Research Article

Blood and enzyme factors change in New Zealand rabbit (*Oryctolagus cuniculus*) as an animal model in response to killed hemorrhagic septicemia virus (VHSv) virulent to *Oncorhynchus mykiss*

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Abstract

The objectives to this research were to determine the haematological changes in *Oryctolagus cuniculus* as an animal model in response to killed hemorrhagic septicemia virus (VHSV) virulent to *Oncorhynchus mykiss*. This study composed of 5 groups; Treatment 1 (T1) with 1^{cc} virus, treatment 2 (T2) with 0.5^{cc} virus and 0.5^{cc} adjuvant, treatment 3 (T3) with 0.75^{cc} virus and 0.25^{cc} adjuvant, treatment 4 (T4) with 0.25^{cc} virus and 0.75^{cc} adjuvant, and a control for a period of 4 months, once a month IM injection in 5 group of three New Zealand rabbits with health certification prepared by Razi Vaccine and Serum Research Institute. After 15 days, blood parameters, hematocrit (HCT), hemoglobin (HGB), mean hemoglobin concentration in red blood cells (MCH), red blood cells (RBC), average concentration of red blood cell (MCHC), white blood cells (WBC), neutrophils (SEG), lymphocytes (Lyn), eosinophil (Eos), aspirate transaminase (AST) and alanine transaminase (ALT).The results showed that ALT, AST, HCT, HGB and WBC concentrations of all rabbits significantly were difference ($p < 0.05$). T2 (0.5^{cc} virus and 0.5^{cc} adjuvant) and T3 (0.75^{cc} virus and 0.25^{cc} adjuvant) showed higher ALT, AST, HCT and WBCs among other treatments in all 4 blood samplings and among these treatments, T2 with lower levels of ALT and AST (indicating less pressure on the liver) and higher levels of WBCs which can lead to greater amount of neutralizing polyclonal antibodies indicated better results.

Keywords: Viral Hemorrhagic Septicemia virus, Rabbit, Hematology, Enzymes

Introduction

VHS is a disease of farmed rainbow trout, farmed turbot, farmed Japanese flounder as

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well as a broad range of wild freshwater and marine species (Meyers *et al*., 1995; Skall *et al*., 2005) caused by VHSV, a virus belonging to the genus Novirhabdovirus, within the family Rhabdoviridae (Walker *et al*., 2000). The causal agent of VHS is the Egtved virus (syn: VHS virus, VHSV), a Lyssauirus of the rhabdovirus family first isolated in 1963 (Jensen, 1965). VHSV is sensitive to temperatures above 25°C and to a wide range of disinfectants (Ahne, 1982). Viral Hemorrhagic Septicemia causes one of the most important fish diseases worldwide, and it is listed as a noticeable disease by the World Organization for World Organization Animal Health (OIE) (Walker *et al*., 2000; Tordo *et al*., 2005).

Virions are bullet-shaped (approximately 70 nm in diameter and 180 nm in length), contain a negative-sense, single-stranded RNA genome of approximately 11,000 nucleotides (Snow *et al.,* 2004) and possess an envelope that contains the membrane glycoprotein, which is the neutralising surface antigen. The genome encodes six proteins: a nucleoprotein, N; a glycoprotein, G; a phosphoprotein, P (formerly designated M1); a matrix protein, M (formerly designated M2); a non-virion protein, NV and a polymerase, L. as well as the traditional host, rainbow trout, in which VHSV can cause severe disease outbreaks, several marine fish species are also susceptible to the disease. The VHSV was detected in European Cultured Fish in 1938 (Rasmussen, 1965; Jørgensen, 1980), and isolated as the causal agent of a severe disease outbreak in farmed rainbow trout (*Oncorhynchus mykiss*) of Denmark in 1962 (Jensen, 1963). The most discriminatory way of typing VHSV is by nucleic acid sequencing. Sequence comparisons of many VHSV isolates by several laboratories have shown that genetic differences appear to be related more to geographical location than to year of isolation or host species (Skall *et al*., 2005). Four major genotypes have been identified, based on sequencing of full-length and/or truncated genes from the N-gene (Einer Jensen *et al*., 2005; Snow *et al*., 2004 and 1999), G-gene (Einer Jensen *et al*., 2004) and NV-gene (11): Genotype I: several sub lineages (Ia–Ie) containing European freshwater VHSV isolates, from the Black Sea area and a group of marine isolates from the Baltic Sea, Kattegat, Skagerrak, the North Sea and the English Channel Genotype II: a group of marine isolates from the Baltic Sea Genotype III: Isolates from the North Atlantic Sea from the Flemish Cap (LÓpez-Vazquez *et al*., 2006) to the Norwegian coast (Dale *et al.,* 2009), the North Sea, Skagerrak and Kattegat. Genotype IV: North American and Japanese/Korean isolate (two sub lineages IVa and IVb (Elsayed *et al*., 2006). Genotype I is divided into several sub lineages, where the marine isolates from wild fish fall into sub lineage Ib. The best resolution of genotype I sub lineages is obtained when analyzing the full-length Gene (Einer Jensen *et al*., 2005). Knowledge of the mechanism of virus transmission has come mainly from studies of rainbow trout VHSV isolates from Europe; this has shown transmission to be horizontal through contact with other infected fish or contaminated water, etc. Virus is shed

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from infected fish via the urine (Smail *et al*., 1999) and reproductive fluids and can also be transferred by piscivorous birds as external mechanical vectors (Olesen *et al*., 1982; Peters *et al*., 1986,). Transmission readily occurs in the temperature range 1–15°C but can occur up to 20°C. Incubation time is dependent on temperature and dose; it is 5–12 days at higher temperatures during and immediately following an outbreak, virus can be isolated readily in cell culture (Wolf, 1988). Kidney, heart and spleen tissues yield the highest viral titers. In carrier (clinically healthy) fish, detection of VHSV is more difficult. VHSV multiplies in a range of fish cell lines, with the BF-2 cell line being the most sensitive to infection by freshwater European strains (Skall *et al*., 2005).

Cell susceptibility is ranked in the order BF-2, FHM, RTG-2, and EPC (Lorenzen *et al*., 1999), but other fish cell lines, such as CHSE-214 and SSN-1, are also susceptible. Susceptibility of a cell line to infection will depend on a range of parameters including cell-line lineage and viral strain differences; it thus appears that the EPC cell line may be more susceptible to VHSV genotype IV isolates than to type I to III isolates (Skall *et al*., 2005).

This disease was detected for the first time in the country in 1383 with the start of monitoring and care of farmed trout by the Veterinary Organization and in 1385 it was officially reported to the World Animal Health Organization (Haghighi Khiabanian Asl *et al*., 2008). Outbreaks of the disease were reduced by control measures, extermination and disinfection and were not observed for 7 years, but in 2012, the occurrence of the disease was reported again in about 60% of the country's provinces (Bokai *et al*., 2016). Scientist all around the world would like to use animal models for different viral and bacterial diseases in order to have a better understanding of how they cause disease and specially to extract antibody against them. Rabbits are one of the most used experimental animals for biomedical research, particularly as a bioreactor for the production of antibodies and serum biological changes (Rahbarizadeh *et al*., 2015). The present study is aimed to describe changes of blood and enzyme factors in New Zealand rabbit (*Oryctolagus cuniculus*) as an animal model in response to the injection of killed viral hemorrhagic septicemia virus (VHSv) of trout fish (*O. mykiss*). Table 1 shows the classification of viral hemorrhagic septicemia virus.

Table 1. Classification of hemorrhagic septicemia virus (OIE.2021).

Realm	**Riboviria**
Kingdom	*Orthonaviriae*
Phylum	*Negarnaviricota*
Class	*Monjiviricetes*
Family	*Rhabdoviridae*
Genus	*Novirhabdovirus*
Species	*Piscine novirhabdovirus*

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Materials and methods

Preparation of hemorrhagic septicemia virus

The initial samples are associated with preparation of culture medium, cell line, virus replication and virus titration. The 6.9 grams of Eagle's Minimum Essential Medium plus 10% bovine serum (EMEM-10), 100 IU/ml penicillin and 0.1 mg/ml streptomycin (Gibco BRL, Life Technologies, UK) were prepared. It was then filtered by a 0.2 micrometer filter made by CHEM Lab. under a sterile microbiology hood and after ensuring that pH is neutral started to prepare monolayer cell culture. Mono layer *Epithelioma papulosum* cyprinidae (EPC) is used as optimal cell culture for viruses (Fijan *et al*., 1983). The EPC cell line is the cell line which is recommended by OIE for the cultivation and titration of VHSV (OIE, 2021). In order to prepare monolayer cell culture, $25cm^2$ flasks were used and sterile EMEM was added to the flasks. Then the flasks were incubated for 24 hours followed by Haenen and Dacidse, (1993) with some modification. at a temperature of 28°C and after this period they were examined under an inverted microscope to check the growth of cells and the formation of a monolayer. After ensuring proper growth of cells, all flasks were kept at 15°C. In this study, the stock virus VHSV- Ia strain DK-5151 with gene bank code (Gen Bank Accession no: AF345859) prepared from the European Union Reference Laboratory was used (Table 2). In order to replicate the virus, after melting the vial containing the lyophilized VHSV using sterile distilled water, the prepared viral solution was filtered using 0.45µ needle filters and 1.5-2ml was added to the flasks containing EPC cells. All the flasks were incubated at a temperature of 15°C and examined for seven days to observe the Cytopathic Effect (CPE). Then, 5ml of trypsin was added to the medium in order to separate the cells, which led to cell lysis and virus leaked into the culture medium, then the culture medium was centrifuged for 10 minutes at 4°C at 200 rpm and the supernatant containing The virus was isolated and stored at -80°C (Haenen and Dacidse, 1993). For the purpose of titration, after performing successive passages in order to reduce the pathogenicity of the virus and maintain its immunogenesis, 96-well plates used for determining virus titration. The virus sample was serially diluted using EMEM medium with a dilution factor of 0.1 from 10^{-1} to 10^{8}. Then, the diluted samples were inoculated in six replicates into 96-well plates containing EPC cell lines without FBS. In addition, empty plates were considered as negative control in each dilution. After inoculation, the titration plate was located in a humid chamber and transferred to a temperature of 15°C. All the inoculated plates were checked daily for seven days for signs of cell damage and their results were recorded. At the end of the seventh day, virus titration was performed according to the method proposed by Reed and Muench, (1938) and the viral titer was expressed as TCID50/ml. Then Bradford method was used to measure viral concentrated protein by Amicon 3KD column (Bradford, 1976). After the same time, viral protein bands were observed with SDS-PAGE gel in SDS-PAGE electrophoresis (Mustafaei, 2014).

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Table 2. The main characteristics of the isolated source of hemorrhagic septicemia virus used in this study (Emmenegger *et al*., 2013)

Genotype	Identifier	Geographic origin	Isolation date	Fish species	Water environment	Host background
Ia	(F1 variant) European	Denmark	1962	Rainbow trout	Freshwater	Farm/ Epidemic

Preparation of rabbits, injection and blood sampling

To perform this test, 15 New Zealand male rabbits with an average weight of 1500 ± 0.25g and the required amount of special pellet food were purchased from Razi Vaccine and Serum Research Institute and housed in 5 cages at the Veterinary Polyclinic of the Islamic Azad University of Tehran Science and Research Unit, located in Shahryar District, Tehran Province. They were kept separately (all three rabbits in each cage) and the adaptation process was carried out for 10 days at a temperature of 25°C. The rabbits were given one daily meal (in terms of 3% of body weight) with rabbit pellets and sufficient water and were prepared for the first injection which was carried out in March, 2022. Then the virus as an antigen to stimulate the immune system with Freund's complete adjuvant (with different ratios of the negative control treatment of 1^{cc} of distilled water, (T1) with a concentration of 1^{cc} of complete virus, (T2) with a concentration of 0.5^{cc} of virus and 0.5^{cc} of Freund's adjuvant, (T3) with a concentration of 0.75^{cc} of virus and 0.25^{cc} of adjuvant, (T4) with a concentration of 0.25^{cc} of virus and 0.75^{cc} of adjuvant were injected for four months, each month as IM to 5 groups of Three New Zealand rabbits with a health certificate prepared from the Razi Vaccine and Serum Institute in Karaj and after 15 days, blood was taken from the marginal vein or the side of the ear (Mojabi *et al*., 2019). Table 3 shows the most common methods of immunization and standard blood sampling protocol in rabbit as animal research model.

Table 3. Common methods of immunization, blood sampling from suggested blood vessels and blood volumes that can be obtained from rabbits as a laboratory model (Rahbarizadeh *et al*., 2015)

1st immunization	Rest	1st booster	Rest	Other booster	Total blood volume	The percentage of the body that contains blood	(Plasma volume) (ml/kg)	Blood sampling
50-250 µg Ag with (CFA) or other adjuvant with SC, ID or IM administration route	3-4 weeks	50-250 µg Ag with (IFA), PBS or other adjuvant with SC, ID or IM administration route	4 or more weeks	µg Ag with (IFA), PBS or other adjuvant with SC, ID or IM administration route	45-75	5.7%	28-51	Side or middle ear vessels

CFA: complete Freund's Adjuvant
IFA: Incomplete Freund's Adjuvant
SC: Subcutaneous
ID: Intra dermal
IM: Intra Muscular
PBS: Phosphate Buffered saline

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Blood and serum measurement

After blood collection, the blood samples of each rabbit in two vials containing anticoagulant to check blood factors and one without anticoagulant to check liver enzymes were sent to the laboratory of the Faculty of Veterinary Medicine, Islamic Azad University, Science and Research Unit and then centrifuged at 3000 revolutions. The preparation of blood spread in order to observe Eos. Mono., PLT and Lymph. On the slide was done by the method of two glass slides and then dyeing with Wright dye was done. A Newbar slide was used to observe and count the number of red and white blood cells. Microhematocrit method was used to measure blood hematocrit. Hemoglobin Cyan met hemoglobin method was used to measure hemoglobin. MCV, MCH and MCHC measurement was a measured according to Mojabi formulation (Mojabi *et al*., 2019).

ALT and AST test

For ALT and AST enzyme assays, the commercial kits of Darman Faraz Kav. Company were used one volume of reagent number 2 with four volumes of reagent number 1 was mixed. The contents of each tube are heated at 37°C for 10 minutes, then one microliter of the working solution was poured into each micro tube and 100 microliters of each rabbit's serum sample was added to it. Then the photometer at a wavelength of 340 nm was set and zero the photometer with air and put the animal control serum as a standard in the photometer and calibrated the device with it. The standard wavelength according to the program of the device for ALT enzyme is in the range of 95 up to 151 nm and the standard wavelength according to the program of the device for AST enzyme is in the range of 118 to 188 nm. After calibration, we read each rabbit's serum sample (www.darmanfarazkave.com).The standard levels of ALT and AST through on Darman Faraz Kav protocol is according to table 4.

Table 4. Enzyme standards based on Darman Faraz Kav. treatment brochure (www.darmanfarazkave.com)

Components	Test way		Unit	Evaluate rate	Maximum	Unit	Evaluate rate	Maximum
ALT(GPT)	PSP in 37°C without	(12701)	U/L	123	95-151	KAT/Lμ	2.05	1.58 2.53
AST(GOT)	PSP in 37°C without	12601)	U/L	152	118-188	KAT/Lμ	2.55	1.94 3.14

Results

The initial titration of the virus-based Reed and Munch method (1983) was considered to be 4 $\times 10^7$ tissue culture infectious dose TCID 50/ml.

The Optic density (OD) of the virus and its concentration are according to Table 5. The final concentration of the virus after concentration is 274 μg/ml, which was also investigated by SDS-PAGE electrophoresis (Bradford, 1976; Mustafaei, 2014).

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Table 5. Determination of virus-infected cell soup concentration by Bradford (1976)

Virus sample	Optical density (OD)	Concentration (µg/ml)
Virus-infected cell soup without concentrate	0.207	137
Amicon filter supernatant (concentrated virus)	0.331	274
Amicon filter bottom liquid	0.0567	29

Table 6. Molecular weight of VHS virus constituent proteins (Nishizawa *et al*., 1991)

Protein	VHS
L	156
G	68.5
N	43.5
M1	24.8
M	--
M2	20.6

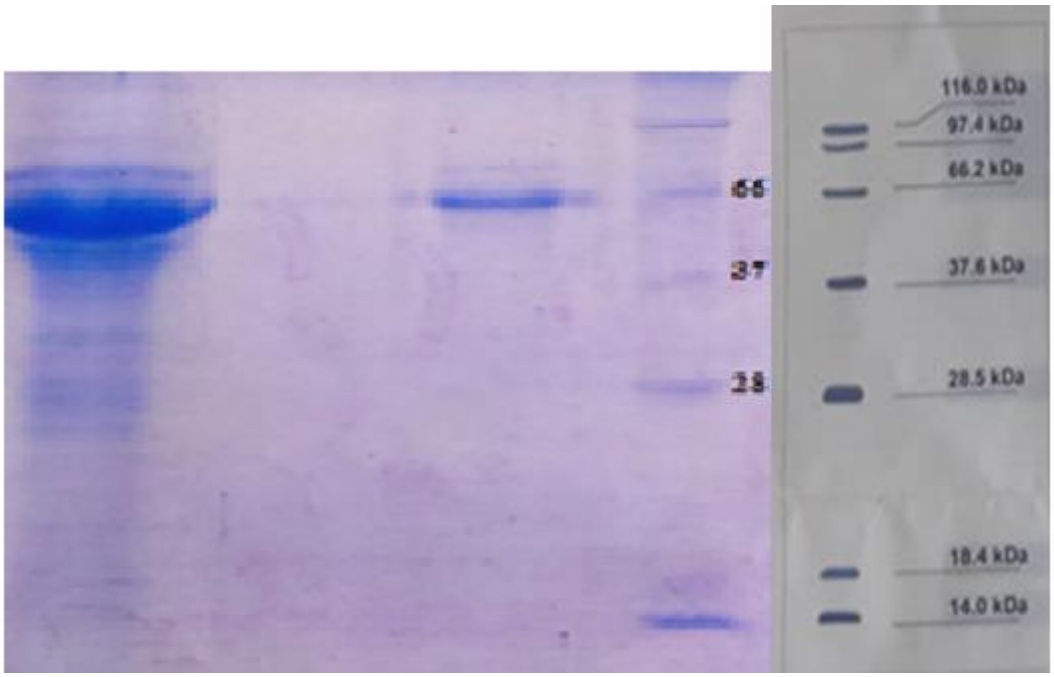

Figure 1. Presence of a single protein band of VHS virus with a concentration of 274 mg/ml (the left blue line referred to final concentrated VHS protein and the thin line on the right shows virus protein concentration before using Amicon ultra centrifugal filter device.

Figure 2 shows the protein bands separated according to the molecular weight of VHS virus by electrophoresis in the process of conducting this study. The molecular weight of these proteins is indicated by the ladder on the right side. Figure 2, which has standard proteins, is compared and also according to the molecular weight of protein G in table 2, it shows that after concentrating the VHS virus, protein G with a molecular weight of 66 kilo Daltons is similar to the molecular weight in table 2, which is approximately 66.8 kilo Daltons. Also, after concentration, a strong band is formed in the region of 66 kilo Daltons, but no band is formed in the sample below the filter. In fact, the 66 kilo Dalton band is related to the G protein of the virus, which is a structural and

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surface protein, and this group of proteins is involved in connecting the virus to host cells. This number is in the range of G protein trout hemorrhagic septicemia virus. As stated earlier, this virus has 6 types of proteins, the most abundant of which is protein G, which can be seen in electrophoresis (Nishizawa *et al*., 1991).

SPSS version 20 software was used to analyze production of antibody and other blood factors against VHS antigen. For this article, one-way ANOVA and Duncan's analytical test were used. The minimum significant level in all these statistical analyzes is considered below 0.05.

Table 7. The hematological parameters on first sampling (March, 1400)

The evaluated index	Negative control	Treatment 1	Treatment 2	Treatment 3	Treatment 4
ALT(IU/L)*	31.0 ± 0.00 [a]	34.0 ±1.15 [b]	45.5± 0.86 [c]	48.5± 0.28 [d]	44.5 ± 0.28 [c]
AST(IU/L)*	42.0 ± 0.57 [a]	43.00 ± 0.57 [a]	47.50 b±5.86	57.0 ± 0.00 [c]	47.0 ± 0.57 [b]
HCT(%)	40.00±0.57	35.00±0.57	34.66±2.02	33.66±3.17	39.00±0.57
HGB(g/dl)	12.65±0.66	11.95±0.08	11.60±0.69	11.25±1.06	13.43±0.34
RBC(X 106/µL)	6.30±0.17	5.60±0.00	5.65±0.37	5.20±0.57	6.25±0.14
MCV(FL)*	61.00 ± 0.57[a]	64.00 ± 0.00 [bc]	62.50± 0.28 ac	64.40 ± 0.80 [b]	62.5 ± 0.43 [a]
MCH(pg)	20.40±0.34	20.45±0.54	20.65±0.37	21.40±0.34	21.35±0.20
MCHC(%)*	23.55±0.08[a]	32.00± 0.57 [b]	31.85± 0.20 b	33.35± 0.20 [c]	33.65± 0.02 [c]
WBC(X 103/µL)*	3520.0±46.18[acd]	2275.0 ±72.16[a]	5475.0 ±1371.2[bc]	3150.0±577.3[ad]	4470.0±98.15[bd]
Eos(%)*	2.33± 0.57[a]	16.0± 6.00 [b]	24.5± 9.50 [bc]	19.5± 9.50 [b]	35.83± 0.28[c]
Lymph(%)*	95.0± 2.00 [a]	79.66± 4.40 [b]	68.0± 5.19 [bc]	77.66± 5.48 [b]	64.16± 0.16 [c]
Seg(%)*	2.66± 3.78 [a b]	4.33± 2.18 [ab c]	7.50± 0.50 [c]	2.83±0.28[a b]	0.0± 0.00[a]

*Different superscript letters in each row indicate significant differences in different groups ($p<0.05$).ALT: Alanine aminotransferase. AST: Aspartate aminotransferase. HCT: Hematocrit. HGB: Hemoglobin. RBC: Red Blood Cell. MCV: Mean Corpuscular Volume. MCH: Mean Corpuscular Hemoglobin. MCHC: Mean corpuscular Hemoglobin Concentration. WBC: White Blood Cell. Eos: Eosinophil. Lymph: Lymphocyte. Seg: Segmented neutrophil.

Table 8. The hematological parameters on second sampling (April, 2022)

The evaluated index	Negative control	Treatment 1	Treatment 2	Treatment 3	Treatment 4
*ALT(IU/L)	56.0± 0.57 [a]	72.0 ±1.73[b]	76.5 ±0.28[c]	86.5 ±1.44[d]	69.0 ±0.57[b]
*AST(IU/L)	46.5± 0.86 [a]	46.50±0.28[a]	54.0±0.57[b]	55.50±0.86[b]	50.50±0.86[c]
*HCT(%)	34.10± 0.81 [a]	34.70±1.15[a]	34.03±2.86[a]	39.75±0.43[b]	35.85±0.31[ab]
*HGB(g/dl)	12.15±0.08[ab]	11.65±0.37[a]	11.35±0.95[a]	13.25±0.14[b]	12.00±0.11[ab]
RBC(X 10^6/µL)	5.95±0.02	5.85±0.20[a]	5.70±0.46[a]	6.60±0.05[b]	6.00±0.05[ab]
MCV(FL)	59.15±0.08[a]	59.25±0.08[ab]	59.70±0.17[bc]	59.60±0.23[ac]	59.5±0.17[ab]
MCH(pg)	29.00±0.00[a]	29.75±0.02[b]	30.00±0.00[c]	30.00±0.00[c]	29.85±0.02[d]
MCHC(%)	33.65±0.08[a]	33.55±0.03[ad]	33.30±0.00[b]	33.35±0.02[bc]	33.45±0.02[cd]
* WBC(X 103/µL)	9050.0±57.73[ac]	8275.0±389.7[ac]	6450.0±2915.6[ab]	8000.0±1760.9[c]	8665.0±733.10[ac]
*Eos(%)	2.33± 0.33[a]	16.0± 3.46[b]	24.5± 5.48[c]	19.5± 5.48[d]	35.83± 0.16[e]
*Lymph(%)	57.0± 1.15[a]	71.16± 9.27[b]	74.50± 10.10[c]	60.00± 0.57[d]	63.33± 1,76[e]
*Seg(%)	40.5± 0.28 [a]	26.83± 8.12[a b]	23.00± 8.66[b]	39.00± 0.57[ab]	35.66± 1.76[ab]

*Different superscript letters in each row indicate significant differences in different groups ($p<0.05$).ALT: Alanine aminotransferase. AST: Aspartate aminotransferase. HCT: Hematocrit. HGB: Hemoglobin. RBC: Red Blood Cell. MCV: Mean Corpuscular Volume. MCH: Mean Corpuscular Hemoglobin. MCHC: Mean corpuscular Hemoglobin Concentration. WBC: White Blood Cell. Eos: Eosinophil. Lymph: Lymphocyte. Seg: Segmented neutrophil.

On the first day of sampling, ALT, AST, MCV, MCHC and WBC showed a significant difference with each other in different groups ($p<0.05$). In this regard, the

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minimum and maximum levels of ALT on the first day of sampling were 31.0±0.00 and 48.5±0.28 in control negative group and T3 (virus 75% and adjuvant 25%), respectively ($p<0.05$). This pattern was also the same in the case of AST, so that its minimum and maximum values were observed in C group and T3 with values of 42.0±0.57 and 57.0±0.00 ($p<0.05$). MCV also indicated significant changes among the research groups ($p<0.05$). In this regard, the highest value was observed in T3, 64.40±0.80, ($p<0.05$), and the lowest value was observed in the C group, 61.00±0.57. MCHC index also witnessed significant changes among the research groups ($p<0.05$). In this regard, the highest value was 33.65±0.02 in T4 and its lowest value was recorded in the C group 23.55±0.08. WBC shows significant changes among the research groups ($p<0.05$). In this regard, the highest value was 5475.0±1371.02 in T3 and the lowest level was 3520.0± 46.18.

On the second day of sampling, AST, ALT, HGB and HCT and the amount of WBC in different groups showed significant differences with each other ($p<0.05$). The minimum AST is 46.50±0.28 in T1 and its highest value is 55.50±0.86 in T3 ($p<0.05$). The minimum level of ALT in the C group is 56. 0±0.57 and the maximum value is 86.5±1.44 in T3 ($p<0.05$). HCT, the highest value in T3, 39.75±0.43 and the lowest value in the C group with 34.10±0.81 has a significant difference ($p<0.05$).

The highest value of HGB in T3 is 13.25±0.14 and the lowest value in T2 is 11.35±0.95, there is a significant difference ($p<0.05$). WBC shows significant changes among the research groups ($p<0.05$). In this regard, the highest value was 9050.0±57.73 in C group and the lowest level was 6450.0±2915.6 in T3.

Table 9. The hematological parameters on third sampling (June, 2022)

The evaluated index	Negative control	Treatment 1	Treatment 2	Treatment 3	Treatment 4
ALT(IU/L)*	58.0± 0.57^{a}	70.5±2.02^{b}	77.0±1.15^{c}	75.0±0.28^{c}	66.0±0.57^{d}
AST(IU/L)*	49.50± 0.86^{a}	48.0±0.57^{a}	57.0±2.30^{b}	55.0±0.57^{b}	55.0±0.57^{b}
HCT(%)*	34.25± 072^{a}	41.00±2.30^{ac}	38.00±1.57^{ac}	42.00±0.57^{bc}	36.5±2.02^{ab}
HGB(g/dl)	12.10±0.11	13.65±0.77	12.65±0.20	14.00±0.17	12.15±0.66
RBC(X 10^{6}/µL)	5.95±0.02	6.23±0.02	5.70±0.46	6.60±0.05	6.00±0.05
MCV(FL)	59.15±0.08	59.25±0.05	59.50±0..28	59.20±0.17	59.30±0.23
MCH(pg)	21.25±1.01	19.65±0.02	19.30±0.17	19.75±0.08	19.79±0.05
MCHC(%)	33.65±0.08	32.75±0.31	33.25±0.02	33.30±0.05	32.75±0.14
WBC(X 10^{3}/µL)*	7575.±331.97^{a}	8375.0±1602.1^{a}	19000.0±490.7^{b}	8700.0±1558.8^{c}	6575.0±1140.2^{a}
Eos(%)*	5.50± 0.86^{a}	7.50± 2.02^{b}	7.33± 0.88^{b}	6.0± 1.15^{a}	7.50± 0.86^{b}
Lymph(%)*	60.0± 4.00^{a}	69.50± 5.48^{b}	64.00± 1.00^{c}	60.00± 1.15^{a}	61.16± 7.82^{a}
Seg(%)*	31.16± 1.09^{a}	23.0± 3.46^{b}	28.6± 0.33^{c}	33.00± 1.00^{d}	31.33± 6.96^{a}

*Different superscript letters in each row indicate significant differences in different groups ($p<0.05$). ALT: Alanine aminotransferase. AST: Aspartate aminotransferase. HCT: Hematocrit. HGB: Hemoglobin. RBC: Red Blood Cell. MCV: Mean Corpuscular Volume. MCH: Mean Corpuscular Hemoglobin. MCHC: Mean corpuscular Hemoglobin Concentration. WBC: White Blood Cell. Eos: Eosinophil. Lymph: Lymphocyte. Seg: Segmented neutrophil.

On the third day of sampling, AST, ALT, HCT indices and the amount of WBC showed significant differences in different groups ($p<0.05$). The minimum AST is 48.0±057 in T1 and its highest value is 57.0±2.30 in T3 ($p<0.05$). The minimum level of ALT in the C

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group is 58. 0±0.57 and the maximum value is 75.0±0.28 in T3 ($p<0.05$).

HCT also witnessed significant changes ($p<0.05$). Its highest value was 42.00±0.57 in T3, and it had a significant difference with the control group ($p<0.05$) and its lowest value was in the C group with a value of 34.25±0.72. The MCHC was recorded with a maximum of 33.65±0.08 in C group and the lowest level was 32.25±0.02 in T2. WBC in different groups showed a significant difference, so that its maximum value was recorded as 19000.0 ± 490.7 in T2 and it showed a significant difference with other groups ($p<0.05$). The lowest amount of white blood cells in the amount of 6757.0 ± 1140.2 was observed in T4.

Table 10. The hematological parameters on fourth sampling (July, 2022)

The evaluated index	Negative control	Treatment 2	Treatment 3
ALT(IU/L)*	51.0± 0.00 [a]	70.50±0.28[b]	84.5±0.28[c]
AST(IU/L)*	44.50± 0.28 [a]	41.5±0.86[a]	52.0±1.73[b]
HCT(%)*	34.25± 0.72 [a]	38.00±0.57[b]	42.00±0.57[c]
HGB(g/dl)	11.85± 0.08	11.85±0.08	12.30±0.00
RBC(X 10^6/µL)	6.05±0.08	5.90±0.00	6.30±0.00
MCV(FL)	59.15±0.08	60.15±0.49	59.00±0.00
MCH(pg)	20.40±0.34	19.80±0.00	19.80±0.00
MCHC(%)	33.40±0.00	33.35±0.02	33.20±0.00
WBC(X 10^3/µL) *	5900.0±490.74[a]	14125±1428.9[b]	8600.0±2338.3[ab]
Eos (%)*	8.00± 0.57 [a]	7.00± 0.57 [a]	5.00± 0.00 [b]
Lymph (%)*	62.0± 0.57 [a]	53.50± 2.02 [b]	66.00± 1.73 [a]
Seg (%)*	25.00± 0.57 [a]	40.00± 1.15 [b]	28.00± 1.15 [a]

*Different superscript letters in each row indicate significant differences in different groups ($p<0.05$). ALT: Alanine aminotransferase. AST: Aspartate aminotransferase. HCT: Hematocrit. HGB: Hemoglobin. RBC: Red Blood Cell. MCV: Mean Corpuscular Volume. MCH: Mean Corpuscular Hemoglobin. MCHC: Mean corpuscular Hemoglobin Concentration. WBC: White Blood Cell. Eos: Eosinophil. Lymph: Lymphocyte. Seg: Segmented neutrophil.

This research is in line with the article (Hafezieh et al., 2021) that investigated the production of polyclonal antibodies in rabbits against viral hemorrhagic septicemia. Hence, in the fourth blood sampling, in order to reduce costs, only the 2nd and 3rd treatments that produced the highest amount of neutralizing antibodies were taken. On the last day of sampling, AST, ALT, HCT and WBC showed significant differences ($p<0.05$). The minimum AST is 41.5±0.86 in T2 and its highest value is 52.0±1.73 in T3 ($p<0.05$). The minimum level of ALT in the C group is 51. 0±0.00 and the maximum value is 84.5±0.28 in T3 ($p<0.05$). HCT also witnessed significant changes ($p<0.05$). Its highest value was 42.00±0.57 in T3, and its lowest value was in the C group with a value of 34.25±0.72. The WBC in different groups showed a significant difference, so that its maximum value was recorded as 14125.0 ± 1428.9 in T2 and it showed a significant difference with other groups ($p<0.05$). The lowest amount of white blood cells in the amount of 5900.0 ± 490.74 was observed in C group.

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Table 11. Standard blood parameters of New Zealand adult male rabbits (Melillio, 2007)

Indicator	Mature male rabbit
(X 10^6/µL) RBC	7.94-5.46
PCV(%)	50-33
Hgb(g/dL)	17.4-10.4
MCV(fL)	66.5-58.5
MCH(pg)	22.7-18.7
MCHC(%)	50-33
(X 10^3/µL) Platelets	656-304
(X 10^3/µL) WBC	12.5-5.5
Neutrophil(%)	54-34
Lymphocyte(%)	50-28
Eosinophil(%)	3.5-0.5
Basophil(%)	7.5-2.5
Monocyte(%)	12-4
ALT(IU/L)	80-45
AST(IU/L)	130-35

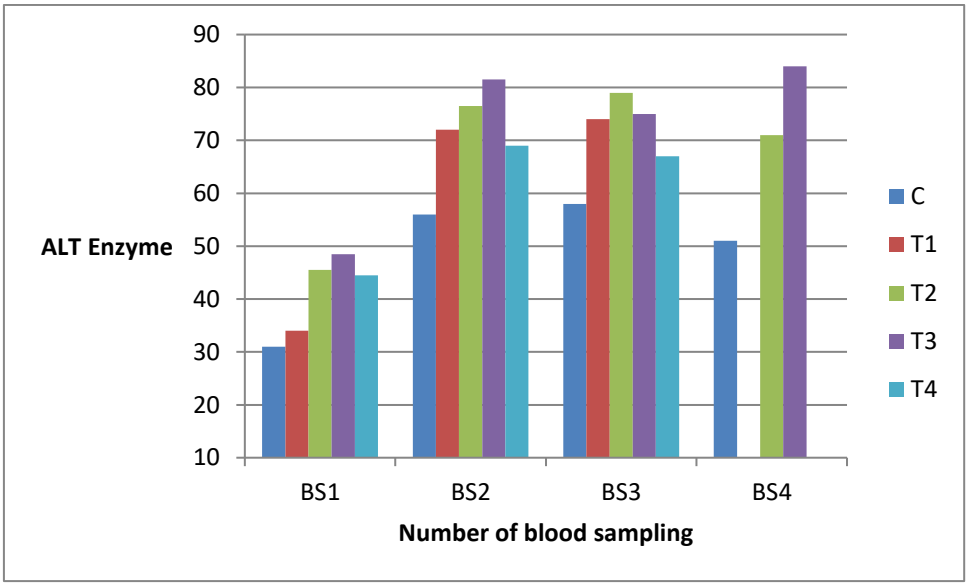

Figure 3. The process of ALT liver enzyme production during 4 blood sampling

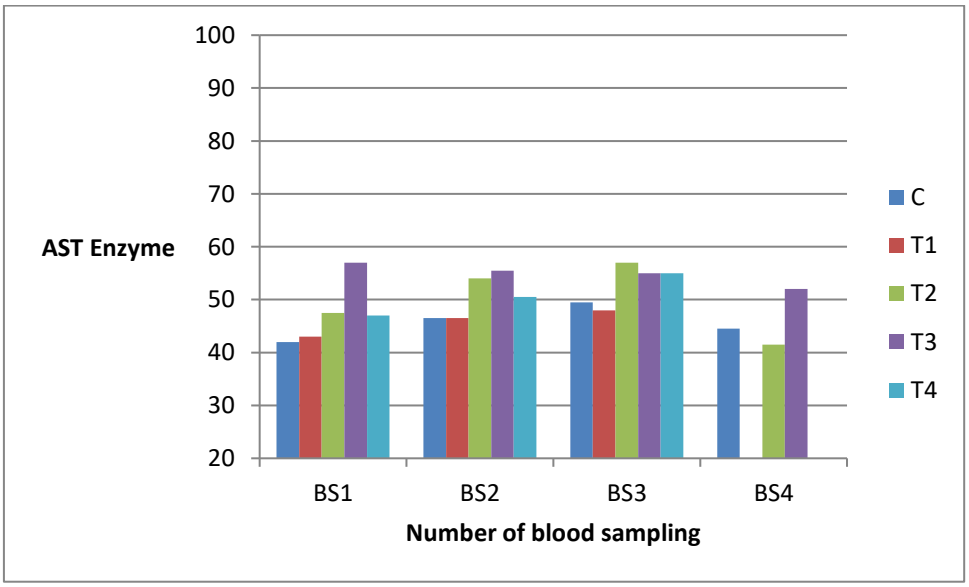

Figure 4. The process of AST liver enzyme production during 4 blood sampling.

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Discussion

Hemorrhagic septicemia virus of trout fish is not pathogenic to rabbit as animal model, but as an antigen (Foreign body) it can lead to immune system stimulation and MHC Class1 activation (Olesen *et al*., 1999), which ultimately leads to produce antibodies (Hafezieh *et al.*, 2021) Other factors such as AST, ALT, WBC, and RBC and indicators related to red blood cells, MCH, MCHC, HGB, and HCT were also measured. In the meantime, on the first day of blood sampling, AST, ALT, MCV and WBC factors underwent changes due to the introduction of this virus into the rabbits' bodies. Table 10 contains standard blood rabbit parameters.

According to table 11, which shows the blood standards in male rabbits, in the first blood sampling, high levels of lymphocytes was observed in all groups (lymphocytosis), even in the control group in the first blood draw, can be considered due to the stress caused by the injection (Table 6). During stress, high adrenaline in the animal's body prevent the return of lymphocytes from the blood circulation to the lymphatic vessels and also the release of lymphocytes from the thoracic duct into the bloodstream causes lymphocytosis, or it can be caused by the presence of high amounts of hemorrhagic septicemia virus antigen and start stimulating the humoral immune system of the rabbit's body to produce plasma cells. In the continuation of blood sampling, changes in ALT, AST, WBC and MCHC factors were observed. The increase in liver enzymes ALT and AST is due to inflammation caused by the presence of a foreign agent (Olesen *et al*., 1999).

According to the research of Elmas *et al*., (2008), with the injection of *Escherichia coli* bacteria in rabbits, which leads to endotoxemia, the levels of AST and ALT enzymes increased in successive injections and blood sampling.

In the first blood sampling, high amount of eosinophil was observed in compare with other blood sampling(Table 6), which can be considered due to the injection of different doses of the virus and Freund's complete adjuvant, sensitivity to the adjuvant occurred, and this was not observed in the other blood samplings due to the injection of incomplete Freund's adjuvant Freund's adjuvants often cause hypersensitivity reactions in laboratory animals after injection, but they are still used due to the addition of immune response along with immunogenic antigen and the production of a large amount of antibodies that are much stronger than when the antigen being injected alone. However, instructions to reduce side effects such as skin ulcers and dryness in booster injections are used with incomplete Freund's adjuvant (Rahbarizadeh *et al*., 2015).

In the second blood sampling, the amount of MCH in the treatments increased (Table 7). In most infections, cytokines and inflammatory mediators are produced, which can directly or indirectly cause damage to the red blood cell membrane and change its structure, leading to changes in factors related to red blood cells such as: HGB, PCV, MCH, MCHC and HCT (Gao *et al*., 2016). It seems that in the second

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blood sampling, among all blood draws, much higher amounts of MCH and WBCs, including neutrophils, was observed, which indicates an acute phase of inflammation caused by the injection of virus and adjuvant in rabbits.

The amount of WBCs in the third blood draw also increased greatly (Table 8), which is a confirmation of the activity and response of the immune system to the presence of immunogen in the body and related to the production of Immunoglobulin and causing leukocytosis. In T2, the amount of white blood cells increased from 6450±2915. In the second blood draw and reached 19000.0±490.7 in the third blood draw, which indicates the presence of inflammation. This number reached almost 14125.0±1428.9 in the fourth blood draw (Table 9), which, despite the little drop, was still higher than the T3 treatment and, naturally, the control group. An increase in the amount of neutrophils and was observed without any significant change in the number of lymphocytes during the second, third and fourth blood sampling. In the course of microorganism invasion and the occurrence of inflammation, neutrophil production-producing growth factors are released by macrophages, T cells, and bone marrow stromal cells and lead to the production of more neutrophils. Growth stimulating factors such as stem cell factors G-CSF, GM-CSF and interleukins play an essential role in the production of neutrophils (Dragoo *et al*., 2012).

This research was carried out based on study of Hafezieh et al in 2021 with the title of investigation of polyclonal antibody production against VHS in the rabbit as an animal model, the results of this research is compared with the antibody titer results obtained in the study of Hafezieh which stated that the treatment 2 (0.5 cc virus and 0.5 cc adjuvant) and T3 (0.75 cc virus and 0.25 cc adjuvant) had higher ALT, AST, HCT and WBCs among other treatments in all 4 blood samplings. Between T2 and T3, T2 with a lower amount of liver enzymes (AST, ALT), indicating less pressure on the liver meanwhile producing higher levels of white blood cells and antibodies (Hafezieh *et al*., 2021), indicated that T2 had more acceptable reaction toward VHS.

Conflict of interest

Authors have no conflict of interest on this work.

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