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Research Article

Evaluation of killed vaccine efficacy on hematology parameters and IgM assay of *Acipenser stellatus* (Pallas, 1771) challenged with Nervous Necrosis Virus (NNV)

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Abstract

Vaccination is considered the most effective, environmentally friendly and cost-effective way of protecting commercial finfish species against infectious diseases with high economic impacts. Efficacy of a new inactivated Viral Nervous Necrosis (VNN) vaccine through heat-inactivated nervous necrosis virus (NNV) combined with three types of adjuvants was assessed in 7 g juvenile of *Acipenser stellatus* (Pallas, 1771) as a laboratory model. Ten groups of 30 juveniles were divided in 18 aquariums to vaccinate via immersion and injection routes using 4 types of vaccines. Vaccination was performed in two stages one month apart and one month after the second replication, exposure to the live virus was performed. During this period, the mortality rate of immersion and injection groups was 12.9% and 19.8%, respectively, compared to 100% mortality in the control group. Sampling was performed in four stages before the first vaccination in the adaptation period, after the first vaccination, after the second replication, and after exposure to live virus. Blood samples were measured to evaluate changes in blood factors (red blood cell count, white blood cell count, hematocrit and hemoglobin) and immunity factors (immunoglobulin M (IgM). In order to prove the virulence of the viral sample (supernatant) used in exposure, the pathogenicity of VNN virus isolated from infected Golden grey mullet (*Chelon auratus*) with ($TCID_{50}$ 10^4) was examined in several guppies. One month after the second repetition of fish, all treatments were exposed to the acute virus sample (supernatant), and after one month, their survival percentage, blood factors and safety were measured.

The results of this study revealed that immersion vaccination in the vaccinated group containing IMS 1312 SEPPIC adjuvant significantly showed higher levels of IgM compared to other groups equal to $0/35 mg\ dl^{-1}$ (p<0.05). This is consistent with the results of hemoglobin 40.6 $g\ dl^{-1}$ (p<0.05), which proves the better efficacy of this type of vaccine compared to other vaccines tested in this study. Based on the results of this study, a killed vaccine with 75% IMS 1312 SEPPIC adjuvant can be recommended for vaccination against VNN.

Keywords: *Acipenser stellatus*, ELISA, Immunization, Nervous Necrosis Virus, Vaccination

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Introduction

Due to the economic damages of fish infectious diseases in cage cultures and the development of this industry, vaccination could be considered as an effective and practical tool in controlling fish infectious diseases in aquaculture and mariculture (Buonocore *et al*., 2019). This worldwide destructive infectious disease is the cause of the most threats to the health of various amounts of fish species. Sturgeon is one of the valued and economical species in the world that is susceptible to (VNN) (Bandín *et al*., 2020). VNN which was known as Viral Encephalopathy and Retinopathy (VER) (Munday *et al*., 1992), seabass (*Centropristis striata*) encephalopathy (Bellance, R., 1988), turbot (*Scophthalmus maximus*) encephalomyelitis (Bloch *et al*., 1993), and fish encephalopathy (Comps *et al*.,1996), is called a neuropathy situation can affect several species of fishes. Betanodavirus causes VNN disease in various fish species worldwide (OIE, 2019).

According to various studies, formalin-inactivated virus, an antiviral particle in Baculovirus, recombinant C protein, synthetic peptides, and Betanodavirus recombinant coat protein that was expressed in *Escherichia coli*, and recently a DNA vaccine, have been reported (Kai and Chi., 2008; Doan *et al*., 2017; OIE, 2019). There is no history of heat inactivation of this virus. In this way, only one unsuccessful experience was reported by Thiéry *et al*. (2006). They reported that heat inactivation at high temperature (100°C) of the virus used in their previous study may have destroyed the multimeric structure of the virions; thus, this condition can explain the main reason for the reduction in antibetanodavirus antibody titer.

Despite adequate data on the immune responses of several finfish towards NNV antigens, no sufficient data is available on the sturgeon immune responses subjected to the NNV antigens, therefore sturgeon fish was selected for this experimental assay. This research aims to assess heat-inactivated NNV vaccine in *Acipenser stellatus*.

Materials and methods

Vaccine preparation and immunization

About 540 juveniles 7 g of *A. stellatus* were transferred from Ghaem Inclusive Economic Development Company of Guilan Province, Anzali port to the main laboratory in Tehran with a closed circulation of water. Physicochemical parameters of aquariums water were measured such as total dissolved solids (TDS): 752 mg/L, temperature: 21.18 °C, pH: 7.38, electrical conductivity (EC): 1497 µs/cm, salinity: 0.76 g/L, and dissolved oxygen (DO): 6.4 mg/L. Also, Sturgeons were fed twice a day by BioMar (France). Ten groups consisting of 30 numbers of *A. stellatus* were placed in eighteen 80-liter aquariums, and aquarium water was replaced twice a day.

Four types of vaccines were designed and prepared in a laboratory in two forms; immersion and injection. Also, Montanide IMS 1312 VG containing liquid-dispersed Nanoparticles, which is an immune system stimulant in fish

vaccines was used for the immersion vaccination method.

In immersion form, there was only one type of adjuvant (50% virus+50% 1312 Seppic Adjuvant) with two different percentages 50% and 75%. In injection form, there were two types of adjuvants, 30% viruses+70% Pasture Adjuvant IPA and 70% virus+30% $Al(OH)_3$, also there were two immersion control groups (100% inactivated virus and 1312 Seppic Adjuvant 100%) and two injection control groups (Inactivated virus100% and Adjuvant 100% IPA).

The inactivated Betanodavirus (ARG/VIR/2016-01) belonging to the Red Grouper Nervous Necrosis Virus (RGNNV) genotype was kindly donated by the Moredun Research Institute (Kim *et al*., 2018). The virus was cultured in SSN-1 cells derived from striped snakehead *(Ophicephalus striatus*) fry (Frerichs *et al*., 1996), propagated in pre-formed SSN-1 cell monolayers, prepared by splitting the cells at 28°C until monolayer attained 70% of confluence.

The growth medium, L-15 Glutamax-I (Gibco, Paisley, Scotland) supplemented with 5% FBS (Foetal Bovine Serum, Gibco, Paisley, Scotland), was removed and the virus was added and allowed to adsorb for 60 minutes at 25°C. The conditioned medium, L-15 Glutamax-I supplemented with 2.5% FBS, was added to the tissue culture flasks and incubated at 25°C until an extensive cytopathic effect (CPE) was observed. (Kim *et al*., 2018). The concentration of the virus was determined by calculating the virus titre (number of infectious units per unit volume) by $TCID_{50}$. Infectivity titrations were performed in flat bottom 96 well plates (Corning) by adding 90 µL of Hanks Buffered Saline Salts (HBSS) (Gibco, Paisley, UK) to each well. Virus (10 µL) or HBSS (negative control) was added to the first well of each row and were diluted ten-fold across the plate. The SSN-1 cells (100 µL) were added to each well of titration plates. Plates were then sealed with Parafilm and incubated at 25°C for 7 days when monolayers were visually assessed for CPE (Kim *et al*., 2018). Virus titres were calculated by the method of Spearman-Karber (Hierholzer and Killington, 1996). Meanwhile, the vaccine was prepared in two forms; immersion and injection applications. In the immersion form, heat-inactivated VNNV at two concentrations (50% and 25%) contained Montanide adjuvant IMS1312 at 1:1 and 1:2 (v/v, antigen/Montanide), were used. The immersion immunization was performed by adding 10 mL of each vaccine in 5 L clean freshwater before fish being vaccinated for 60 minutes at 20°C. In the injectable form, heat-inactivated VNNV at two concentrations 30% contained antigen at 30:70 v/v (antigen: IPA) and 70:30 (antigen: aluminum hydroxide) were used. Fish were vaccinated intraperitoneally with 0.1 mL per fish after being anesthetized by clove extract at 2.2 g/L. The booster immunization was followed by one-month post-primary vaccination. Blood samples were taken from the caudal vein of the immunized fish before and after vaccination. Ten days after the first vaccination, ten days after the second

vaccination, and ten days post-challenge with NNV (Table 1).

Table 1: Sampling days before and after vaccination.

Sampling day	30	70	100	130
Sampling time	First sampling Adaptation (1)	Second sampling 10 days after the first vaccination (2)	Third sampling 10 days after the second vaccination (3)	Fourth sampling 10 days after a challenging day (4)

Note: 30 days after a challenging day, the mortality rate of sturgeon juveniles was assessed (Until day 150).

Hematological assay

The blood samples were obtained from the caudal vein and processed for hematological works according to the procedure described (Zarejabad *et al*., 2010). A part of collected blood samples was used for serum preparation, and the second part was used to determine hematological indices (Blaxhall *et al*., 1973). The obtained dried blood smears were subjected to 96% ethanol before being stained with Giemsa stain.

IgM measuring using ELISA assay

One-step Zell Bio GmbH kit ELISA (ZB-10025C-F9648) was used to titrate specific IgM. Sera samples were added to wells of ELISA plate, which were pre-treated with the anti-IgM monoclonal antibody. The anti-IgM antibodies labeled with biotin combined with streptavidin-HRP were then added to wells. Unbound enzymes were removed after incubation at 37ºC for 60 min and washing the plates. After adding chromogen A and B into the wells, they were incubated at 37ºC for 10 min. The kit substrates of A and B were then added before reading the optical density (OD) of the reactions at 450 nm by ELISA reader.

Challenge experiment

The immunized sturgeons were challenged with the strain of NNV previously isolated from the first outbreak in Golden grey mullet (*Chelon auratus*) by Zorriehzahra *et al*. (2005). To make sure of virus activation [($TCID_{50}=10^4$, (1500× *g* for 10 min)], 50 guppies were exposed and by providing a new supernatant of the virus from dead guppies that were obtained from centrifuging at 3000 × *g* for 20 min and filtered from 0.45 nm Bohner funnel filter, they were diluted 5 cc in 45 cc of PBS (Nazari *et al*., 2014). Sturgeons were challenged with 0.1 cc of this supernatant through intraperitoneal.

Statistical analysis

The ANOVA was used to determine the differences between treatments in terms of the dependent variables (IgM and hematology parameters) as a significant level $p<0.05$. The Tukey was followed to determine the differences two by two $p<0.05$. The tests were carried out using the SPSS software version No. 26 (SPSS Inc., Chicago, IL, USA).

Results

Immune response measuring by IgM

The mean OD of fish antibodies before the first vaccination in the negative

control group (group 10; Table 1) was approximately 0.33±0.03, which did not differ from the values in other groups (p>0.05). On the 70 days of storage (10 days after the first vaccination), the average minimum OD of fish antibodies in the negative control group was 0.17±0.02. The OD in the positive control treated with 100% PBS solution was 0.20±0.02 (group 9). On day 70, the average OD in group 5 or the control group vaccinated with the killed virus (without Adjuvant) was 0.32±0.02, which was higher than fish that received killed virus plus adjuvant. On day 100 of the study (10 days after the second vaccination), the minimum average of OD was measured in the negative control (0.21±0.01). In contrast, the highest value of OD (0.41±0.01) was seen in group 6 followed by groups 7 (0.38±0.01) and 5 (0.37±0.01). Ten days after challenging fish with the live virus (day 130), minimum (0.24±0.01) and maximum (0.35±0.01) OD were measured in groups 8 and 2, respectively.

Based on numerical statistics of the extracted Mauchly test, the interaction of sampling days (time) and vaccination groups was significant on changes in OD of antibody titer. The effect of different sampling times on the antibody titer for each vaccinated group is given in Table 2 and Figure 1.

Table 2: Estimated value of measured antibody (OD) at 450 nm by interaction effects of vaccination group and time of sampling.

Immunized groups*	Sampling day**	Standard error ± average (mg/dL)
1	1	0.33 ± 0.31^{Aa} ***
	2	0.28 ± 0.22^{Ba}***
	3	0.31 ± 0.28^{Aa}
	4	0.31 ± 0.27^{Aa}
2	1	0.33 ± 0.31^{Aa}
	2	0.28 ± 0.23^{Ba}
	3	0.29 ± 0.25^{Ba}
	4	0.35 ± 0.31^{Ba}
3	1	0.34 ± 0.31^{Aa}
	2	0.30 ± 0.24^{Ba}
	3	0.30 ± 0.26^{Ba}
	4	0.28 ± 0.24^{Ca}***
4	1	0.33 ± 0.30^{Aa}
	2	0.30 ± 0.24^{Ba}
	3	0.30 ± 0.27^{Ba}
	4	0.27 ± 0.23^{Ca}
5	1	0.33 ± 0.31^{Aa}
	2	0.32 ± 0.26^{Aa}
	3	0.38 ± 0.35^{Ba}
	4	0.28 ± 0.24^{Ca}
6	1	0.33 ± 0.31^{Aa}
	2	0.25 ± 0.19^{Aa}
	3	0.41 ± 0.38^{Aa}
	4	0.26 ± 0.23^{Aa}

Table 2 (continued):

Immunized groups*	Sampling day**	Standard error ± average (mg/dL)
7	1	0.33±0.31^{Aa}
	2	0.33±0.20^{Aa}
	3	0.38±0.35^{Aa}
	4	0.32±0.29^{Aa}
8	1	0.33±0.31^{Aa}
	2	0.31±0.25^{Aa}
	3	0.35±0.32^{Aa}
	4	0.24±0.21^{Aa}
9	1	0.33±0.31^{Aa}
	2	0.20±0.15^{Aa}
	3	0.35±0.32^{Aa}
	4	0.25±0.22^{Aa}
10	1	0.33±0.31^{Aa}
	2	0.18±0.12^{Aa}
	3	0.21±0.18^{Aa}
	4	0.27±0.24^{Aa}

*Based on Table 1 information- **Based on table 2 information- ***Values with non-similar letters to lowercase fonts indicate a significant difference in each vaccination group at different times and with similar letters with uppercase font indicating a significant difference on the same day in different groups (p<0.05).

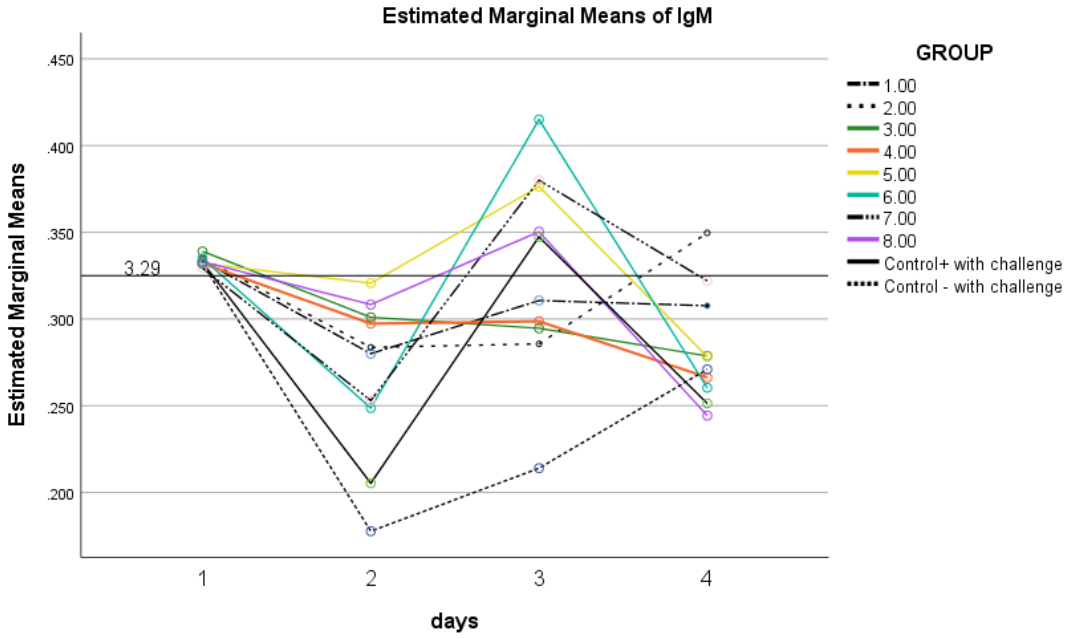

Figure 1: The trend of the interaction of vaccination groups and the elapsed time on the immunoglobulin change of young fish.

Hematology assay findings

Hemoglobin

According to Table 3, the Maximum amount of hemoglobin on day 70 (7.32) was seen in group3 that was no significant difference between group 2 (6.53) and group 5 (6.81) (p>0.05). The lowest hemoglobin content (3.67) was observed in group 4, 10 days after the second vaccination (day 100), the maximum amount of hemoglobin (8.26) was observed in group 8, which was not significantly different from groups 1 to 6 (p>0.05). The lowest hemoglobin content (4.70) was observed in group 7.

According to Table 3, HCT, MCV, MCH, MCHC, and RBC index, the contrast of groups did not affect their rate (p>0.05), but sampling times did affect it (p<0.05).

Table 3: Estimation of the average amount of indicators related to hemoglobulin due to the reciprocal type of vaccination groups × sampling time.

Index	Days	Groups										SEM
		1	2	3	4	5	6	7	8	9	10	
Hb (g/dL)	Av1 5. 69±0.3^{A}	4.23^{aA}	6.53 ^{bA}	7.32^{Ba}	3.67^{aA}	6.81^{bA}	4.60^{aA}	5.34^{aA}	4.88^{aA}	4.13^{Aa}	9.36^{cA}	0.97
	AV2 6.86±0.3^{A}	7.27^{acB}	7.20^{acA}	6.58^{acA}	5.96^{aB}	5.78^{aA}	6.63^{acB}	4.70^{bA}	8.26^{acB}	7.33^{acB}	8.89^{dA}	0.97
	ACH 4.2±0.3^{B}	3.14^{aA}	6.40^{aA}	2.38^{Ab}	3.53^{aA}	3.10^{aB}	3.97^{aA}	2.47^{aB}	4.53^{aA}	6.30^{Ab}	6.48^{aB}	0.97
Hct (%)	Av1 17.9±0.9^{A} AV2 20.7±0.9^{B} ACH 12.6±0.9^{C}	Interaction & Groups N.A.										1.6
MCV	Av1 163.3^{A} AV2 171.1^{B} ACH 208.81^{C}	Interaction & Groups N.A.										17.1
MCH	Av1 49.5^{A} AV2 48.3^{A} ACH 87.8^{B}	Interaction & Groups N.A.										9.1
MCHC	Av1 32.3 ^{A} AV2 33.9^{B} ACH 33.8^{B}	Interaction & Groups N.A.										0.37
RBC (10^{6} mm-^{3})	Av1 17409.9^{A} AV2 18361.4^{B} ACH 6640.2^{C}	Interaction & Groups N.A.										1707.8

Note: Hemoglobin concentration (Hb); Hematocrit (Hct); Red Blood Cells (RBC); Mean Corpuscular Volume (MCV); Mean Corpuscular Hemoglobin (MCH); Mean Corpuscular Hemoglobin Concentration (MCHC). Data with non-similar letters with lowercase fonts indicate a significant difference on the day of sampling (p<0.05)

The maximum amount of hematocrit was observed in group 2 followed by group 7 with a significant difference (p<0.05). The amount of hematocrit increased after the second vaccination compared to the first vaccination, but profoundly after exposure to live virus decreased (p<0.05). The rate of MCV increased after the second vaccination compared to the first vaccination, especially after exposing fish to the live virus (p<0.05). MCH levels did not change significantly after the second vaccination compared to the first immunization (p>0.05). MCH and MCHC were significantly increased after exposure to live viruses (p<0.05). The rate of RBC after the second vaccination changed significantly compared to the first vaccination

(p<0.05), and its level was significantly reduced after exposing fish to live viruses (p<0.05) (Fig. 2).

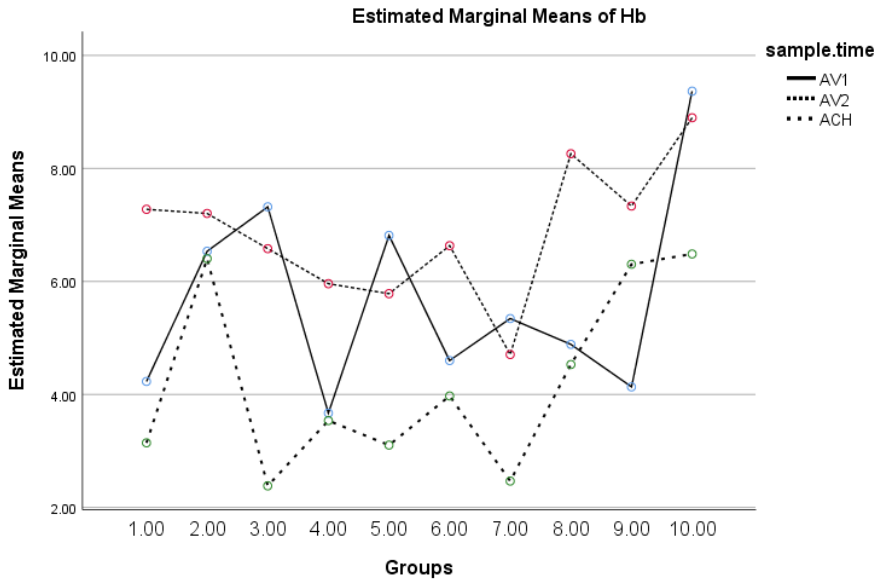

Figure 2: Average marginal trend of the evaluated hemoglobin value due to the interaction of 10 vaccination groups × sampling time.

Complete Blood Count findings (CBC)

Based on Table 4 Lymphocytes and WBC levels did not change significantly after the second vaccination compared to the first vaccination (p>0.05). However, their values decreased significantly after challenging the live virus (p<0.05). The dual effect of sampling time variables and different vaccination groups on mean SEG and EOS was evident. The maximum value of Seg. was (20533). Moreover, the minimum amount of (6157) was observed in groups 7 and 8, respectively, on day 70. Ten days after the second vaccination, the maximum amount of Seg (14646) was observed in group 5, which was not significantly different from the second group (p>0.05). The minimum amount of Seg (5568) was observed in group 8. After challenging with live virus, the maximum amount of Seg (4400) was observed in group 6. The minimum amount of Seg (1517) was observed in group 7. As shown in Table 4, the number of Seg after the challenge is deeply reduced. The maximum value of EOS (1904.3) and the minimum amount of (148) was observed in groups 1 and 8, respectively, on day 70. Ten days after the second vaccination, the maximum amount of EOS (3608) was observed in group 5, which was significantly different from others (p<0.05). The minimum amount of EOS (445.6) was observed in group 6 with no significant difference from groups 7 and 3 (p>0.05). After challenging with live virus, the maximum amount of EOS (158) was observed in group 4 with no significant difference from groups 5 and 1 (p>0.05). The minimum amount of EOS (1.3) was observed in group 6 with no significant difference from group 7. Monocyte and

Band levels significantly decreased after the second vaccination compared to the first vaccination (p<0.05), but their values decreased significantly after fish were exposed to live virus (p<0.05).

Table 4: Estimation of the average amount of indicators related to white blood cells due to the reciprocal type of vaccination groups × sampling time.

Index	Days	Groups										SEM
		1	2	3	4	5	6	7	8	9	10	
WBC (/µL)	Av1 10209.2^{A} AV2 10679.3^{A} ACH 2882.9^{B}	Interaction & Groups N.A.										1012.4
LYM (/µL)	Av1 6459.2^{A} AV2 6301.3^{A} ACH 3355.9^{B}	Interaction & Groups N.A.										
SEG (/µL)	Av1 11348.2^{A} AV2 10494.8^{A} ACH 2727.7^{B}	13976^{aA}	9647^{bA}	12741^{Ca}	11687^{Ca}	8636^{Ba}	17501^{dA}	20533Ea	6157^{fA}	5251^{Fa}	7349^{Fa}	3193
		6710^{Ab}	12929^{Bb}	6604^{Aa}	13763^{bB}	14646^{Ba}	8025^{cB}	5616Ab	5568^{aA}	12057^{Bb}	19028^{dB}	
		1957^{Ac}	2625^{aC}	3393^{Bc}	3556^{bC}	1776^{Ac}	4400^{cC}	1517^{Ac}	2519^{aB}	3193^{Bc}	41358^{cC}	
EOS (/µL)	Av1 490.2^{A} AV2 993.9^{B} ACH 269.1^{C}	1904.3^{aA}	389.6^{bA}	638.3^{Ca}	299.0^{bA}	443.0^{Ba}	379.6^{bA}	160.6^{dA}	148.0^{dA}	179.0^{Da}	360.0^{bA}	9.457
		794.3^{Ab}	1071.0^{Bb}	515.0^{Ab}	1365.6^{bB}	3608^{Bb}	445.6^{cB}	504.6^{aB}	562.3^{aB}	229.0^{Bb}	843.3^{bB}	
		125.0^{Ac}	102.3^{aC}	90.6^{Ac}	158.0^{bC}	144.3^{Bc}	1.3^{cC}	5.0^{cC}	310.6^{dC}	815.6^{Ec}	938.3^{fC}	
MON (/µL)	Av1 224.8^{A} AV2 190.6^{B} ACH 35.0^{C}	Interaction & Groups N.A.										47.1
BAND (/µL)	Av1 365.1^{A} AV2 67.1^{B} ACH 29.7^{C}	Interaction & Groups N.A.										3.129

Note: White Blood Cells (WBC); Lymphocytes (LYM); Segmented Neutrophils (SEG); Eosinophils (EOS); Monocytes (MON); Banded Neutrophils (BAND).

According to Fig. 3, groups 1 and 2 have been the lowest mortality rate among other groups. Mortality started a few days after a challenging day. Before challenging *A. stellatus* with the live virus, there was no significant mortality among treatment groups. On the other hand, according to the 0% mortality in the negative control group before challenging and 100% mortality in this group after challenging with live virus, it can be concluded that challenging with the live virus is the result of these mortalities.

These mortality rates are in line with the results of hematology and ELISA assays. Based on these results and mortality rates, a vaccine with IMS 1312 adjuvant has the highest effectiveness against live virus.

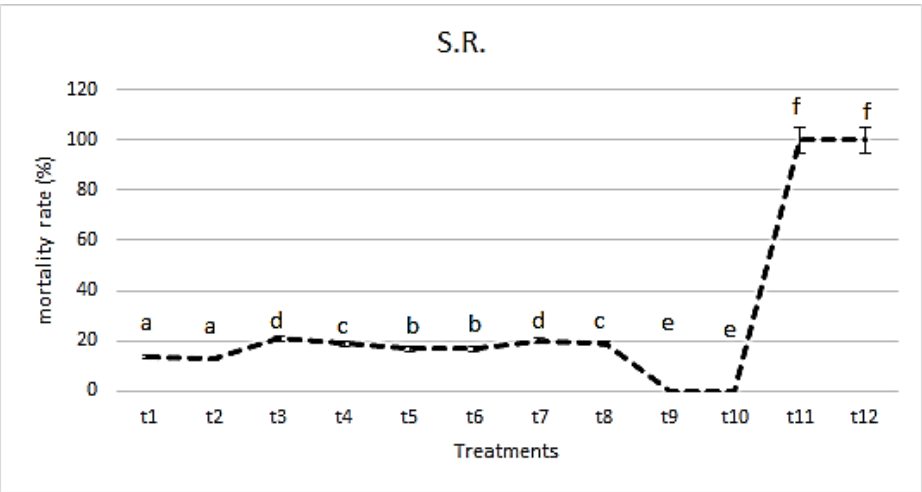

Figure 3: The mortality rate of sturgeon groups. Values with non-similar letters indicate a significant difference in each vaccination group (p<0.05) and with similar letters indicating a non-significant difference in different groups (p>0.05).

Discussion

Betanodavirus is the main pathogen of the VNN disease that causes high mortality in the larval stage of several marine fish species (Kim *et al*., 2018). Also, VNN mortality in larva is always more than in juvenile or adult fish (Kai and Chi., 2008). Meanwhile, no effective treatment has been found for VNN disease so far (Zorriehzahra *et al*., 2019). So, vaccination could be considered as an effective process in controlling VNND. Due to the low activity of the fish immune system in the early stages of life, the use of various vaccines has been reported. Several vaccines including, formalin-inactivated virus, an antiviral particle in Baculovirus, recombinant C protein, synthetic peptides, and Betanodavirus recombinant coat protein expressed in *E. coli*, and recently a DNA vaccine has been reported (Kai and Chi., 2008; Doan *et al*., 2017; OIE, 2019). These vaccines have been mostly examined at larval or juvenile stages than adult fish. Most outbreaks of VNN usually occurs in the first stage of fish larvae and juvenile, and due to incomplete immune system, vaccine preparation and administration are serious scenarios in mariculture (Patel *et al*., 2014). From a practical aquaculture point of view, it could be feasible to immunize the fish by immersion route at larval and juvenile stages against VNN (Lobb 1987; Nakanishi, T. and Ototake, M., 1997). For instance, in a study by Kai and Chi., 2008), three months after vaccination, sturgeon immunized by immersion route had only a 10% mortality rate, while 60% mortality was observed in the sturgeon group immunized via injection route (Kai and Chi., 2008).

Montanide IMS 1312 VG adjuvant facilitates initial immunization and long-term immune response through antibody formation or T cells cytotoxic in the host. (O'Hagan, D.T., Singh, M., 2003) . Aquatic adjuvants may help the cellular and humoral immune system respond by improving the absorption of antigens through the fish surface sites. It has been revealed that skin, lateral line, gills,

stomach, and intestine are the main antigens absorption places in the fish (Khimmakthong *et al*., 2013).

Some researchers stated in their study that all the adjuvants improve antigen absorption and also vaccine performance (Hwang *et al*., 2017). Also, in the other study, it was shown that Montanide IMS 1312 VG increases the effect of the killed vaccine against *Yersinia ruckeri* causative agent of Enteric Redmouth Disease (Yersiniosis) in Rainbow trout (*Oncorhynchus mykiss*) not only by stimulating acquired immune but also by the forming of the innate immune system in the host (Soltani *et al*., 2014). The vaccine is absorbed from the skin and mucosal surface, which are exposed to the vaccine contents. The immersion method is effective for mucosal immunity induction, but humoral immunity has less impact (Dhar *et al*., 2014).

Among adjuvants used in aquatics vaccination, Montanide IMS 1312 VG has the best impression in increasing the immune system. Hwang *et al*., (2017) found that VHSV vaccination by immersion method in Montanide IMS 1312 VG protect fish against VHSV and 10 gr dose of this adjuvants protect them better than 50 g dose. Nevertheless, in the present study, 75% dose, Montanide IMS 1312 VG was protected better in young sturgeon (*Acipenser stellatus*). The best result in this study was found from immersion vaccine in the presence of Montanide IMS 1312 VG on the vision of making higher antibody and hemoglobin titer and blood parameters. Nishizawa *et al*. (2011) believed that immunity by immersion method has interesting effects on epidermal immunity responses against RGNNV. They also found that immunity by immersion needs higher doses of the virus compared with injection form that is the negative point of immersion vaccination method because, by increasing doses of the virus in the vaccine, risks of the disease which occur in fishes with lower immune system will be increased. Vaccination by immersion method is more practical but has less power creation of immunization because of incomplete antigen absorption by mucosal membranes compared with injection form (Bøgwald, J. and Dalmo, R.A., 2019). Increasing consumption of Montanide IMS 1312 VG adjuvant helps the effectiveness of the VHSV vaccine in the immersion method.

According to the result of this study, it is inferred that among consumed adjuvants, only Montanide IMS 1312 VG had positive effects on the immune system, increasing its percentage up to 75%. Also, the findings of this study revealed that IPA and Aluminum hydroxide adjuvants had no ideal effects on the immune system. According to the findings of this study, the highest immunization rate was in the immersion vaccine group. In this study indirect sandwich, ELISA was used for IgM antibody measuring (Jaramillo *et al*., 2016).

Based on the results of this study, second vaccination causes more stability in hemoglobin and sometimes increases this rate in the groups. After challenging with the acute virus, the maximum amount of hemoglobin in sturgeons was observed in the second group

(Montanide IMS 1312 75%) (Table 3) that there was a significant increase compared to the other groups. The minimum rate of hemoglobin in sturgeon was (2.38 g dL) that were observed in group 3 or injectable adjuvants IPA 70%. In conclusion, it seems that group 2 has more influence in preserving hemoglobin than other groups. The second group had the highest IgM titer and the highest survival rate. Because of the differentiation of adjuvant and virus percentage, the titer of the antibody changed.

According to the results of antibody titer and survival rate obtained among treatments groups, group 3 has the highest mortality rate (Fig. 3). It means that the mean OD of IgM in group 3 in the second sampling session (0.30±0.02), which was administered 10 days after the first vaccination with the vaccine, consisted of 70% IPA adjuvant of Pasteur Institute of Iran and 30% of the inactivated virus was injected and showed a significant decrease compared to its value in the initial sampling (0.33±0.03) before vaccination (Fig. 1). This decrease in IgM can be due to its lack of adequate production due to a weaker immune response system with the first injection of the vaccine. After the second vaccination, the amount of IgM of fish (0.29±0.01) compared to the first vaccination, showed no significant change (p>0.05) which can be due to proper response of the immune system, adequate production, and more IgM compared to the antigens in fish plasma. However, in the exposure of group 3 with live virus and considerable consumption of plasma IgM, the results of sampling after challenge showed that the amount of fish immunoglobulin OD (0.27±0.01) showed a significant decrease, which can be a sign that the contents of the vaccine group 3 are not effective compared to the contents of the vaccine group 2. The titer of antibodies between groups was different only because of the difference in adjuvants. According to the results of ELISA about antibody titer and mortality rate between vaccinated groups (1-4) and control groups (5-8), it seems that the effect of IMS 1312 adjuvant on the efficacy of vaccine had been better than other groups due to more stimulation and better stability in the immune system.

Up to now, there is no official report about VNN vaccination in Sturgeon from the viewpoint of hematology. But according to some research results, it is expected that hemoglobin rate should be increased as an immune indicator with vaccination and booster. (Kim *et al.*, 2011). Meanwhile, no study for evaluating the killed vaccine against Viral Nervous Necrosis in *A. stellatus* has been done. The present research about this type of vaccine effectiveness on blood parameters could be the first report in Acipenseridae. As a result of this study, and concerning the early update of this disease in the larval stage and no possibility of using injectable vaccines in this period, immersion vaccines by adjuvant Montanide IMS 1312 VG are recommended.

It is better to use vaccines according to each farm condition in the future. Based on the hematological results of this study, blood parameters are different from each other and depend on several

factors. Also, concerning sea pollution and the increasing development of cage culture in the region, it is better to vaccinate susceptible fish before sending them to the cage to increase immunity system against VNN to upgrade the percentage of survival rate.

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