An inactivated infectious pancreatic necrosis virus immersion vaccine used in rainbow trout (*Oncorhynchus mykiss*) (Walbaum, 1792) and the effect of Montanide IMS 1312 VG as adjuvant on the vaccine efficacy

S Rashidi Monfared[1], M Akhlaghi[1*], S. A Pourbakhsh[2], R Fallahi[2], S Soltanian[1], A Yektaseresht[1]

[1] Department of Veterinary, Shiraz University, Shiraz, Iran

[2] Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran

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Abstract

Since infectious pancreatic necrosis virus (IPNV) is the most predominant strain causing fatal viral disease in Iranian fish farms, an inactivated vaccine for this fatal pathogen was developed. Additionally, the effect of the above-mentioned vaccine was investigated against IPNV using Montanide 1312 VG adjuvant. Two hundred and forty healthy fish with an average weight of 3.00 ± 0.45 g were vaccinated using immersion method with ethylene amine-inactivated IPN vaccine in combination with Montanide IMS 1312 VG for 5 min at 11 ± 1°C. The control group was vaccinated with sterile phosphate-buffered saline. The immersed vaccine with Montanide IMS 1312 VG adjuvant increased the expression of immune system genes such as IgM, MX, and CD8 within 30 days of vaccination period. The expression of IgM, Mx ($P<0.05$) and CD8 ($P>0.05$) genes was higher for the vaccine containing 10g of Montanide than that of 50g of Montanide. In fish vaccinated with the adjuvant, the relative percent survival was notably higher than of fish vaccinated without the adjuvant and the control group. Results showed that the ethylene amine-inactivated IPNV vaccine by the immersion method with Montanide IMS 1312 VG significantly has a strong protective immunity against IPNV in rainbow trout.

Correspondence: M Akhlaghi, Department of Veterinary, Shiraz university, Shiraz, Iran (e-mail: akhlaghi@shirazu.ac.ir).

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Introduction

First isolation report of infectious pancreatic necrosis virus (IPNV) was in 1958 by Wolf *et al.* This viral disease annually causes irreparable economic losses to the salmonid fish breeding industry around the world (Raissy, Momtaz, Ansari & Moumeni 2010; Ballesteros Rodriguez Saint-Jean, Encinas, Perez-Prieto & Coll 2015). The viral agent of the disease is a RNA virus with two 60 nm-strands containing two genomic regions A and B, belonging to the Aquabirnavirus of Birnaviridae (Biering, Melby & Mortensen 1997; Roberts & Pearson 2005; Lokka & Koppang, 2016). The IPNV is known as a systemic and acute infectious agent in rainbow trout (*Oncorhynchus mykiss*), especially in fish with an age of six months and a weight range of 1-3 g (Wolf 1988; Mutoloki, Munang'andu & Evensen 2015). The target tissue of this virus is the exocrine region of the pancreas which causes tissue lesions. However, the virus in the absence of lesions in tissues such as the kidney, liver and spleen has been detected by molecular methods (Ahmadi, Oryan, Akhlaghi & Arsalan 2013). The vaccination is one of the anticipated way to prevent and reduce the disease spread. Many studies have been conducted to develop an effective vaccine against IPN virus in rainbow trout, both forms of inactivated and recombinant and subunit vaccines (Brudeseth, Wiulsrød, Fredriksen, Lindmo, Løkling, Bordevik, Steine, Klevan, Gravningen 2013; Gudding, Lillehaug & Evensen 2014; Embregts and Forlenza 2016). Nonetheless, many of these vaccines are not agreed for the commercial use (Heras, Rodriguze & Perez-prieto (2010)). The large-scale production native vaccines are important because of the varying levels of surface antigens of the virus as well as the dominant species in each the ecosystem region. Immersion vaccination is a suitable method for mass vaccination of a high number of small fish. No commercial immersion vaccine for viral disease has been developed because of the low rate of efficacy obtained during the experimental studies (Hawang, Kwon, Kim, Jung, Park & Son 2016). Nowadays, most vaccines are relying on adjuvants as platforms to promote the immune response (Hawang *et al*., 2016). There are several commercial adjuvants available to vaccinate salmon and rainbow trout (Aucouturier, Ganne & Laval 2012; Hawang *et al*., 2016). Montanide 1312 VG (SEPPIC, France) is considered as an antigenic fish is recommended for the immersion and bathing vaccination (Hawang *et al*., 2016; Soltani, Shafiei, Yosefi, Mosavi & Mokhtari 2014). Montanide IMS 1312 VG is composed of water-dispersed liquid nanoparticles as a means of stimulating vaccine delivery. It is also suitable for the mass immersion vaccination. The early onset of immunity is facilized by the use of adjuvants, long duration of effector responses, for instance, antibody formation or cytotoxic T cell activity, and render booster immunizations dispensable (Hawang *et al*., 2016). As a result of using aqueous adjuvants, both cellular and humoral immune responses in fish are enhanced, since they improve antigen uptake

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via fish surface. It has been shown that the skin including the lateral line canal, gills, stomach and posterior intestine act as the main uptake sites for both groups of inactivated and live bacteria (Khimmakthong, Deshmukh, Chettri, Bojesen, Kania & Dalsgaard 2013); Therefore, the aim of this work was to produce an IPNV-killed vaccine in relation to the prevalent strain of the virus in Iran and to investigate the potential of adjuvant volume used to improve the vaccine efficacy.

Materials and Methods

Fish

Two hundred and forty rainbow trout (*O. mykiss*) with an average weight of 3.00 ± 0.45 g were provided by a farm in the Karaj province, Iran, to conduct this experiment. These fishes did not present any sign of infection neither by IPNV nor by other pathogens. Before the beginning of each experiment, six fish were randomly used for reverse transcription polymerase chain reaction (RT-PCR) analysis. Kidney and spleen tissues were collected for the RT-PCR examination to verify IPNV-free fishes.

Fish handling

Fishes divided into four groups, each group with sixty animals. One of the groups was considered as a control. These groups were kept in three 1000 L fiberglass containers with 900 L of fresh water and a renewal rate of 9 L min^{-1}. These containers were kept under the same conditions. The containers were filled with the continuous flow and aeration and the cleaning performed by a suction operation on a daily basis. During the experiment, the average temperature and oxygen concentration were 12 ± 1 ˚C and 8.7 ± 0.5 mg L^{-1}, respectively. The level of dissolved oxygen was daily measured using a DO meter (WTW Oxi3205, Munich, Germany). The fish were fed twice a day with extruded ration 3% of live weight per day. The fish were adapted during 10 days before the beginning of experiments.

Preparation of ethylene amine-inactivated IPNV vaccine

A prevalent isolate of the IPNV in Iranian trout farms was propagated in CHSE-214 cells (Chinook salmon embryonic) (Spence, Fryer & Pilcher, 1965) to produce a new vaccine. The cells were cultured in minimal essential medium (MEM) supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (GIBCO-BRL, USA), 100 IU ml^{-1} penicillin G and 100µg mL^{-1} of streptomycin. The virus was titrated according to the described procedure of Reed and Muench (1938). Following the beginning of cytopathogenic effect (CPE), the supernatant was harvested and stored at -70 ºC until the time is was supposed to be used (Fig.1). IPNV (50% tissue culture infective dose, median tissue culture infectious doses ($TCID_{50}$): 10^7 mL^{-1} titer) was inactivated in ethylene amine for 30 h at room temperature (23 ± 1°C) and cultured again in the (CHSE-214) cell line for 10 days to confirm the inactivation.

The design of vaccine formulation with adjuvant

The ratio of the premix adjuvant used with the antigen was 10 and 50 g of Montanide IMS 1312 VG to 10^7 $TCID_{50}$ of IPNV vaccine in 10 mL

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phosphate-buffered saline (PBS). Montanide IMS 1312 VG was added to ethylene amine-inactivated IPNV vaccine, while the mixing of a complete batch was carried out at a low shear rate using a magnetic stirrer (low speed) at the room temperature to obtain the sufficient homogeneity. The premix was diluted in 5 L of fresh water for 5 min, whereas the final concentration of IPNV vaccine was 10^7 $TCID_{50}$ $5L^{-1}$ (Hawang *et al*., 2016).

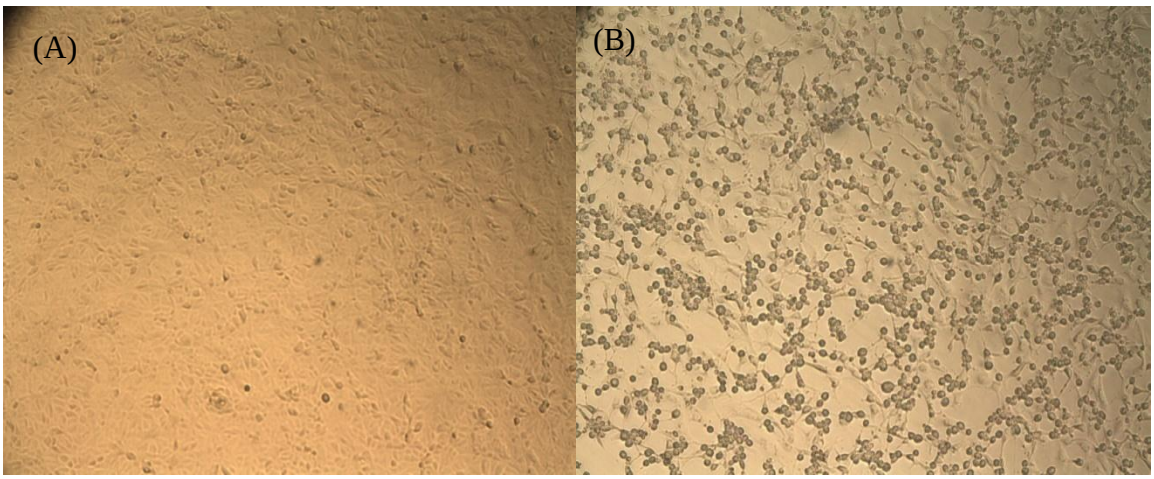

Figure.1. CHSE-214 cell culture (40x); A- control cell culture and B- infectious CHSE-214 cell with IPN virus.

Vaccination and sample collection

Four fish groups were immersed 5L of fresh water for 5 min under the aeration as follows: group 1, PBS (no vaccination, control); group 2, IPNV vaccine 10^7 $TCID_{50}$ in 5 L of fresh water; Group 3, IPNV vaccine 10^7 $TCID_{50}$ and 10 g Montanide 1312 VG in 5 L of fresh water, and group 4, vaccine 10^7 $TCID_{50}$ and 50 g Montanide 1312 VG in 5 L of fresh water (Table 1). On 2, 7, 15 and 22 days after the vaccination, three fishes were randomly collected from each group and sacrificed with overexposure to clove oil and then tissues of spleen and head kidney were collected from each fish to be used for RT- PCR expression of Mx-1, CD8, IgM genes.

Real-time quantitative PCR gene expression analysis

The total RNA was extracted from the head kidney and spleen tissues using a High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer`s instructions. The purified RNA was stored at -80 ºC for 24 h. The total RNA concentration was determined using a Nano Drop TM. One µg RNA was reverse-transcribed into cDNA using a AccuPower RocketScriptTM cycle RT PreMix with oligo (dt) 20 (Bioneer, Korea). Each the RT-quantitative PCR (qPCR) reaction was performed using (7.5 µL 2×SYBER Green PCR Master Mix and 200 nM of each primer pairs specified in table 1 and 1 µL of cDNA in a final volume of 15 µL by using AccuPower 2X GreenStar PCR Master Mix (Bioneer, Korea). Amplification parameters were as follows: 1 cycle (15 min at 95 ºC), 40 cycles (15 s at 95 ºC), and the final cycle (59 ºC at 1 min). All melting curves of related amplicons were analyzed. The endogenous control gene (EF-1 α) was used as reference for the expression pattern analysis of target genes. The data

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analysis was according to the relative quantitation method and subsequently ΔΔCT values were used to determine the relative fold changes in expression (Livak and Schmittgen, 2001). All amplifications were performed in duplicated. RT-qPCR was performed using primers mentioned in Table 2.

Table 1. Control and vaccinated groups information.

Group name	Number of Fishes	Inoculation
Control group	60	PBS
Treatment group 1	60	10^7 $TCID_{50}$ IPNV
Treatment group 2	60	10^7 $TCID_{50}$ IPNV with 10 g Montanide 1312 VG in 5 L of fresh water
Treatment group 3	60	10^7 $TCID_{50}$ IPNV with 50 g Montanide 1312 VG in 5 L of fresh water

Table 2. Primer sets used for RT-qPCR analysis of differential gene expression

Gene	Primer sequence 5`–3`: Forward-Reverse	Accession number
IgM	ACCTTAACCAGCCGAAAGGG TGTCCCATTGCTCCAGTCC	X65263.1
CD8α	AGTCGTGCAAAGTGGGAAAG GGTTGCAATGGCATACAGTG	AF178053
Mx	AGCGTCTGGCTGATCAGATT AGCTGCTCGATGTTGTCCTT	OMU30253
EF1α (house-keeping gene)	GATCCAGAAGGAGGTCACCA TTACGTTCGACCTTCCATCC	AF498320

IPNV challenge

At 30 day-post vaccination, 45 fish from each group were challenged by intraperitoneal (IP) injection of 0.2 mL $fish^{-1}$ with IPNV at 10^7 $TCID_{50}$ mL^{-1}. The relative percent survival (RPS) (Amend, 1981) was calculated using the end cumulative mortalities, as follows:

$$RPS\ (\%)= (1-\frac{Mortalitey\ of\ vaccinated\ group}{Mortality\ of\ unvaccinated\ control\ group})\times 100$$

Transcription of immune-related genes (RT-qPCR)

The effect of inactivated IPNV vaccine by the immersion method of vaccination on the expression of Mx gene related to the innate immune response and IgM, CD8 genes belonged to the adaptive-immune response in the head kidney and spleen tissues of vaccinated and the control group was evaluated by RT-qPCR analysis (Livak and Schmittgen, 2001).

Statistical analysis

Data was statistically analyzed by the relative expression software tool (REST, 2009 V2.0.13) analysis. Differences were considered statistically significant a p-value of < 0.05.

Results

Efficacy of IPNV-inactivated vaccine

The survival percentage of the vaccinated fish and the rate of protection and efficacy of studied vaccines by increasing the expression level of the studied immunity genes in the vaccinated groups were compared to the

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control group after the inoculation of IPNV on the test fish. The vaccinated and control groups were infected with live virus in order to evaluate the survival rate of vaccinated fish after 30 days. Cumulative mortality was recorded for 22 days after the challenge (Fig. 2). The cumulative mortality rate in the control group was 88.9%, while this parameter for vaccinated groups with inactivated virus without adjuvant, inactivated IPNV containing 50 g Montanide 1312 adjuvant and, inactivated IPNV containing 10 g Montanide 1312 adjuvant was 75.50, 53.30, and 40.12% respectively. The survival rate for corresponding vaccinated groups was 15.23, 40.31 and 66.13%, respectively. In this study, the effect of the vaccine produced with the defined doses (10g and 50 g) for the adjuvant Montanide IMS 1312 VG was investigated on immune system genes including Mx-1, IgM and CD8 in rainbow trout. To test this, the spleen and anterior kidney tissues were collected from three fish per groups on 2, 7, 15 and 22 days after the immersion vaccination and their qPCR experiments were conducted to access expression levels of the three immune-associated genes. The expression of genes in immersion vaccinated groups with two different doses of adjuvant and non-adjuvant is shown in Fig. 3. The RT-qPCR analysis showed a meaningful increase in the IgM expression on 7, 15 and 22 days after the vaccination in the kidney and, on 15 and 22 days after the vaccination in the spleen for the vaccinated group with 10 g of Montanide adjuvant, which was higher than that of the vaccinated group with 50 g of Montanide adjuvant. The rate of IgM expression significantly increased on the 15^{th} and 22^{th} day after the vaccination in both kidney and spleen tissues in the vaccinated group with 10 g of Montanide adjuvant. The expression level of CD8 gene in the kidney and spleen tissues of the vaccinated group with 10 g of Montanide 1312 adjuvant increased compared to the vaccinated one with 50 g of 1312 Montanide adjuvant. This increase was not significant for the kidney tissue compared with the control group, but it was significantly higher in the spleen tissue than the control group on the 15^{th} and 22^{th} day after the vaccination ($P<0.05$). In groups of non-adjuvant and the vaccinated with 50 g of Montanide, the CD8 gene expression did not show any significant increase in both kidney and spleen tissues. The expression of Mx gene in the vaccinated group with 10 g of Montanide 1312 adjuvant showed a significant increase in all four days of sampling compared with the control group in the kidney tissue, while this increase was significant only on days 2, 7 and 15 after the vaccination in the spleen tissue ($P<0.05$). In the vaccinated group with 50 g of adjuvant significant increase was observed for the expression of Mx gene compared to the control group on days 2, 7 and 22 after the vaccination, while the increase in expression of this gene was insignificant on 15^{th} day after the vaccination. Also, the expression of Mx gene in the spleen tissue showed a significant increase on 2, 7 and 15 days of vaccination. In the vaccinated group without the adjuvant, the increased expression of Mx gene was significant only on 2^{th} and 7^{th} days after the vaccination.

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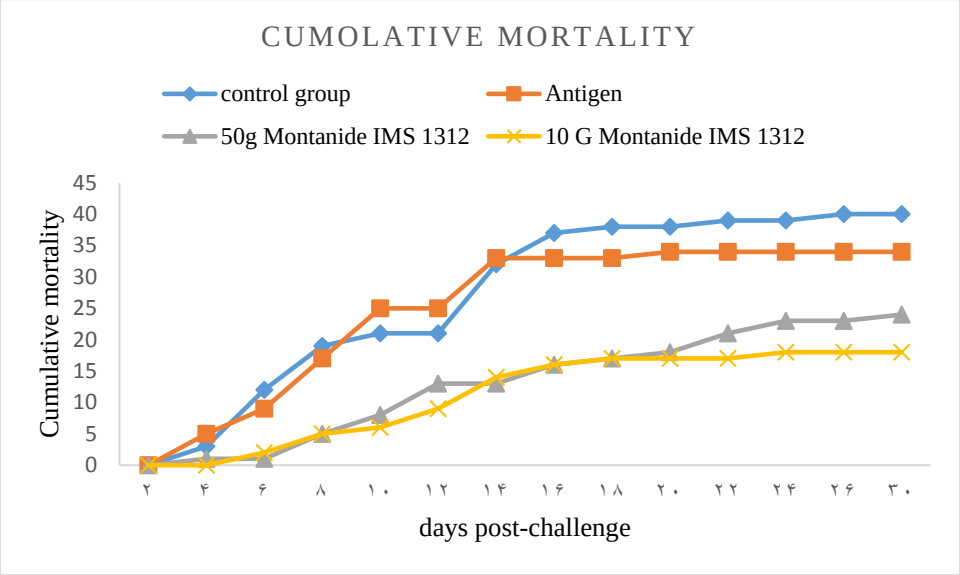
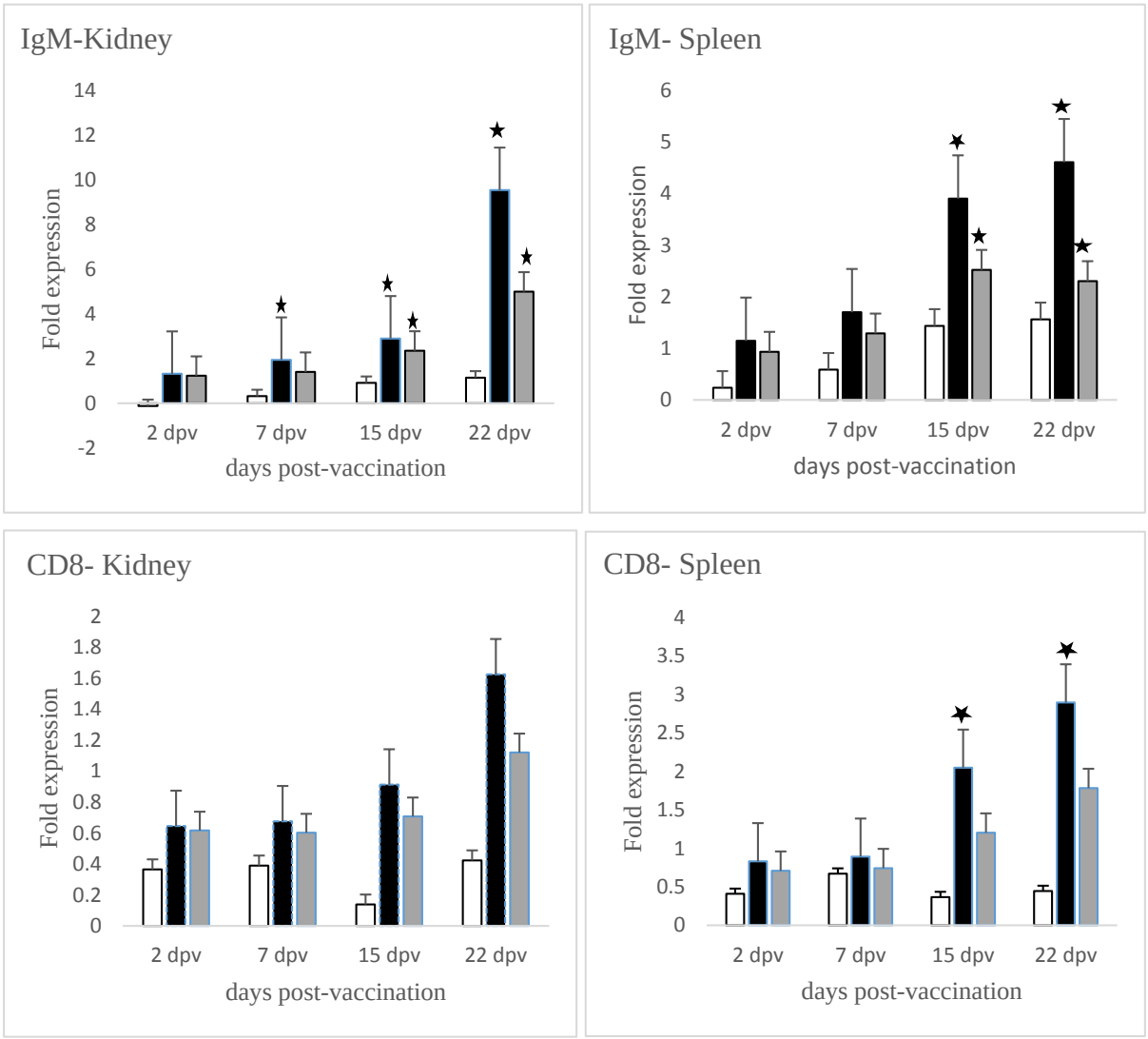


Figure 2. Cumulative mortalities of immersion immunized rainbow trout with inactivated-IPNV at 2×10^7 $TCID_{50}$. Control group fishes were vaccinated with PBS. Mortality was daily recorded during 30 days.



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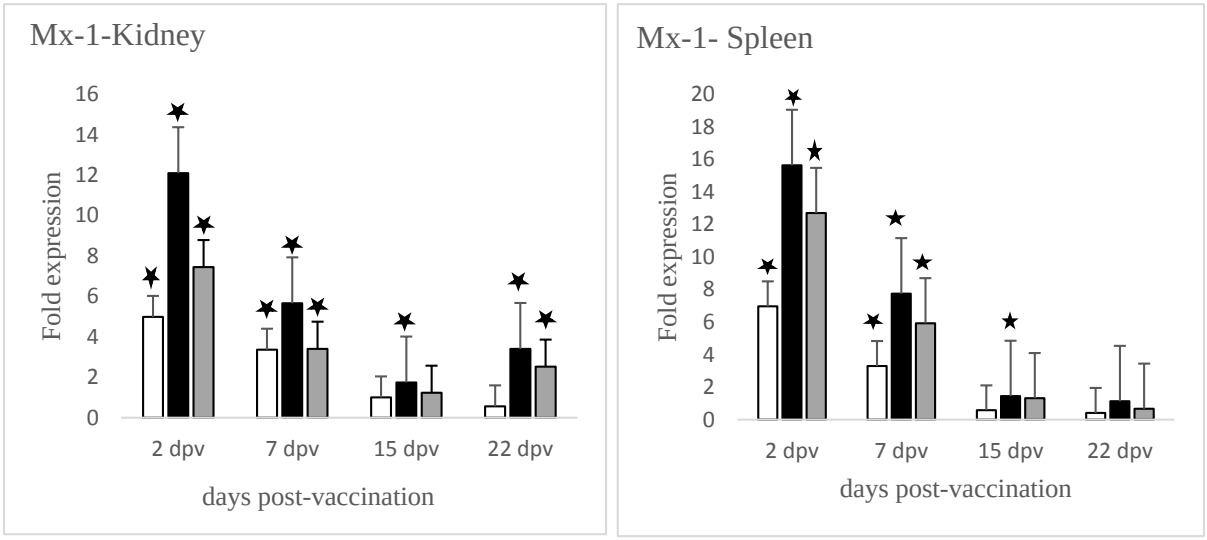

Figure 3. The ratio expression of IgM, CD8, and Mx genes in vaccinated groups with inactivated-IPNV without Adjuvant (white chart), 10 g of Montanide 1312 adjuvant (black chart), 50g of Montanide 1312 adjuvant (gray chart) to the control group. the results of the expression of the desired genes in the immersion vaccinated groups (Table 1) were compared to the control group in spleen and kidney tissues on days 2, 7, 15 and 22 after the vaccination by RT-qPCR. The data were normalized based on the EF-1α gene as a homogenous gene. Significant changes ($P < 0.05$) in the level of expression in vaccinated groups were shown in comparison to the control group with a star label.

Discussion

Currently, there is no available commercial vaccine against IPNV, despite the IPN spread is one of important viral disease of rainbow trout. Different types of vaccines such as live, formalin or heat-inactivated whole virus, fusion protein, subunit, virus-like particles have been experimentally studied for their defensive roles in some viral diseases of fish (Gudding *et al*., 2014; Munang'andu & Evensen 2015). The immersion vaccination is most convenient for small fish, fry, and fingerlings that are impractical to handle during the injection (Evensen 2009). The injection method for vaccination is a stressful method and it is very difficult to vaccinate large amounts of small fish (Embregts & Forlenza 2016). Therefore, it is very important to use a method with less manipulation and stress for fish (Urquhart, Murray, Gregory, O'Dea, Munro, Smail, Shanks & Raynard 2008). In the present research, the immunity and efficacy of ethylene amine-inactivated IPNV vaccine were evaluated by the immersion method in combination with an IMS Montanide 1312 adjuvant. Results showed that the IPNV immersion vaccination in the presence of Montanide IMS 1312 VG protects fish against the infection and disease caused by IPNV so that a 10 g dose of Montanide IMS 1312 VG provides a better protection than a 50 g dose of this adjuvant. No toxicity has been reported to use the IMs 1312 VG adjuvant in hematological and histopathological studies (Hawang *et al*., 2016).

The expression of three immunity genes was investigated for 22 days after the vaccination. The Mx expression was significantly increased in the vaccinated groups on the second and seventh day after the

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vaccination in both spleen and head kidney tissues compared to the control group. Also, it was observed that this increase was higher in the vaccinated group with 10 g of Montanide IMS 1312 VG than that of the group received a 50 g dose of the adjuvant. Changes in the Mx gene expression of on the 15th day were not significant in the vaccinated group with IPN in combination with 50 g of Montanide IMS 1312 VG and also in the non-adjuvant vaccine recipient group. Increasing the expression level of Mx gene in the kidney tissue of the vaccinated group with a 10 g dose of Montanide in the second and seventh days was remarkably higher than that of the vaccinated group with 50 g of Montanide IMS 1312 VG, which was (12.1 fold vs. 7.45 fold) and (5.66 fold vs. 3.4 fold) more than the control group. These changes were also significant in the spleen tissue as it was higher on the second (15.6 Fold vs. 12.7 Fold) and seventh (7.7 Fold vs. 5.9 Fold) days in the group receiving 10 g of Montanide than that of the group received 50 g of Montanide. There was no significant difference in the expression level of this gene in the two groups treated with different doses of Montanide on the 15th and 22th days after vaccination, but the difference was significant when compared these two groups with the non-adjuvant group.

The CD8 gene expression level did not show any significant difference in both groups after the vaccination on the 2nd, 15th and 23rd days after the vaccination, despite an increase in the expression level compared to the control group. The expression level of CD8 gene in the spleen tissue increased significantly in the two vaccinated groups compared with the control group, so that this increase significantly was more on 15 and 24 days (2.04 fold vs. 2.9 fold, respectively) after the vaccination in the group receiving 10 g of Montanide than that of control group. However, no significant difference was observed in the expression level of CD8 gene between the vaccinated groups with 10 g and 50 g of Montanide adjuvant.

The expression rate of IgM gene in the kidney tissue was higher in the vaccinated group with 10 g Montanide IMS 1312 VG than that of the control. However, this rate was more in vaccinated group with 50 g Montanide IMS 1312 VG than that of the control only on 15 and 21 days after the vaccination. Changing the expression level of IgM gene for the kidney tissue in the vaccinated group with 10 g of Montanide IMS 1312 VG was significantly higher that of vaccinated group with 50 g of the applied adjuvant. Moreover, the expression of IgM gene in kidney tissue was higher on 15 (3.9 fold vs. 2.52 fold) and 22 (4.6 fold versus 2.3 fold) days than that of the control group. Changes in the IgM expression level showed a significant difference between the vaccinated group with 10 g and 50g of Montanide IMS 1312 VG only on day 22 (twice as high in 10 g adjuvant). However, no significant difference was found between the vaccinated group without the adjuvant and the control group.

According to the findings of this study, the uptake rate of antigen particles can be improved by the employment of adjuvant that my result in increasing of immunization efficacy. Soltani et al. (2014) reported that the using of Montanide IMS 1312 VG adjuvant may increase the efficacy of Yersinia vaccine in rainbow trout by

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stimulating the specific immune system and also the innate immune responses. Also it is reported in the previous studies that the efficacy of viral hemorrhagic septicemia virus (VHSV) vaccine was improved in rainbow trout by using the Montanide IMS 1312 VG adjuvant (Hawang *et al.*, 2016). The elucidation of the additional mechanisms underlying these protective effects should finally lead to an improved IPNV immersion vaccine with Montanide IMS 1312 VG, but it needs further studies. Finally optimizing the concentration of employed adjuvant will also help to increase the efficacy of IPNV immersion vaccine.

To conclude, the efficacy and safety of the immersion vaccine with Montanide IMS 1312 VG adjuvant against IPNV in Rainbow trout were tested and verified. Results of this study indicated that the dominant strain of the IPNV in Iran's farms can be vaccinated by designing appropriate changes in the expression level of the immune-related genes along with using the optimal concentrations of the adjuvant Montanide IMS 1312 VG that can be used to vaccinate the sensitive populations of rainbow trout especially at earlier stages of fish lives. As any improvements made in immunization rate can result in significant economic advantages on the local and global aquaculture, further studies should be conducted to increase the fish vaccination efficacy against IPNV.

Conflict of interests

The authors declare that there is no conflict of interest.

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واکسن کشته شده ویروس نکروز عفونی پانکراس به روش غوطه وری برای ماهی قزل آلای رنگین کمان (*Oncorhynchus mykiss*) (Walbaum, 1792) و بررسی تاثیر ادجوانت مونتاناید IMS 1312 VG بر اثربخشی واکسن

سمیرا رشیدی منفرد[1]، مصطفی اخلاقی[1*]، سید علی پوربخش[2]، روزبه فلاحی[2]، سیاوش سلطانیان[1]، آزاده یکتاسرشت[1]

[1] دانشکده دامپزشکی، دانشگاه شیراز، شیراز، ایران

[2] موسسه تحقیقات واکسن و سرم سازی رازی، سازمان تحقیقات، آموزش و ترویج کشاورزی، کرج، ایران

چکیده

از آنجایی که ویروس نکروز عفونی پانکراس (IPNV) شایع‌ترین عامل بیماری کشنده‌ی ویروسی در مزارع پرورشی ایران است، واکسن کشته شده برای این ویروس کشنده ساخته شد. علاوه بر این اثرگذاری واکسن ساخته شده علیه ویروس IPN همراه با ادجوانت مونتاناید IMS 1312 VG مورد بررسی قرار گرفت. تعداد دویست و چهل عدد ماهی سالم با وزن ۰/۴۵± ۳/۰۰ گرم با ویروس کشته شده‌ی IPN توسط اتیلن آمین در ترکیب با ادجوانت مونتانابد IMS 1312 VG ، به روش غوطه وری به مدت ۵ دقیقه در دمای ۱ ± ۱۱ درجه‌ی سانتی‌گراد واکسینه شدند. گروه شاهد PBS استریل دریافت کردند. واکسن ساخته شده همراه با ادجوانت مونتاناید IMS 1312 VG سبب افزایش بیان ژن‌های سیستم ایمنی از جمله ژن‌های IgM، Mx و CD8 در طول دوره‌ی ۳۰ روز پس از واکسیناسیون شد. سطح بیان ژن های Mx، IgM و CD8 ($P < 0.05$) در واکسن حاوی ۱۰ گرم مونتاناید بیشتر از واکسن حاوی ۵۰ گرم مونتاناید بود. درصد نسبی بقای ماهیان در گروه های واکسینه شده با ویروس کشته شده همراه با ادجوانت مونتاناید بیشتر از ماهیان گروه شاهد و گروه واکسینه شده بدون ادجوانت بود. نتایج نشان داد که واکسن ویروس کشته شده ی IPN با اتیلن آمین در ترکیب با ادجوانت مونتاناید IMS 1312 VG ایمنی و حفاظت قوی در برابر ویروس IPN در ماهیان قزل آلای رنگین کمان ایجاد می‌کند.

کلمات کلیدی: ویروس نکروز عفونی پانکراس (IPNV)، قزل آلای رنگین‌کمان، واکسن غوطه‌وری، مونتاناید IMS 1312 VG

***نویسنده مسئول:** akhlaghi@shirazu.ac.ir

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