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Investigation of the bacterial effects of *Aeromonas hydrophila* along with Freund adjuvant on the expression of lysozyme-c gene in common carp (*Cyprinus carpio*)

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Abstract

Among the bacterial diseases of the aquatic animals, *Aeromonash ydrophila* is one of the known pathogenic factors in fish causing complications such as fin rot, skin sores, and deadly haemorrhagic septicaemia. Therefore, the use of vaccines along with various synthetic and natural adjuvants for better immunization against this disease is important and is examined in different studies. In this study, the bacterial effects of *Aeromonas hydrophila* along with Freund adjuvant on the expression rate of lysozyme-c gene in the anterior kidney tissue of the common carp fish (*Cyprinus carpio*) were measured. 180 fish specimen with an average weight of 50g were divided up into 3 groups and each group into 3 repetition of 20 specimen to this end. Groups 1 to 3 were respectively immunized by using physiology serum (as control), bacteria without adjuvant and bacteria along with Freund adjuvant in the form of intraperitoneal. Three groups each were challenged with the deadly concentration of *Aeromonas hydrophila* in the 28th day of the research. Sampling the anterior kidney tissue was done before the challenge and intervals of 12, 24, 72 hours and also 7 days after the challenge. The expression rate of lysozyme-c in the taken samples was finally measured by doing PCR experiment in the real time. According to the obtained results, the level and the procedure of the studied gene expression in both immunized groups showed a significant increase than the control group ($P<0.05$). It can generally concluded that immunization by the bacterial *Aeromonas hydrophila* in carp (*Cyprinus carpio*) caused an increase in the level and the improvement of the procedure of the gene expression of lysozyme-c after facing the bacterium causing the disease.

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Introduction

Common carp with the scientific name of *Cyprinus carpio* is considered as the most important hydrothermal rearing fish resulting in about 5.6 percent of the world aquaculture production (FAO, 2010).

Aeromonas hydrophila is a gram-negative bacterial pathogen and is considered as one of opportunist pathogenic bacteria in a lot of fresh water and brackish water fish species and especially Cyprinidae in Asian countries and on the other hand ever increasing growth of rearing aquatic animals in the concentrated form caused more spread of these diseases including contaminating with this bacterium. complications and damages arising from contamination with this bacterium including a broad spectrum of skin injuries to common contamination such as rotting skin and tail and also hemorrhagic septicemia resulting in a big economic loss in aquaculture throughout the world (Lee, 2001).

By reason of the high antigen difference of *Aeromonas hydrophila*, a successful commercial vaccine for this bacterium isn't presented to the market so far. From last years alongside the attempt to make a vaccine against various diseases (in different animal species), the use of the materials known as adjuvant or the vaccine assistance in improving the effects and the functions of vaccines are always considered as well. Utilizing aiding immunization materials along with vaccines cause a decrease in using vaccine, to help with enough absorption of vaccine, to slow the process of vaccine absorption, to help with presenting the vaccine in low temperature and decrease the side effects arising from the processes of preparing vaccines.

Freund adjuvant is one of the most famous and common used adjuvants in aquatic animals' vaccines. This adjuvant cause the improvement of response to the vaccine through the slow presentation of antigen, and also stimulation of immunization system especially an increase in T helper lymphocytes (Freund, 1956). The disadvantages of this adjuvant are the toxic effects, tissue stimulation and Granuloma and pain production. The other more important bad effects such as Carcinogenesis in mouse has been reported as well (Byars & Alison, 1990; Gupta *et al.*, 1993; Petrovsky & Aguilar, 2004; Evensen *et al.*, 2005; Sivakumar *et al.*, 2011).

The exact information obtained by examining the mechanisms relating to immunization system in molecular level (gene expression) can be used to better and exact understand the relationship between immunization and the host protection against invasive factor and widely in promoting prevention and treatment of the diseases. That is why the investigation of the immunization mechanisms of the animals including fish in molecular level has been considered in recent years and is increasingly enhancing (Raida *et al.*, 2011).

Lysozyme (EC.3.2.1.17) is one of the three hydrolase enzymes that has a role in defensive and immunization system of the body directly influencing on the gram-positive bacteria and the internal layer of the gram-negative bacteria and consequently causing the destruction of the external wall by the other complement system and enzymes (Palaksha *et al.*, 2008).

In fish, lysozyme is mainly in kidney, skin, and gills where there are the high risk of the attack of pathogens. Lysozyme has a general role in the defensive mechanism against infectious diseases and its capability in the digestion of peptidoglycan bacteria presents it as an important factor in defense against pathogen bacteria (Montet & Ray, 2009).

The purpose of the present research is the effects of vaccination by using the bacterial *Aeromonas hydrophila* along with Freund adjuvant on the expression rate of the lysozyme-c gene.

Materials and methods

Fish and grouping

180 common carp fish with an average weight of 50g and an age lower than 1 year were provided from one

of the hydrothermal fish rearing centers (Shoushtar, Khouzestan, Iran). They were kept in 500 liter plastic (fiberglass) tanks for 2 weeks to be adapted to the laboratory conditions.

Fish were divided into 3 groups and each group into 3 repetition (each repetition consists of 20 fish) in separate 120 liter glass aquaria with the same conditions. Groups were immunized as follows:
Group 1 control.

Group 2 were vaccinated in the form of intraperitoneal by using the bacterial *Aeromonas hydrophila* without adjuvant.

Group 3 were vaccinated in the form of intraperitoneal by using the bacterial *Aeromonas hydrophila* in combination with Freund adjuvant.
During this period and the experiment period, the quality conditions of the tank water were kept in a desirable span by using the biological and mechanical filter, heaters equipped with thermostat and periodic replacement of the water (and replacing it with water having the same and suitable condition). Some of the quality factors of the water consisted of the temperature of 26 - 28° C, pH= 7.8, dissolved oxygen in water 8 - 10 ppm, ammonia and nitrite about 0 and nitrate 10 mg/l.

The used food was the fish special granule food and the amount of daily feeding was considered about 3% weight of the biomass in two to three separate meal.

Preparing and injecting vaccine
After culturing *Aeromonas hydrophila* in TSB environment for 48 hours in a temperature of 37° C, 1% formalin was added to the environment and was kept in a 4° C refrigerator. After passing 24 hours, cultured bacteria were separated from the culture medium by doing centrifuge in rotation of 3500 within 10 minutes and the sediment of the bottom of the pipes was washed by physiology serum three times in order to ensure the complete deletion of the culture medium materials and added formalin (Rohovec *et al*., 1981; Nayak *et al*., 2004; Bastardo *et al*., 2012). First by McFarland turbidity meter, its turbidity was regulated to the extent of number 7 pipe (number 7 pipe has a concentration equal to 2.1×10^9) and then the concentration was regulated to the extent of 10^9 bacterium in per milliliter by diluting it (Sun *et al*., 2011).

In the next stage, bacterium with Freund adjuvant were mixed at a 1:1 ratio and were completely homogenized by carrying out the vertex and the transmission with pressure from syringe needle with 60 cc volume. As a result, the final concentration of the prepared bacterium was equal to 5×10^8 (Habeeb *et al*., 2007).

Vaccination
Immunization for each group was the 1st and the 14th days of the experiment. In the first and the second turn of the immunization of group number 3 the complete adjuvant and incomplete Freund were respectively used. And the injection for all fish was in the form of intraperitoneal and to the amount of 1% weight of the body (Evensen *et al*., 2005). Fish were anaesthetized before injecting the anaesthetic material MS222 into dishes equipped with aerator.

Challenge and sampling
After culturing bacterium in a TBS medium for 48 hours in a temperature of 37° C, separation of the bacterium was carried out using centrifuge in the rotation of 3500 for 15 minutes. The separated bacteria were washed by sterile PBS. The successive dilution (10^5 to 10^9) from bacterium was prepared using the McFarland pipes. After infecting the fish in this dilution, first the fish of each group were anaesthetized using MS222 by the concentration of 40 ppm, then 0.1 ml from the bacterial dilution was injected in the form of intraperitoneal and the casualties of each dilution were recorded for three days and after the fish death, the cause of death as a result of bacterial infection was ensured by reculturing the bacterium from the internal organs, and LD_{50} was measured using the Pribit software. The obtained 50 percent deadly dose for this bacterium was equal to 10^8 (Alishahi *et al*., 2010).

Extracting RNA and manufacturing cDNA

Extracting RNA was carried out using acid guanidinium isothiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 2006).

In the last phase of extraction, 50 microliter sterile buffer Tris was added to the RNA sediment and was kept in a -70° C freezer by the manufacturing time of cDNA.

After extracting RNA, its concentration was calculated by using spectrophotometer NanoDrop (Eppendorf, Germany) instrument and the absorption ratio 260/280 beyond 1/8 was confirmed to manufacture cDNA.

cDNA synthesis was carried out using random Hexamers method, commercial kit (Amplisense, Russia). The mentioned kit consists of 3 reserve solution including RT-mix, RT-G-mix, and Revetase enzyme.

Polymerase chain reaction (PCR)

In order to confirm the expression of the studied gene, first the reaction of PCR was carried out the synthesized cDNA samples of kidney tissues before performing PCR in real time. A list of the used primers in this study, succession and the length of the fish which was reproduced by them is presented in table 1.

The volume of PCR reactions considered 25 microliter. In order to determine the suitable temperature of the primer connection and also the most suitable concentration of $Mgcl_2$ for lysozyme-c and ß-actin, the concentrations of 1, 2 and 3 milli molar $Mgcl_2$ and the temperatures 52-60° C were tested.

PCR reaction was carried out in thermocycler instrument. 35 cycles were considered as the number of cycles doing the PCR reaction for the gene reproduction. A sample of PCR reaction lacking cDNA was used as the negative control in each PCR reaction.

PCR reaction in real time

In order to evaluate the expression changes of Lys-c gene, the PCR test in real time, comparative method ΔΔCt and the instrument ABI Step One Plus (America) were used. The test method was based on the use of cybergreen color.

In this study the ß-actin gene of the common carp fish was used as the internal control, and the expression changes of the target gene were evaluated in accordance with the fixed expression of this gene. The samples of the negative control lacking cDNA were considered in each reaction.

50 cycles were considered as the number of cycles doing the reaction for each gene and the reaction steps were as follows:

The used method in analyzing data was based on the ΔΔCt method. The comparative amounts of the expression of the considered gene in comparison with the expression of ß-actin in each tissue were measured by using StepOne software and were reported based on the ΔΔCt method. The comparison of the gene expression of Lys-c in groups and various times were calculated based on the $2^{-\Delta\Delta Ct}$ formula (Pfaffi *et al.*, 2001).

Statistical analysis

SPSS®16 software was used for statistical analysis of the data. All data were indicated based on the mean ± standard deviation. The significant level of the statistical tests was considered $P<0.05$ for all tests and ANOVA test and LSD test were used for comparison among various means.

Results

Investigation of the comparison of the expression rate of Lys-c gene among the tested groups

Figure 1 indicates that no significant differences were observed among groups before the challenge ($P>0.05$).

12 hours after the challenge, the expression rate for both vaccinated groups was significantly higher than

the control group (P<0.05). Between both vaccinated groups the expression level of the group receiving Freund was higher than the group vaccinated without adjuvant as well (P>0.05).

24 hours after the challenge, both groups receiving the vaccine have the higher amounts of the expression in comparison with the control group (P<0.05). But no significant differences were observed between the two vaccinated groups (P>0.05).

In the third day after the challenge, the gene expression rate in group vaccinated by adjuvant was significantly higher in comparison with group 1 and 2 (P<0.05).

One week after the challenge, no significant differences were observed among groups (P>0.05) (fig. 1).

Table 1. Succession and the length of the fish which was reproduced by the used primers in this study along with the temperature of the primer connection.

Gene	Length	Succession	Temperature
β-actin	260 bp	Pioneer: AGACATCAGGGTATGGTTGGT	58° C
		Reverse: CTCAAACATGATCTGTGTCAT	58° C
		Pioneer: GTGTCTGATGTGGCTGTGCT	58° C
Lys-c	332 bp	Reverse: TTCCCCAGGTATCCCATGAT	58° C

The study of the process of the expression changes of lysozyme-c gene in each one of the groups in different hours of sampling

Figure 2 shows that no significant increase or decrease processes were observed in different stages of sampling in the control group (P<0.05).

In vaccinated group, the gene expression rate in a stage before the challenge was in the lowest level. In the stage 12 hours after the challenge the gene expression significantly increased in comparison with the stage before the challenge (P<0.05). After this stage, the expression process descended and it continued by the seventh day.

Table 2. The number of carried out cycles for each gene.

Stages	Temperature	Time	Cycles
Initial denaturation	94° C	5 min	1
Denaturation	94° C	15 sec	50
Primer binding and elongation	60° C	45 sec	50

In vaccinated group + Freund, the gene expression rate in the stage before the challenge was in the lowest level. In the stage 12 hours after the challenge, it significantly increased in comparison with the stage before the challenge (P<0.05). After this stage, the expression process descended and it continued by the seventh day and on the seventh day after the challenge had lower significant difference among the other steps after the challenge (P<0.05), but with the step before the challenge had no significant differences (P>0.05) (Fig. 2).

Discussion

In this survey the bacterial effects of *Aeromonas hydrophila* along with Freund adjuvant on the expression of Lys-c gene in common carp fish were studied.

In the present study we observed the expression increase of the Lys-c in vaccinated groups in 12 hours after the challenge.

Kozinska and Guz (Kozinska and Guz, 2004) reported the increase in macrophage activity and the

concentration of serum total antibody after the vaccination of the common carp by *Aeromonas* bacterium.

Vaccination of the tilapia fish by some species of *Aeromonas* and *Pseudomonas* was separately reported by Osman *et al.* (Osman *et al.*, 2009).

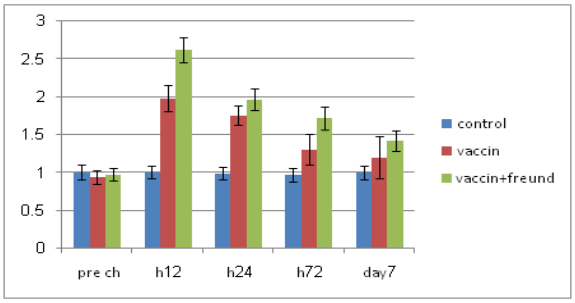

Fig. 1. Comparing the gene expression of Lys-c in the anterior kidney tissue of the various group in different times of sampling. The heteronymous letters in charts related to each step show significant differences (P<0.05).

Bastardo *et al.* (Bastardo *et al.*, 2012) successfully used a bivalent vaccine of *Aeromonash ydrophila* and *Lactococcus garvieae* in rainbow trout (*Oncorhynchus mykiss*).

The above studies correspond with the results of the present research from the view point of the possibility of stimulating the immunity system of the fish using different immunization including bacterium to produce better immunity response.

The used adjuvant in this study is Freund and alongside the carried out studies in the field of aqua animal vaccination, the role of different adjuvant were also studied along with the vaccine types.

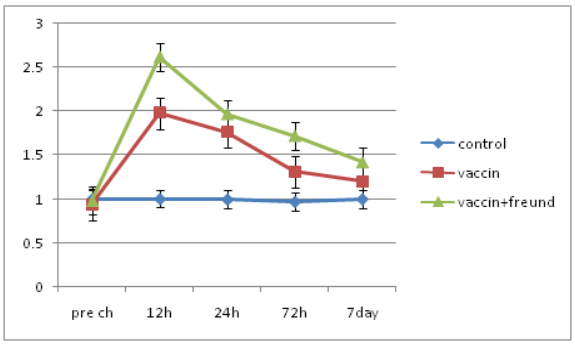

Fig. 2. increase and decrease process of the Lys-c gene during the sampling period for each group.

The results of this study indicated that the use of the Freund's complete adjuvant caused faster induction of the expression of Lys-c gene in 12 hours after the challenge in the kidney anterior tissue. These results also correspond with the results of the other researchers.

The obtained results of Paterson and Fryer (Paterson & Fryer, 1974) and Oliver *et al.* (Olivier *et al.*, 1985) studies based on the improvement of the immunity response as a result of using the Freund's complete adjuvant along with *Aeromonas salmonicida* in salmon are among the first reports about the effects Freund adjuvant in fish. In the next researches an increase in the Phagocytosis activity, respiratory explosion and the natural deadly cells as a result of the resistance increase against infection arising from *vibrio anguillarum* after the injection of Freund adjuvant in trout fish were reported (Kajita *et al.*, 1992).

Sun *et al.* (Sun *et al.*, 2011) obtained the most protection of the *Euryglossa orientalis* against the disease by the bivalent vaccine of *Edwardsiella tarada* and *vibrio anguillarum* along with Freund's incomplete adjuvant.

Jiao *et al.* (Jiao *et al.*, 2010) confirmed the positive function of the Freund adjuvant in the Japanese *Euryglossa orientalis* and in comparing it with the produced adjuvant from Aluminium hydroxide, he reported the Freund adjuvant function more efficiently.

Concerning the obtained results of the Lys-c gene expression in comparison with the control group, both immunized groups show significantly higher levels of the gene expression in all steps of sampling. Mohanty and Sahoo (Mohanty & Sahoo, 2010) reported the expression changes of genes including IL-1ß, TNF-α, lysozyme-c and g after the infection with *Edwardsiella*. According to the results of this study Lysozyme-c and g during 6 to 12 hours after the infection reached the maximum expression and it was the same in IL-1b but TNF-α in the challenged group

not only it showed no increase but also from the step 48 hours after the challenge the expression decrease of TNF-α gene was observed. The present study also correspond with the carried out research by Mohanty and Sahoo (Mohanty & Sahoo, 2010) since 12 hours after vaccination Lysozyme-c had the most expression rate.

Saurabh *et al.* (Saurabh *et al.*, 2011) examined the expression of genes related to immunity in three organs such as kidney, skin, and liver in *rohu lebeo rohit*a during the infection with *Argulus siamensis* so that the expression of the lysozyme-c gene in kidney had no significant differences between unpolluted control group fish and different degrees of the fish infected with lice, and the same results were obtained in skin and liver which were different from the obtained results of the present study that probably the difference reason is for the stimulus kind of the body immunity system which is the killed bacterium (vaccine) in the present study.

In Freund adjuvant group which had the best function in the expression rate of lysozyme gene, the lowest level of the expression was observed in the step before the challenge. 12 hours after the challenge the gene expression significantly increased in comparison with the step before the challenge and it reached the highest level among all fivefold steps of sampling ($P<0.05$) that probably this expression increase is due to the increase in the activity of lysozyme serum of common carp after the challenge with pathogen bacterium.

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