut into pellets. In the case of bacterial suspension, the doses are the same for microparticles formulation.

Immunization Procedure

Juvenile rainbow trout obtained from a farm with no history of lactococcosis were maintained at concrete raceway cage of 2x2x1m for 4 weeks for acclimation. Water temperature was 18±1°C and dissolved oxygen amount was 7.5 mgL^{-1}. The experimental design included eight fish groups (50 fish per group having with 20 g initial average weight were used on each challenge application). Intraperitoneal immunization was performed by injection of 0.1 ml of the formalin-killed bacterin ($1x10^{10}$ cfu/ml). For oral immunization, oral vaccine was included in the feeding material and fish were fed with the mixture over 7 day-period with a daily feeding rate of 1% of the body weight. Immunization groups were as *Table 1*. The tests were done in duplicate.

Challenges were carried out on the 30, 60, 90 and 120 days after vaccination, fish from all groups were IP (injection of 0.1 ml) challenged with the homologous strain *(Table 1)*. Mortalities were recorded daily over a 3-weeks period and all the dead fish were examined to confirm by the isolation of the inoculated strain from the internal organs. Protection was evaluated by determining the relative percent of survival (RPS) according to Amend [28] in each group using the formula:

RPS = 1 - (% mortality in vaccinated/% mortality in control) x 100

Table 1. *Experimental groups used for the evaluation of vaccines against Lactococcus garvieae in rainbow trout*

Tablo 1. *Gökkuşağı alabalıklarında Lactococcus garvieae'ye karşı hazırlanan aşıların etkinliklerinin değerlendirilmesinde kullanılan deneme grupları*

Fish Group	Number of Fish*	Challenge Dose (cfu/ml)
I. Chalange (30 Days)		
Group 1	50	$1.2x10^5$
Group 2	50	$1.2x10^5$
Group 3	50	$1.2x10^5$
Group 4	50	$1.2x10^5$
II. Chalange (60 Days)		
Group 1	50	$1.5x10^5$
Group 2	50	$1.5x10^5$
Group 3	50	$1.5x10^5$
Group 4	50	$1.5x10^5$
III. Chalange (90 Days)		
Group 1	50	$2.3x10^5$
Group 4	50	$2.3x10^5$
Group 5	50	$2.3x10^5$
Group 6	50	$2.3x10^5$
Group 7	50	$2.3x10^5$
Group 8	50	$2.3x10^5$
IV. Chalange (120 Days)		
Group 1	50	$1.8x10^5$
Group 4	50	$1.8x10^5$
Group 5	50	$1.8x10^5$
Group 6	50	$1.8x10^5$
Group 7	50	$1.8x10^5$
Group 8	50	$1.8x10^5$

Group 1: *Intraperitoneally vaccinated with formalin inactivated bacteria on day 0 (positive control)*
Group 2: *Orally vaccinated with PLGA microspheres on day 0*
Group 3: *Orally vaccinated with SA microbeads on day 0*
Group 4: *Non-vaccinated group (negative control)*
Group 5: *Fish were vaccinated on the 0 day as Group 1 and then subjected to oral booster vaccination with PLGA microspheres on 61st day*
Group 6: *Fish were vaccinated on the 0 day as Group 1 and then subjected to oral booster vaccination with SA microbeads on 61st day*
Group 7: *Fish were vaccinated on the 0 day as Group 2 and then subjected to oral booster vaccination with PLGA microspheres on 61st day*
Group 8: *Fish were vaccinated on the 0 day as Group 3 and then subjected to oral booster vaccination with SA microbeads on 61st day*
** Test were done duplicate and totally 2000 fish were used in the study. Arithmetic means of dead fish were used for RPS calculation.*

Statistical Analysis

Results were analyzed by ANOVA and significances were determined by Duncan's test (SPSS 9.0 package for Windows).

RESULTS

The levels of protection were given in *Table 2*. The vaccine groups resulted in a greater level of protection, determined by a lower level of mortality compared with that of the control group (Group 4). The protection remained high over the time as significant differences in mortality were observed between the control and the treatment groups on the 30th, 60th, 90th and 120th post-vaccination days. As seen in *Table 2*, "statistically" significant level of protection was achieved in rainbow trout within 30 days when the vaccine (bacterin) was administered via IP injection compared with non-immunized trout ($P<0.05$).

A significantly high level of protection with a RPS value of 95.34% was achieved in 20 g rainbow trout within 30 days when the bacterin was administered by IP injection *(Table 2)*. Protections obtained with oral PLGA microspheres and SA microbeads were 62.79% and 53.48%, respectively on the 30th day of vaccination *(Table 2)*.

On the basis of these results, a second experiment including booster vaccination by using PLGA microspheres was designed. The results obtained are shown in *Table 2*.

Table 2. *Protective efficacy of the different vaccine formulations administered intraperitoneally (IP) or by the oral route against L. garvieae in rainbow trout*

Tablo 2. *Gökkuşağı alabalıklarında Lactococcus garvieae'ye oral ve İP yolla uygulanan farklı aşı formulasyonlarının koruma etkinliği*

Fish Group	Challenge Dose (cfu/ml)	Number of Fish	Mortality (%)	RPS
I. Chalange (30 Days)				
Group 1	$1.2x10^5$	50	4 ^{c}	95.34
Group 2	$1.2x10^5$	50	32 ^{b}	62.79
Group 3	$1.2x10^5$	50	40 ^{b}	53.48
Group 4	$1.2x10^5$	50	86 ^{a}	-
II. Chalange (60 Days)				
Group 1	$1.5x10^5$	50	16 ^{c}	82.22
Group 2	$1.5x10^5$	50	50 ^{b}	44.44
Group 3	$1.5x10^5$	50	56 ^{b}	37.77
Group 4	$1.5x10^5$	50	90 ^{a}	-
III. Chalange (90 Days)				
Group 1	$2.3x10^5$	50	40 ^{b}	52.38
Group 4	$2.3x10^5$	50	84 ^{a}	-
Group 5	$2.3x10^5$	50	8 ^{e}	90.47
Group 6	$2.3x10^5$	50	12 ^{de}	85.71
Group 7	$2.3x10^5$	50	24 ^{cd}	71.42
Group 8	$2.3x10^5$	50	28 ^{bc}	66.66
IV. Chalange (120 Days)				
Group 1	$1.8x10^5$	50	50 ^{c}	42.85
Group 4	$1.8x10^5$	50	84 ^{a}	-
Group 5	$1.8x10^5$	50	14 ^{d}	83.33
Group 6	$1.8x10^5$	50	16 ^{d}	80.95
Group 7	$1.8x10^5$	50	30 ^{bc}	64.28
Group 8	$1.8x10^5$	50	32 ^{b}	61.90

Significant difference ($P<0.05$) letters shows differences between the groups

RPS value obtained with PLGA microspheres in the first challenge was 62.79% and then decreased to 44.44% in the second challenge. Protection in IP vaccinated animals was noted as 95.34% on the 30th day while it decreased to 82.22% on the 60th day. Thereafter, this value declined until 42.85% in the fourth challenge that was performed on the 120th day post-vaccination. However, increase in RPS was observed in a fourth challenge group that was vaccinated firstly by IP administration of the bacterin and revaccinated on the 61st day with the oral encapsulated vaccine, reaching values of 83.33% *(Table 2)*.

Significant level of protection was achieved on 90th and 120th days with fish immunized intraperitoneally followed by booster vaccination on 61st day with oral PLGA microspheres (90.47% and 83.33% respectively) and SA microbeads (85.71% and 80.95% respectively) as shown in *Table 2*.

DISCUSSION

It has been observed that good protection levels are only achieved when vaccines are intraperitoneally (IP) administered [7]. Immersion procedures produce a lesser degree of protection against Gram-positive bacterial infections in salmonids [7,8]. Romalde et al.[8] have reported the efficacy of vaccination in rainbow trout against lactococcosis [8]. They applied a combined strategy consisting of a primary immunization with an aqueous bacterin followed by a booster immunization (3 months later) with an oral alginate-encapsulated vaccine [8].

In fact, the way of delivery may reflect the efficiency of transferring the immunogenic constituents of the vaccine to the important recognition and effector components of the fish immune system. As in the case of other Gram-positive fish pathogens such as *S. iniae* or *S. parauberis* [7,8,29] good protection results were also achieved against *L. garvieae* infections by the IP administration of vaccines but not by immersion procedures [7]. However, the formulations assayed until now to prevent fish streptococcosis caused by *S. iniae* or *L. garvieae* rendered protection for short periods of time (approximately 3-4 months) [8]. Similar results were obtained in the present work for the *L. garvieae* bacterin, since 120 days after vaccination, the protection level dropped to RPS values of 42.85%.

Oral vaccination is less stressing for animals and requires less time for application. Another advantage of this technique is that regardless the size of the fish mass vaccination of a pool is possible [8,30]. Since the first contact between animal and pathogens in oral immunization occurs through mucosal immune system oral vaccination becomes more promising [31]. It has been reported that oral vaccination may induce the appearance of antigen-specific antibodies in skin mucus, bile or intestine in fish species [32]. On the other hand, oral vaccination has some disadvantages. High acidity of fish stomach inactivates antigens and prevents absorption in the lower gut [33]. To overcome this problem efficient delivery methods should be improved.

In our study, 95.34% RPS has been obtained 30 days after IP administration of the prepared bacterin. Eldar et al.[10] have obtained 90.0% RPS against *Streptococcus iniae*; Romalde et al.[11] obtained 83.3% RPS against L. garvieae, and Ravelo et al.[33] obtained 82.6% RPS against *L. garvieae* after IP immunization of fish. This complies with other research results.

There are only one data available regarding the usefulness of oral vaccines in the prevention of fish streptococcosis, regardless of its causative agent. Oral vaccination with encapsulated and non-encapsulated antigens was preliminary evaluated as alternative immunization procedures against trout lacotococcosis [8]. Autors reported that several microparticle systems for protection of the antigen and efficient oral vaccine delivery were tested in trout in comparison to a vaccine produced by adding directly inactivated bacterial cells to the fish food. Only the formulation including bacteria encapsulated in alginate-acetone microspheres rendered significant levels of protection (RPS of 50%), which indicates that these microparticles seem to avoid the antigen degradation, due to low pH and proteases, in the anterior part of the digestive tract. Similar results were obtained in oral vaccination experiments against *Vibrio anguillarum* [19].

Romalde et al.[8] reported the efficacy of oral immunization using alginate-microparticles for booster vaccination in rainbow trout to prevent lactococcosis. The fish were initially IP vaccinated with the aqueous bacterin and they received an oral booster vaccine 90 days later. On the 30th day of revaccination protection reached to 87% RPS.

Although protection obtained in the present study with the alginate microparticles provides good booster vaccination, this vaccination can not be used as primary immunization method According to the European Pharmacopoeia [34].

Similar results were obtained in the present work for the *L. garvieae* bacterin, revaccination of fish 61 days after a first IP immunization resulted in an increase in the protection, RPS levels rising from 42.85 to 83.33%. Although RPS value was under 63% with single oral

administration of either encapsulated SA or PLGA vaccine, application of a booster vaccination increased the protection level until 60% in both systems. Fish vaccinated IP exhibited an RPS value of 82.22% on the 60th day. Thereafter, this value declined gradually over the time and attained to 52.38% and 41.46% on the 90th and 120th days respectively. Application of initial IP vaccination together with SA and PLGA oral vaccines booster resulted with increased protection level. In that case, while protection with SA booster vaccination were 85.71% and 80.95% on the 90th and 120th days respectively, the RPS obtained with PLGA booster for the same measuring days were 90.47% and 83.33%. Statistically significant difference ($P<0.05$) were obtained when oral application of SA microbead and PLGA microsphere vaccines were used either in combination (oral SA + oral SA; oral PLGA + oral PLGA) or after IP application. These results are in good agreement with those of reported by Romalde et al.[8] and Yazıcı [35].

It can be concluded from the present research that booster application of oral SA and PLGA vaccines provided efficient and sufficient level of immunization against *L. garvieae* and that the protection obtained by rainbow trout lasted up to 120 days.

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