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Effects of Lipopolysaccharides (LPS) of *Yersinia ruckeri* on Immune Response in Rainbow Trout *(Oncorhynchus mykiss)* by Intraperitoneal and Oral Administration

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Abstract

BACKGROUND: *Yersinia ruckeri* (*Y. ruckeri*) is an important fish pathogen, which has received more attention recently due to the increasing economic losses in the rainbow trout industry worldwide. Nowadays researching on novel oral vaccines is expanding due to convenience and ease of administration.

OBJECTIVES: In the present study, the effect of *Y. ruckeri* lipopolysaccharide (LPS) on Protective immunity of the *Y. ruckeri* bacterin was evaluated in rainbow trout.

METHODS: For this purpose, 720 juvenile rainbow trout were randomly divided into 8 groups each in triplicates. Group G1 to G4 received respectively PBS, bacterin, LPS and Bacterin+ LPS by intraperitoneal route. Groups 5 to 8 received respectively PBS, bacterin, LPS and Bacterin+ LPS by meal.

On day 60 after immunization, half of the fish in each treatment was challenged intraperitoneally and the remaining half of fish in the oral receiving bacterin groups were challenged by bath method with 1 LD50 and 1 LC50 of a *Y. ruckeri* local virulent isolate respectively.

RESULTS: The ELISA results indicated that LPS via oral route did not increase the humoral immune response in serum, but LPS with the FKC could increase antibody levels significantly in serum and mucus ($P<0.05$). According to the results after I.P. challenge, in G2 to G4 groups survival rate was 76.47, 58.82 and 52.94 percent respectively, which was significantly higher ($P<0.05$) than G5 to G7 groups with 25, 17.65, 35.29 percent survival rate. The survival rate after bath challenge was 37.5, 49.49 and 29.49 in groups G5 to G7, respectively, which was significantly ($P<0.05$) more than the control group (G1).

CONCLUSIONS: The results showed that LPS has a significant effect on the induction of protective immunity to *Y. ruckeri* bacterin principally via I.P. route. Because of acceptable protection, the above method can be a promising and novel candidate to control yersiniosis in rainbow trout.

KEYWORDS: Immune response, lipopolysaccharide, rainbow trout, vaccination, *Yersinia ruckeri*

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Introduction

Yersinia ruckeri, which belongs to the family *Enterobacteriaceae* causes enteric red mouth disease (ERM) or yersiniosis, leading to significant economic losses in salmonid aquaculture worldwide particularly rainbow trout due to fast growing and robust under farming conditions. This pathogen has also been isolated from several non-salmonid fishes, earthworms, birds, mammals and a human case (Ormsby, M. and Davies, 2017; Kumar et al., 2015; De Keukeleire et al., 2014; Guijarro et al., 2018) as well as in milk, cheese, chicken and minced meat (Özdemir and Arslan, 2015).

Yersiniosis causes septicemia, anorexia, numbness, ascites, splenomegaly, darkening of the skin, and bilateral exophthalmia (Altinok et al., 2001; Tobback et al., 2007; Strøm et al., 2018). It was first isolated in Idaho, USA, from rainbow trout *Oncorhynchus mykiss* and has consequently been found in all trout producing countries including Iran (Kumar et al., 2015; Soltani et al., 2014; Calvez et al., 2014).

Lipopolysaccharides represent the main component of the outer cell wall membrane of Gram negative bacteria and constitute a polysaccharide portion consisting of lipid A, a core polysaccharide and an O-specific chain. Interestingly, it has been demonstrated that unlike mammals, fish are unsusceptible to the toxic effects of Lipid A which confers the endotoxic properties of LPS (Bishop, 2005; MacKenzie et al., 2010). Investigations showed that the main reason for the resistance of fish to endotoxic shock is related to the low sensitivity of fish monocyte-macrophage lineage cells to LPS due to a lack of serum-borne factors which confer the sensitivity to LPS in mammals (Iliev et al., 2005). Bacterial LPS act as a strong stimulator of the immune system including the induction of anti-LPS antibodies, polyclonal stimulation of B-lymphocytes and general enhancement of immunity in diverse eukaryotic species including fish (Boltana et al., 2014; Goetz et al., 2004).

The results of various studies have shown that bacteria can develop antibiotic resistance to most commonly used antibiotics (Capkin et al., 2015). Thus, vaccination can protect fish against yersiniosis and reduce antibiotics use. For this reason, many attempts have been made for vaccinating fish against yersiniosis by using bacterins and subcellular components such as LPS. Researchers clearly had demonstrated the critical role of the LPS as a component of the ERM bacterin vaccine. LPS is a potent immunogen even when applied alone via injection, oral or immersion depending on the age and size of the fish (Altinok et al., 2016; Welch and LaPatra, 2016; Villumsen et al., 2014; Villumsen et al., 2014; Raida, and Buchmann, 2008; Costa et al., 2011).

Formaldehyde-killed bacterin of *Y. ruckeri* serogroup 1 (Hagerman strain), was the first vaccine to be commercialized in the aquaculture industry and induced good protective effect against ERM when administered by immersion or injection routes (Johnson and Amend, 1983).

Although vaccine protection is usually highest when used via parenteral routes, these methods are also related to intensive handling and stress for the fish. There is no hesitation that with respect to animal welfare and handling costs, the mucosal route of vaccination, and in particular the oral route, would be the perfect method of vaccine delivery but the generation of safe and effective oral vaccines is among one of the most dif-

ficult tasks of immunologists (Embregts and Forlenza, 2016).

The development or refinement of targeted vaccines for aquaculture species is imperative to avoid serious economic losses due to disease outbreaks (Nguyen et al., 2018). Thus, in current research we have investigated the effect of LPS on protectivity of *Y. ruckeri* bacterin in rainbow trout.

Materials and Methods

Fish and experimental design

Juvenile rainbow trout (7±1.2 g) from a hatchery without any report of yersiniosis in the past 4 years in Lorestan province, Iran was brought to the aquarium room in the aquatic animal health department of the veterinary faculty, Shahid Chamran University, Ahvaz, Iran. Fish were kept at 14 °C in 200L glass aquaria with filters and fed commercial trout feed (BioMar, Denmark), 4% biomass per day. During adaptation and experiment period chemo-physical parameters of water were maintained in optimum condition by means of aeration pumps, biofilters and frequent water changes for all groups. Water parameters of experiment were as follows: temperature; 14±1.2 °C, dissolved oxygen; 9±1mg L^{-1}, pH; 7.9±0.53, NH_3 < 0.01mg L^{-1}, NO_2 < 0.1 mg L^{-1} and salinity 800 µSc m^{-1}. Fish were adapted for 2 weeks before the experiment. Also, their health status was examined by sampling from brain and kidney. Totally 720 rainbow trout fish were divided randomly into eight equal groups in triplicate (90 fish per group). Group G1 to G4 received respectively 0.1 ml of PBS, FKC bacterin (1×10^{10} cfu/ml), LPS (300 µg/ml) and Bacterin+ LPS by intraperitoneal route. Groups G_5 to G_8 received respectively PBS, bacterin, LPS and Bacterin+LPS by meal according to the method of Villumsen et al 2014; briefly, feed pellets (Biomar, Denmark, diameter = 1 mm) were thoroughly spray coated with PBS (G_5), FKC (1×10^{10} cfu/g) (G_6), LPS (300 µg/kg/Bw daily) (G_7) and FKC enriched with LPS (G_8) and after one hour incubation in 40 ºC, the pellets were coated with fish oil. Fish in each group were fed up to 3% of average bodyweight ad libitum every other week for 14 days (Villumsen et al., 2014; Kadowaki et al., 2013).

Preparation of Bacterin

The Hagerman strain of *Y. ruckeri* (KCW 291153) were grown in Trypticase Soy Broth medium (TSB; Difco) at 28 °C for 48 h. Then bacteria were washed twice using sterile PBS. The bacterial concentration was adjusted to 10^8 cfu/ml using pour plate count method.

Formalin-killed *Y. ruckeri* (FKB) was produced according to Ghosh et al. (2016) with some modifications. The adjusted concentration of *Y.ruckeri* was inactivated by 1% formalin (in neutral buffered) for 24 h. Afterward, the cells were centrifuged (8000 g; for 30 min) and washed twice in phosphate-buffered saline (PBS). To ensure the bacterial inactivation, 100 µL of prepared FKB was inoculated in TSA plates and incubated at 28 °C for 24h. The prepared bacterin was stored at 4 °C until use.

Lipopolysaccharide (LPS) extraction

The phenol-water method was performed for LPS extraction according to Apicella (2008) as follows.

Dried bacterial cells (500 mg) were suspended in 15 mL of 10 mM Tris-Cl buffer (pH 8.0) containing 2% SDS, 4% 2-mercaptoethanol, and 2 mM MgCl2. The mixture was vortexed and placed in a 65 °C water bath until the bacterial cells were sol-

ubilized. One mL of proteinase K (100 µg mL^{-1}) was added to the mixture and stored at 65 °C for an additional hour. Subsequently it was placed in a 37 °C water bath overnight. Twenty-two mL of 3M sodium acetate and 40 mL of cold absolute ethanol was added to the proteinase K-digested cell suspension and was kept overnight at –20 °C. After centrifugation, the precipitate was suspended in 9 mL of 10 mM Tris-Cl (pH 7.4), 100 µg mL^{-1} of DNase I (Cinnagen, Iran) and 25 µg mL^{-1} of RNase (Cinna gen, Iran) at 37 °C for 4 h and equal volume of 90% phenol preheated to 65 °C was added to the mixture and was heated at 65 °C for 15 min. Finally the mixture was centrifuged at 6000 × g for 15 min and the aqueous top layer was removed and dialyzed against distilled water. The extracted LPS was kept at -70 °C until use.

Quantification of LPS

Carbohydrate content of extracted LPS was quantified using phenol-sulphuric acid assay in a microplate format with some modifications (Dubois et al., 1956). Briefly, 30 µl of 5% w/v phenol was added to 50 µl of dilute LPS samples followed by immediate addition of 100 µl of concentrated sulphuric acid. The reaction mixture was incubated at room temperature for 30 min followed by 10 min extended incubation at 90 °C under dark conditions that resulted in the development of dark brown color. The reaction mixture (180 µl) was transferred to a 96 well microplate and absorbance at 485 nm was recorded using a microplate reader (Synergy-H4, BioTek, USA). The amount of carbohydrates (referred as glycans) in purified LPS was estimated from a standard curve generated using D-glucose as reference sugar.

To ensure the purity of extracted LPS, protein concentration was carried measured by the Bradford method.

Enzyme-linked immunosorbent assay (ELISA) antibody titers

Yersinia ruckeri-specific antibody levels in plasma, intestine and skin mucus were measured by ELISA with some modifications (Skov et al., 2018). Concisely, Microplates (Nunc, Denmark) were coated with 50 µl $well^{-1}$ of formalin-killed and sonically disrupted *Y. ruckeri* (100 µg/ml) antigen at a 1:15 dilution in bicarbonate coating buffer (pH = 9.6) for 18 h at 4 °C. Plates were washed with phosphate-buffered saline (PBS) containing 0.05% of Tween-20 (PBS-T) and then non-specific binding sites were blocked with 2.5% skim milk (High media, India) for 1 h at 25 °C. Rainbow trout plasma or mucus samples (100 µl) were then added at a 1:20 and 1:1 dilution respectively in PBS +0.05% Tween-20 (PBS-T) containing 0.1% skim milk. After 90 min incubation at 25 °C, 100 µl of mouse monoclonal mouse anti-rainbow trout immunoglobulin (prepared and kindly donated by Prof. Seyfi in our faculty) at a 1:7500 dilution in PBS-T containing 0.1% skim milk was added to all wells and then shaken for 60 min. After washing in PBS-T (three times), 50 µl of goat anti-mouse IgG HRP conjugate (Sigma-Aldrich) at a 1:2500 dilution in PBS-T containing 0.1% skim milk was added and incubated for 60 min. Plates were washed as above and 50 µl TMB (3,3 ′, 5,5; -tetramethylbenzidine - H2O2) chromogen solution was added to each well for 10 min at 25 °C. The reaction was stopped with 50 µl 2 N H2SO4. Lastly, serum and mucus antibody levels were read spectrophotometrically at 450 nm by an ELISA reader (Accu Reader, Taiwan).

Determination of *Y. ruckeri* LD50 and LC50

The 10-fold (10^5 to 10^8 CFU ml^{-1}) serial

dilutions of *Y. ruckeri* were prepared in PBS and 100µl of each bacterial dilution injected intraperitoneally to 10 fish. The mortality rate was recorded daily for 10 days, mortality rate was analyzed and the LD_{50} was determined by using probit software.

For LC50 determination, the serial (10^6 to 10^9 CFU ml^{-1}) 10-fold dilutions of *Y. ruckeri* were prepared and 10 fish were immersed in each concentration for one hour and returned to the fresh water. The mortality rate was recorded daily for 10 days, and LC_{50} was determined using probit software.

Challenge experiment

Thirty fish of each of the G_1 to G_8 groups (10 fish from each replicate) were challenged by I.P. route and 30 fish of the G_5 to G_8 groups were challenged by bath method Both I.P. and bath challenge were conducted for each group 60 days post-vaccination using live *Y. ruckeri*. For I.P. challenge fish were anesthetized with 50 mg L-1 MS-222 (Sigma—Aldrich, Denmark) and $1LD_{50}=7.5\times10^7$ CFU of *Y. ruckeri* were injected intraperitoneally, whereas for bath challenge the fish were immersed in $1LC_{50}=1.6\times10^8$ CFU ml-1 of *Y. ruckeri* for one hour. Following challenge procedure fish were transferred to 100 L aquaria. Mortality was checked several times daily for 10 days and dead fish were recorded and removed. The cumulative percent mortality (CPM) was determined after 10 days, and the Relative Percentage of Survival (RPS) was calculated using the following equation: (RPS = 1 - (mortality in vaccinates / mortality in controls) × 100 (Ma et al., 2017). Two groups of 10 fish for each treatment were kept non-infected but under similar conditions and served as a negative control group (Ghosh et al., 2016; Villumsen et al., 2014).

After challenge anterior kidneys of dead fish were cultured on blood agar plates for confirmation of the cause of death. Standard PCR using *Y. ruckeri*-specific 16S ribosomal RNA gene primers were used for confirmation of *Y. ruckeri* (Carson and Wilson, 2009).

Statistical analysis

Comparison of differences in mortality between groups was analyzed using the Kaplan– Meier survival analysis test (Log-rank value) (SPSS 22.0 version).

Results

Antibody titer

In all injection groups, the serum ELISA antibody titer increased significantly 30 days after immunization ($P< 0.05$). On day 60, there was significant difference just in FKC+LPS (Fig. 1). Although no significant differences could be determined in the mucus ELISA antibody titer of skin on day 30, the antibody titer of all injection and oral FKC groups significantly increased on day 60 (Fig. 2).

The ELISA antibody titer of intestine mucus did not show a significant increase on day 60. However, 30 days after immunization the antibody titer of FKC+LPS (I.P. and oral), I.P. and oral FKC groups increased significantly compared to fish of other groups and control (Fig. 3).

In bath challenge, *Y. ruckeri* concentration to induce 10, 25, 50, 75 and 90 percent mortality were 6.4×10^6, 2.9×10^7, 1.6×10^8, 9×10^8 and 4.2×10^9 CFU mL^{-1} respectively, whereas after I.P. challenge these amounts were 6.5×10^5, 6.1×10^6, 7.5×10^7, 9×10^8 and 8.5×10^9 respectively. The mortality in challenged fish increased along with increasing bacterial concentration in both challenge procedures.

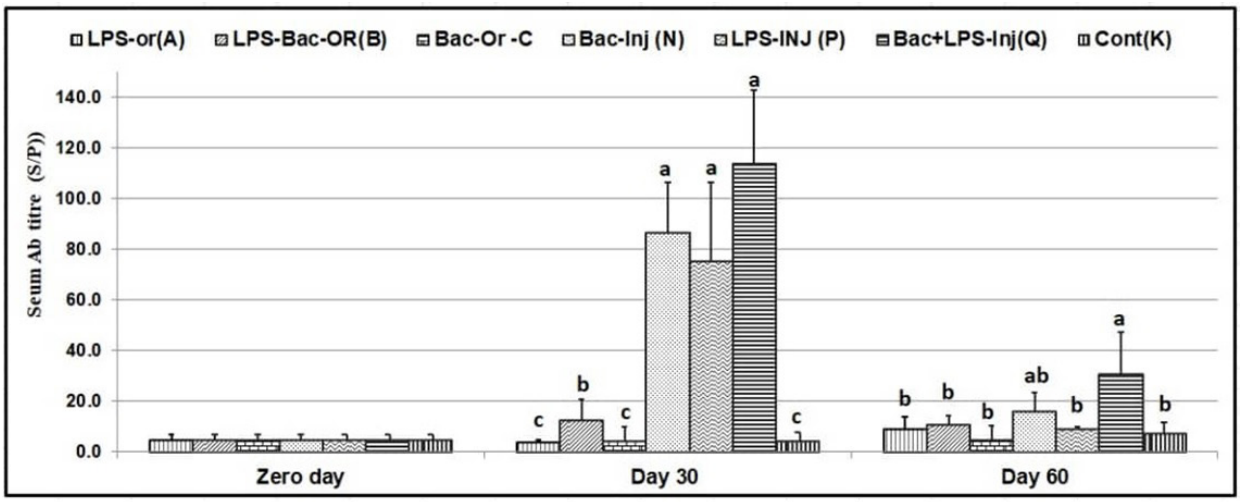

Figure 1. Serum antibody titer against *Y. ruckeri* in experimental groups in different sampling point using ELISA method. Statistical differences between treatment groups are indicated by different letter notations

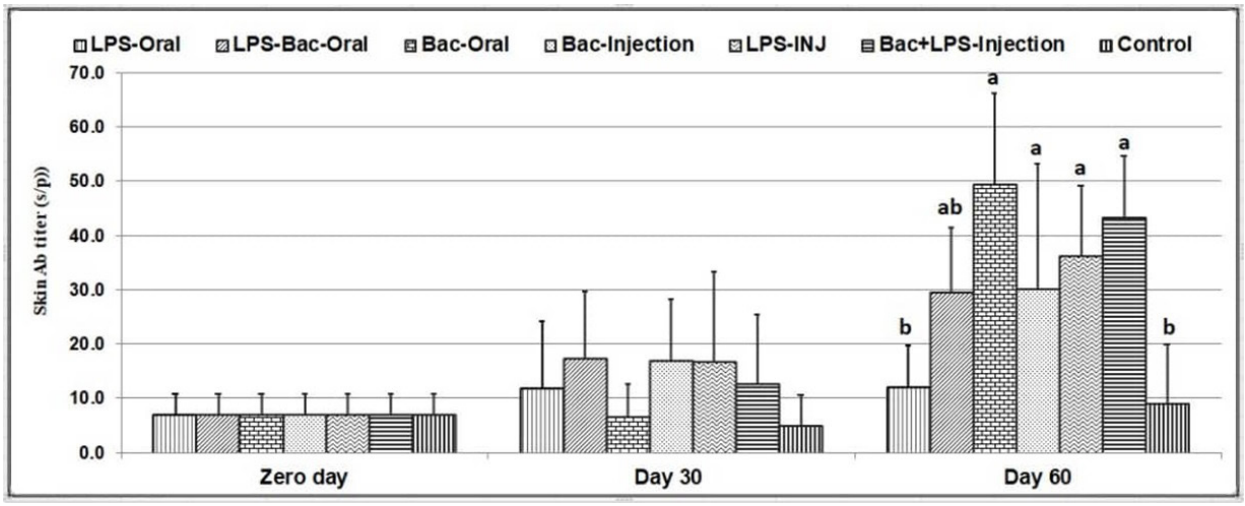

Figure 2. Skin mucus antibody titer against *Y. ruckeri* in experimental groups in different sampling point using ELISA method. Statistical differences between treatment groups are indicated by different letter notations

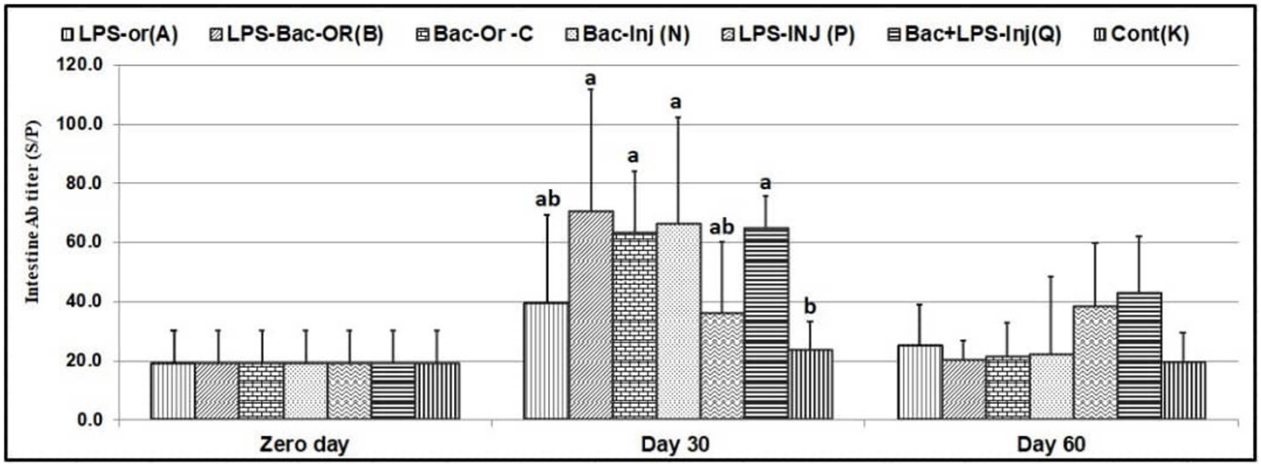

Figure 3. Intestine mucus antibody titer against *Y. ruckeri* in experimental groups in different sampling point using ELISA method. Statistical differences between treatment groups are indicated by different letter notations

The CPM and RPS rates after I.P. or bath challenge are presented in Table 1. The results indicated a significant decrease in mortality was observed in G_7 after challenge via injection and bath methods in comparison to control groups (P<0.05). In the injection receiving bacterin groups (G_2 to G_4) post-challenge survival rate was 76.47 %, 58.82 % and 52.94 % respectively, which was significantly higher (P<0.05) than the control group and oral receiving bacterin groups (G_5 to G_7) with 25, 17.65, 35.29 survival percentage. The survival percentage in bath challenge for G5 to G_7 was 37.5, 49.49 and 29.49, respectively, which was significantly (P <0.05) more than the control group (G_1).

Post-challenge mortality in both injection and bath methods revealed that the lowest mortality was in Bacterin + LPS treatment, which showed a significant difference compared to control treatment (P<0.05). Comparison of injection and bath challenge in oral treatments showed higher RPS in bath method compared to the injection method (Fig. 4-8).

Table 1. The lethal concentration of injection and bath challenge based on the probit analysis output

Lethal concentration (LC)	Bacterial concentration (CFU ml^{-1})
LC_{10}	6.4×10^{6}
LC_{25}	2.9×10^{7}
LC_{50}	1.6×10^{8}
LC_{75}	9×10^{8}
LC_{90}	4.2×10^{9}
Lethal Dose (LD)	
LD_{10}	6.5×10^{5}
LD_{25}	6.1×10^{6}
LD_{50}	7.5×10^{7}
LD_{75}	9×10^{8}
LD_{90}	8.5×10^{9}

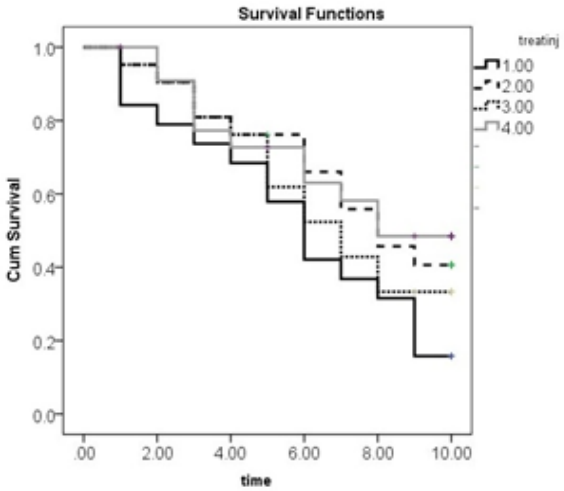

Figure 4. Survival rate of orally immunized fish challenged intraperitoneally with *Y. ruckeri* (Kaplan–Meier test)

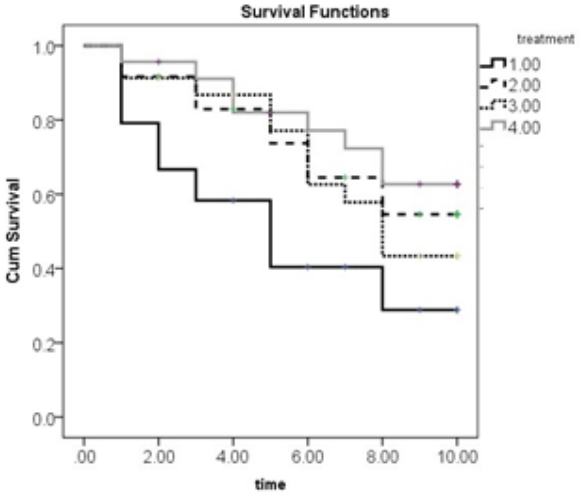

Figure 5. Survival rate of orally immunized fish challenged via bath with *Y. ruckeri* (Kaplan–Meier test).

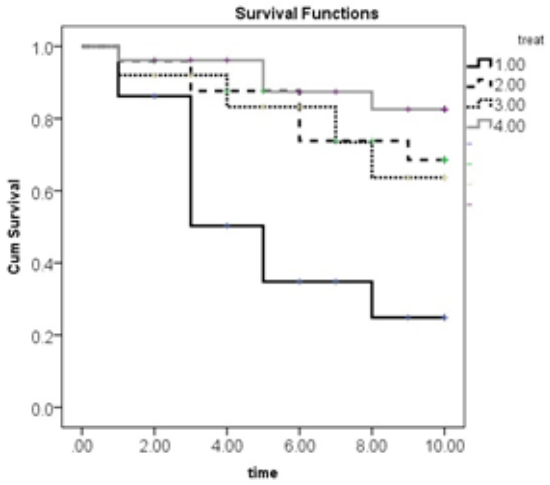

Figure 6. Survival rate of injection immunized fish challenged via injection with *Y. ruckeri* (Kaplan–Meier test)

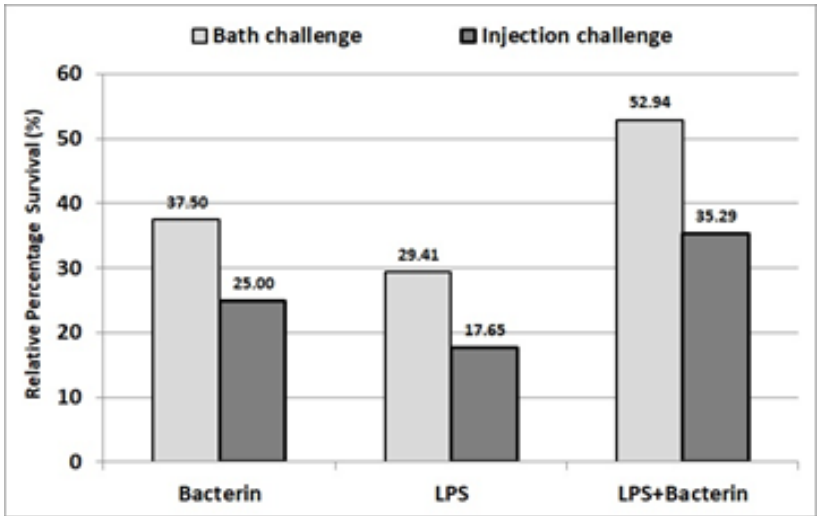

Figure 7. The RPS of orally immunized fish after I.P. and bath challenge by *Y. ruckeri*

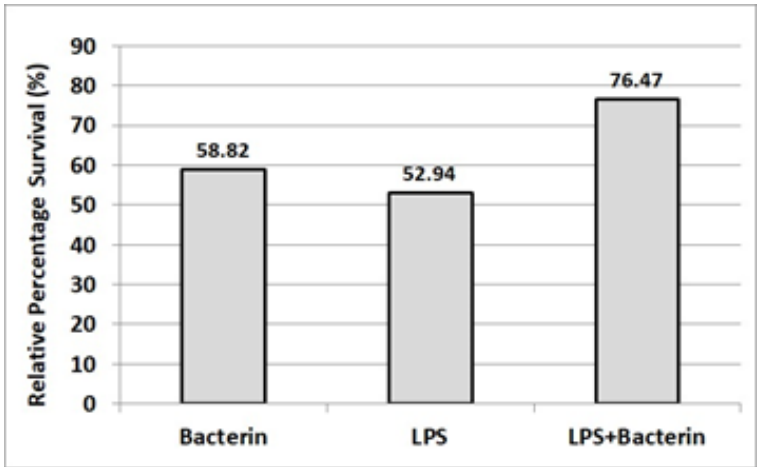

Figure 8. The RPS of injection immunized fish after I.P. challenge by *Y. ruckeri*.

Discussion

Depending on the fish species and production cycle, farmers prefer to select a vaccination regime that mostly confers strong and long-lasting protection such as I.P. injection method. Nevertheless, oral vaccination is the most comfortable and stress-free way of mucosal vaccination and expansive work is still devoted to the upgrading currently accessible vaccines and to design novel and efficient oral vaccination strategies (Embregts and Forlenza, 2016). The present study showed that *Yersinia ruckeri* bacterin could produce immune response in oral procedure but it was not enough. Also, we found an increased mortality in juveniles given LPS by feed during the challenge experiment. Intraperitoneal injection of LPS in juvenile rainbow trout has been shown to result in protection against a disease, but a complex of the LPS and bacterin resulted in a better protection.

The phenomenon of tolerance has been broadly studied in humans. By definition, tolerance is hyporesponsiveness, i.e. while a primary stimulation of an organism with a given antigen will lead to a specific response, a secondary stimulation with the same antigen may lead to only a minimal response or no response at all (Ziegler-Heitbrock, 1995). Tolerance to LPS has been demonstrated in rabbits and rats (Sanchez-Cantu et al., 1989). Oral tolerance was observed in rainbow trout against a soluble protein when the substance was administered simultaneously via the oral and intraperitoneal routes (Davidson et al., 1991).

Zapata et al. (1997) state that tolerance in young fish can only be induced to

T-dependent antigens and make the animal unable to combat an infection. This fact may explain why the LPS feeding fish survived poorly with the bacterial challenge in this study.

O'Donnell et al. (1994) monitored the fate of orally administrated *E. coli* LPS in brown trout (*Salmo trutta*) and revealed that large amounts of the compound were present in the epithelial cells of the lower intestinal segment. Generally, the efficacy of these vaccines in fish needs to be confirmed through pathogen challenge studies (Rombout et al., 2014).

The use of inactivated pathogens undoubtedly poses an advantage with respect to environmental safety but, together with the lack of strong mucosal adjuvants, it might be

one of the causes of the weak and short-term protection provided by these vaccines. Researcher's studies have shown that vaccines containing immunogenic fractions such as LPS or ECP can generate higher protection compared to inactivated whole-cell bacterins. Because whole bacteria cannot be taken up by the enterocytes, the release of higher amounts of LPS in the second gut segment may be an advantage of this antigen delivery system (Anderson and Jeney 1991; Jeney and Anderson 1993; Ispir et al., 2009; Joosten et al, 1995).

The importance of the addition of LPS in vaccine designs has been reported by several authors. Saeed and Plumb (1986), worked on *E. ictaluri* vaccine and showed that mortality rate in LPS-vaccinated channel catfish (via single injection) was reduced (36.7%) compared to control group (70%). In the current study the RPS was followed by exposure to a virulent challenge 60 days after a single injection vaccination with LPS of *Y. ruckeri* was 52.94 % that is partly similar to the previous data. In other research Sun et al. (2011), studied the immune protection in grass carp (*Ctenopharyngodon idella*) against *Aeromonas hydrophila* infection using bacterial LPS as a vaccine and the RPS rate was reduced to 55.6–83.3%. Dehghani et al. (2012) investigated the efficiency of formalin-killed, heat killed and LPS vaccines against *A. veroni* and *A. hydrophila* in rainbow trout and 34% RPS was found for the LPS vaccine. These dissimilarities in survival rate can be due to the differences in injection dose, the method of LPS extraction, challenge time, bacterial species and concentration in the challenge as well as fish species and size.

Mao et al. (2013) used LPS-vaccine for control of *Pseudomonas putida* infection in large yellow croaker, *Pseudosciaena crocea* and their experimental groups showed a significant increase of RPS after challenge. The results of this study are in agreement with the results in previous investigations.

Numerous investigators have argued that LPS can be applied as a feed additive to enhance the nonspecific immunity due to LPS resistance against gastric destruction (Kohchi et al., 2006; Selvaraj et al., 2006; Nya and Austin, 2010). Dietary supplementation of LPS was also effective in preventing disease caused by *A. hydrophila* and in stimulating the innate immune response of rainbow trout (Nya and Austin, 2010). Our results revealed that the LPS+bacterin (injection) is a protective immunogenic compound for rainbow trout against yersiniosis, at least at the assayed concentration, and this is in agreement with previous studies which reported that LPS-enriched vaccines can offer better protection against bacterial diseases in fish. Lately, Welch and LaPatra (2016) demonstrated that *Y. ruckeri*-derived LPS at doses of 1 ng per fish and above resulted in perfect protection against *Y. ruckeri* infection. However, this finding was not obtained in the present study by injection of 30 µg ml^{-1} per fish. In another research, gilthead seabream (*S. aurata*) intraperitoneally injected with purified LPS from *A. salmonicida* and *V. anguillarum* demonstrated increased resistance to pathogen challenge (Boltana et al., 2014; Velji et al., 1990).

To determine the effects of *E. coli* LPS on the regulation of immune response and protein expression of striped catfish (*P. hypophthalmus*) juvenile fish were injected with 3, 15 or 45 mg *E. coli* LPS kg^{-1} and challenged with *Edwardsiella ictaluri* (Hang et al., 2013). The authors found that 3 mg LPS kg^{-1} yielded the best results, whereas higher dose

led to immune suppression. These investigators also described that dietary supplementation of *E. coli* LPS (at 0.01%) enhanced the immunity of striped catfish (*P. hypophthalmus*) and provided higher protection to fish against bacterial infection than that of the 0.05% LPS supplementation group (Hang et al., 2016). In fish, both enterocytes and intraepithelial macrophages have been revealed to play a role in antigen uptake in the second gut segment. Also intraepithelial macrophages can take up and transport antigens particularly when antigens where coupled to enteric adhesion molecules. Besides, the motile non-resident macrophages can potentially transport antigens at peripheral sites (Companjen et al., 2006; Rombout and van den Berg, 1989). Investigators showed that if the antigens can reach the correct inductive sites after oral vaccination, effective stimulation is produced and oral tolerance is not induced (Kim and Jang, 2014).

Mucosal vaccines are most effective when they mimic the natural route of infection and stimulate suitable innate besides adaptive immune responses. In most studies, to evaluate the efficacy of the vaccine challenge, experiment was not always performed using the natural route of infection. IgM is typically the earliest antibody isotype to be made in response to an antigen. In the present study challenge was conducted in both I.P. and bath method, which is closer to the occurrence of the disease in natural conditions. It has already been mentioned that oral vaccination can lead to increased serum IgM levels, and in some infections serum IgM levels correlated to diseases' resistance or susceptibility (Rombout et al., 1989; Tobar et al., 2015). So this can affect the outcome of the challenge. On the other hand, it cannot be accepted that bypassing the mucosa by directly injecting the pathogen into the peritoneal cavity or muscle might also bypass crucial mucosal immune mechanisms. Therefore, the consequent activation of local as well as systemic responses might lead to an underestimation of challenge results. The present study showed that oral administration of antigens increased the titre of mucosal antibodies, especially in the intestine, and the length of mucosal immunity response was longer than blood immunity. Nevertheless, in terms of IgM production, I.P. administered antigens induced a greater IgM titer in serum than orally administered vaccines.

LPS molecules, similar to enterotoxins, might in fact retain the ability to trigger strong local responses and improve the efficiency of suboptimal inactivated vaccines. Not only enterotoxins but also enteric pathogens themselves (e.g. E. *tarda, V. anguillarum, E. coli, Y. ruckeri*), either as live vehicles or as inactivated pathogens, can provide adequate stimulatory signals and act as adjuvants for currently suboptimal oral vaccine formulations.

In the present study, intraperitoneal injection was found to be the most effective method as even the used dose of bacterin + LPS mixture was sufficient to get appropriate protection against the pathogen. This enhanced protection observed might be due to the adjuvant effect of available LPS with bacterin, which could continuously induce the macrophage to elicit nonspecific cellular immune response.

Our consequences revealed that oral vaccine with LPS alone cannot stimulate an appropriate level of protection to that normally observed after challenge The type of antigens and the dosage of the oral vaccines are important to achieve good and long-lasting protection (Tebbit et al., 1981; Johnson

et al., 1982). Because of the reduced labour requirements and reduced stress on the fish during vaccination, efficacious oral vaccines would be preferred by rainbow trout farmers in Iran. However, no such vaccines have been licensed to date.

The results obtained in this study showed that LPS could have an important role in the protection against yersiniosis but this effect becomes significantly higher when used with bacterin. The vaccine candidate (LPS+bacterin by I.P. route) is being further evaluated based on their protective efficacy via various challenges. So, LPS could be considered as protective antigens to design potential vaccines against this pathogenic micro-organism and bath challenge is preferable for assessing the effectiveness of mucosal vaccines. By modifying the doses of LPS and the length of the stimulatory period, we will hopefully achieve more favorable results.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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تأثیر لیپوپلی‌ساکارید *یرسینیا راکری* بر پاسخ ایمنی در ماهی قزل آلای رنگین کمان (*Oncorhynchus mykiss*) به روش تجویز داخل صفاقی و خوراکی

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چکیده

زمینه مطالعه: *یرسینیا راکری* از عوامل بیماری‌زای مهم ماهیان است که اخیراً به دلیل زیان‌های اقتصادی در صنعت ماهی قزل آلای رنگین کمان در سراسر جهان توجه بیشتری را به‌خود جلب کرده است. امروزه تحقیقات در مورد واکسن‌های جدید خوراکی به دلیل راحتی و سهولت تجویز گسترش یافته است.

هدف: در مطالعه حاضر، تأثیر لیپوپلی‌ساکارید بر محافظت‌کنندگی باکترین *یرسینیا راکری* در ماهی قزل آلا رنگین کمان مورد بررسی قرار گرفت.

روش کار: بدین منظور، ۷۲۰ قطعه ماهی قزل آلای رنگین کمان نوجوان به صورت تصادفی به ۸ گروه در سه تکرار تقسیم شدند. گروه‌های ۱ تا ۴ به ترتیب، بافر نمکی فسفات، باکترین، LPS و باکترین + LPS را به صورت تزریق داخل صفاقی دریافت کردند. گروه‌های ۵ تا ۸ به ترتیب بافر نمکی فسفات، باکترین، LPS و باکترین + LPS را به صورت خوراکی دریافت کردند. نیمی از ماهیان موجود در هر تیمار، ۶۰ روز پس از ایمن‌سازی با $LC_{۵}$۱ از جدایه‌ی حاد *یرسینیا راکری* به روش داخل صفاقی و نیم باقیمانده‌ی ماهیان موجود در گروه-های دریافت‌کننده‌ی خوراکی باکترین، با $LC_{۵}$۱ از جدایه‌ی حاد *یرسینیا راکری* به روش داخل حمام نیز چالش شدند.

نتایج: نتایج الایزا نشان داد که LPS خوراکی به تنهایی قادر به افزایش ایمنی هومورال در سرم نبود اما LPS به همراه FKC توانست سطح پادتن در سرم و مخاط را به طور قابل توجهی افزایش دهد ($P<۰/۰۵$). بر اساس نتایج پس از چالش در گروه‌های دریافت‌کننده‌ی تزریقی باکترین ($G_{۲}$ تا $G_{۴}$)، میزان درصد بقاء در چالش تزریقی به ترتیب ۷۶/۴۷ ، ۵۸/۸۲ و ۵۲/۹۴ به‌دست آمد که به طور معنی‌داری ($P<۰/۰۵$) بیشتر از گروه های ($G_{۵}$ تا $G_{۷}$) به ترتیب با درصد بقاء ۲۵، ۱۷/۶۵ و ۳۵/۲۹ بود. میزان درصد بقاء در چالش به‌روش حمام در گروهای $G_{۵}$ تا $G_{۷}$ به ترتیب ۳۷/۵، ۴۹/۴۹ ۲۹/۴۹ بود که به طور معنی‌داری($P<۰/۰۵$) بیشتر از گروه شاهد ($G_{۱}$) بود.

نتیجه گیری نهایی: نتایج نشان داد که LPS دارای اثر معنی داری بر القای ایمنی محافظتی به ویژه در روش تزریق داخل صفاقی در برابر باکتری *یرسینیا راکری* است. به علت حفاظت قابل قبول، روش فوق می‌تواند یک کاندید امیدوارکننده و جدید برای کنترل یرسینیوزیس در قزل آلای رنگین کمان باشد.

واژه‌های کلیدی:

یرسینیا راکری، قزل آلای رنگین کمان، لیپوپلی‌ساکارید، پاسخ ایمنی، واکسیناسیون

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