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Effects of *Aeromonas hydrophila* killed by heat, formalin, and UV on liver enzymes, biochemical factors and gene expression in rainbow trout, *Oncorhynchus mykiss*

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ABSTRACT

Aeromonas hydrophila is one of the most significant and prevalent bacteria in fish farming. In this study, the effects of some killing methods of *A. hydrophila* on liver enzymes, biochemical parameters, and gene expression in rainbow trout, *Oncorhynchus mykiss* were evaluated. Four groups of 20 fish, each an average weight of 5 g with three replicates were exposed to live *A. hydrophila* and heat, formalin, and UV-killed bacteria via intraperitoneal injection. The results showed that the levels of liver enzymes, AST and ALT did not differ significantly among the treatments ($p > 0.05$). Blood biochemical analysis revealed a significant increase in total protein levels in the treatment groups compared to the control ($p < 0.05$), while no significant differences were observed in albumin levels ($p > 0.05$). The expression of immune-related genes (TNF-α and IL-6) varied significantly among treatments, with the formalin treatment showing the highest expression levels ($p < 0.05$), and the control group showing. These findings suggest that inactivation of *A. hydrophila* with formalin is more effective in enhancing the immune response of rainbow trout compared to heat or UV inactivation methods.

Keywords: *Aeromonas hydrophila*, Rainbow trout, Gene expression, Liver enzymes
Article type: Research Article.

INTRODUCTION

The lack of freshwater resources has become a critical global development challenge all over the world. Although the main problems of humans in the past centuries were issues such as disease, food supply, energy, etc., but in the current century, the provision of fresh water for the production of cold-water fish is one of the most important challenges for mankind. Iran is no exception to this rule, but the situation of freshwater resources is more acute for a water-scarce country like Iran (with an average rainfall of 254 mm, Iran is considered one of the semi-arid countries of the world) and it seems that the crisis of shortage of freshwater resources in the future, it will appear as a serious challenge that this issue will cause the lack of development in the production of cold fish, and to deal with such a situation, the productivity of production must increase. Increasing productivity in conditions of lack of surface development causes an increase in density, which causes diseases in the field of fish breeding. The spread of diseases such as streptococcosis in recent years has created serious problems for salmon farming in Iran so the annual damage caused by the disease has been estimated at $3 million (Nafisi Bahadi 2012). Aquaculture production has overtaken total capture fisheries by approximately 8.7% over the past four decades. Antibiotics and chemical drugs are commonly used in aquaculture to prevent diseases, however, this practice leads to significant problems including the rapid spread of antibiotic-resistant pathogens in aquatic ecosystems,

environmental damage, and reduced disease resistance in fish (Abdelkhalek *et al*. 2017; Adel *et al*. 2017; Sarhadi e*t al*. 2020). Consequently, there is an urgent need for the use of non-chemical substances. Special attention is paid to the production of vaccines to control diseases in a cultural condition. For vaccine production, bacteria are inactivated by various methods such as heat, formalin and UV rays, and so on. Then, the fish are injected by killed bacteria as the vaccine (Mai *et al*. 2021; Ahangarzadeh *et al*. 2022). In a study in which Rafiee and Vafadar (2021), used lactobacilli isolated from the intestines of common carp in feeding rainbow trout, they found that this improved their growth parameters and bacterial resistance. Also, in another study, Jafaryan *et al*. (2011) using bacilli isolated from sturgeon fish, were able to increase the growth and survival of rainbow trout larvae against salinity, heat, and acidity stresses. In another study that was conducted to investigate the effect of feeding salmon with aloe vera powder on the basic immune function and their immune response to the unusual *Aeromonas salmonicida* killed with formalin, it was found that long-term feeding of salmon with aloe vera in the aquatic environment. Breeding does not increase their ability to resist bacterial infection or reduce the impact of bacterial diseases once they are infected (Zanuzzo *et al*. 2015). In a study conducted by Radhakrishnan *et al*. (2023), to analyze the increase in innate immune responses in common carp, *Cyprinus carpio* L. in the juvenile stage, after administration of heat-killed *Aeromonas hydrophila* at a dose of 1×10^7 CFU mL^{-1} through encapsulation. They found that oral administration of encapsulated antigens enhances innate immunity in juvenile fish. In a study conducted by Meshkini *et al*. (2015) to investigate the effect of levamisole on the immune system of rainbow trout under conditions of density stress, they found that the use of levamisole can increase immunity in salmon under conditions of density stress. Also, they suggested the use of levamisole with a maximum concentration of 0.1% of the diet as an immune stimulus in rainbow trout in times of high-density stress. Based on the studies by Owehand & Selminen (1998) and Hassan *et al*. (2019), various methods have been used to inactivate or kill the bacteria. In fact, the acquired methods will influence serum as well as immunity system gene expression changes in fish. Regarding to the effect of killing methods on vaccine efficiency, the present study was conducted to evaluate the effect of killing *A. hydrophila* by heat, formalin, and UV on survival, blood biochemical, and expression of immunogenic genes (TNF-α and IL-6) in one of the most culturing species in aquaculture section, rainbow trout.

MATERIALS AND METHODS

Designing the experimental

One hundred twenty rainbow trout with an average initial weight of 5.16 ± 0.08 g were transferred to farming tanks (500 L) with a density of 20 pieces per tank so each treatment included 60 pieces of fish. After adaptation, biometry was done for running a completely randomized design as four trial treatments with three replications. During the experiment, fish were fed with commercial feed for 20 days. To evaluate the immunogenicity of *A. hydrophilic*, killed bacteria by heat, formalin, and UV methods were injected to fish as intraperitoneal on the 20^{th} day of farming with a concentration of 10^5 CFU mL^{-1} (Joseph & Carnahan 1994).

Killed bacteria preparation and injection

Preparing killed bacteria is performed according to the following methods. At the end of the bacterial killing steps by any of the following methods, the solutions were tested in the culture medium to confirm all bacteria were killed.

Formalin. In this method 37% formalin solution was used with a ratio of 3 units of formalin solution to 97 units of distilled water, and the final solution contained 1.11% of the active ingredient then formalin at a rate of 3% of based stock formalin was added to the solution containing bacteria and stored in the refrigerator for 24 hours. After centrifugation (4000 rpm) and washing with buffer phosphate solution, kept at 3 °C (Imani & Akhlaghi 2004).

Heat. The containing bacteria solution placed in a water bath at 60 °C for 4 hours, then kept at 3 °C (Imani & Akhlaghi 2004).

UV. The solution containing bacteria exposed to radiation at a distance of 30 cm of UV lamp with a power of 15 watts for 2 hours at a constant temperature (25 °C; Strunk *et al*. 2011).

Sampling and immunological gene expression

After 4 weeks of breeding, at the end of the experiment, five fish were randomly removed from each replicate. Following an overdose of clove oil anaesthesia (1 mL L^{-1}), the fish were dissected, their liver were removed, and stored in a freezer at -80 °C for further evaluation.

RNA isolation and complementary DNA preparation

The samples underwent total RNA isolation utilizing the RiboEx™ reagent from the miRNeasy Kit (QIAGEN) in accordance with the provided protocol. A Nanophotometer (IMPLEN-P100, München, Germany) was employed to measure RNA concentration and assess purity, while RNA integrity was verified through electrophoresis on a 1.5% agarose gel. To remove any genomic DNA contamination, the isolated RNA was subjected to DNase I treatment using the Fermentas Kit (France). Complementary DNA (cDNA) was then generated through reverse transcription with oligo-dT primers following the Fermentas Kit's (France) standard procedures.

Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR was performed to evaluate expression patterns of the IL-6 and TNF-a genes using Fermentas Maxima SYBR Green qPCR Master Mix (1×) as described by Safari *et al*. (2025). Specific primer sequences are detailed in Table 1. Target gene expression was normalized against the reference gene β-actin, with relative quantification determined through the $2^{-}\Delta\Delta Ct$ calculation method. All analytical procedures were conducted using the Bio-Rad System software (version 2.00, Hercules, CA, USA.) for data processing and interpretation.

Table 1. Primer sequences for qRT-PCR analysis of IL-6, TNF-a, and β-actin Genes.

	Primer sequence	Accession number	Primer efficiency
IL-6	F: TTTCAGAAGCCCGTGGAAGAGA R: TCTTTGACCAGCCCTATCAGCA	DQ875251	96
TNF-a	F: CGCTGACACAGTGCAGTGGA R: TCCCCGATGGAGTCCGAATA	AJ302617	97
β-actin	F: ACGGCCAGAGGCGTACAG R: TTCAACCCTGCCATGT	AF254414	98

Liver enzymes and biochemical parameters

Liver enzymes including AST (aspartate aminotransferase) and ALT (alkaline phosphatase) were measured using a kit from Pars Azmoun Company (Karaj, Iran) and a photometer (model AE-600, EMRA Company, Japan) with an enzymatic method (International Federation of Clinical Chemistry) according to the instructions.

Total protein (mg dL^{-1}), albumin (mg dL^{-1}) were determined using Pars Azmoon kits (Pars Azmoon, Tehran, Iran) and the biochemical auto analyzer instrument (Eurolyser, Belgium).

Statically analyses

The relative expression of the desired genes was analyzed using REST software (Pfaffl *et al*. 2002). Data analysis and determination of significance levels were carried out by applying SPSS 2018 software and the Duncan statistical test with a 95% confidence interval and one-way analysis of variance.

RESULTS

Liver enzymes, and biochemical parameters

The results showed that the mean AST values did not differ significantly between the treatments ($p > 0.05$), although the highest values were observed in the formalin treatment and the lowest in the UV treatment (Fig. 1). The ALT values also did not show a significant difference between the treatments, but the highest values were related to the UV treatment and the lowest values were related to the heat treatment (Fig. 2). The results of biochemical parameters showed a significant increased protein content in the formalin and UV treatments compared to the heat treatment and control group ($p < 0.05$). Nevertheless, no significant difference in blood albumin content was observed in the experimental treatments except the control treatment (Table 2).

Table 2. Effect of different methods of killing *A. hydrophila* on the biochemical parameters of blood serum in rainbow trout (n = 15; mean ± SD)

Biochemical Indices	Experimental treatment			
	Control	Heat	Formalin	UV
Total Protein	3.38±021^{c}	4.53±0.34^{b}	5.15±0.22^{a}	5.33±033^{a}
Albumin	2.43±0.28^{b}	3.02±0.14^{a}	2.88±0.17^{a}	3.15±0.22^{a}

Note: Different letters characterize significant difference ($p \leq 0.05$).

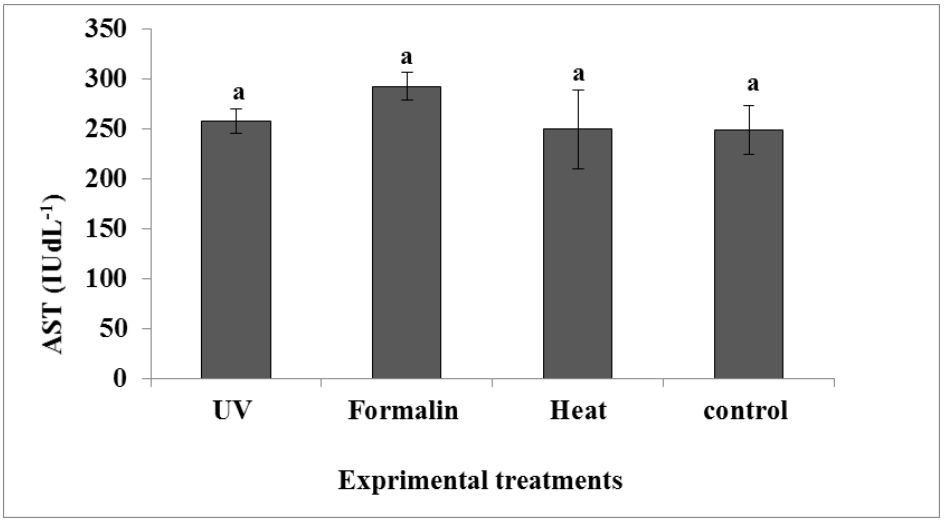

Fig. 1. The effect of different methods of killing *A. hydrophila* on AST activity in blood serum of rainbow trout (n = 15; mean ± SD). Values sharing the same letter are not significantly different ($p > 0.05$).

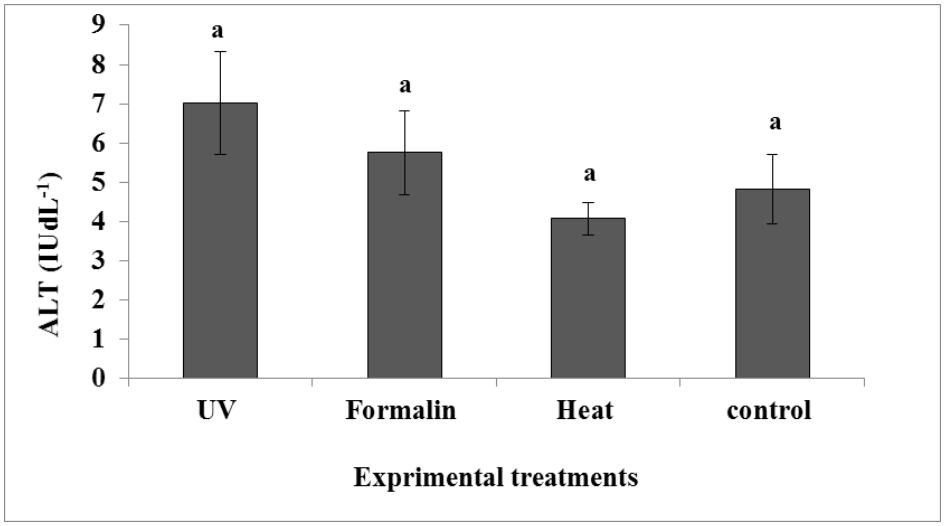

Fig. 2. The effect of different methods of killing *A. hydrophila* on ALT activity in blood serum of rainbow trout (n = 15; mean ± SD). Values sharing the same letter are not significantly different ($p > 0.05$).

Immunity gene expression and survival rate

The results of immune genes expression (TNF-α and IL-6) among different treatments showed that the highest expression of TNF-α and IL-6 genes belonged to the formalin treatment. Furthermore, the lowest expression of immunity genes occurred in the control group ($p < 0.05$; Fig. 3). According to Figs. 4 and 5, the injection of killed *A. hydrophila* in all three methods of heat, formalin, and UV had a significant effect on TNF-α and IL-6 gene expression compared to the control group respectively ($p < 0.05$). In contrast, no significant difference was observed in the expression of the IL-6 gene between UV and heat treatments. The mortality results have been shown in Fig. 3. Based on the results, mortality in the control treatment (85%) was higher than in other treatments ($p < 0.05$).

DISCUSSION

In the present study, *A. hydrophilic* was killed by three methods of heating, formalin, and UV to evaluate the immunization. *A. hydrophila* is a toxic septic agent for cold and warm water fish (Ma *et al*. 2019). Measurement of serum biochemical parameters can be used as an indicator to examine the changes in the immune system of fish (Lamers & Muiswinkel 1986). Inflammation of liver cells may cause the secretion of some liver enzymes into the bloodstream. The results of this study showed that there was no significant difference in the levels of liver enzymes ALT and ALP in different treatments.

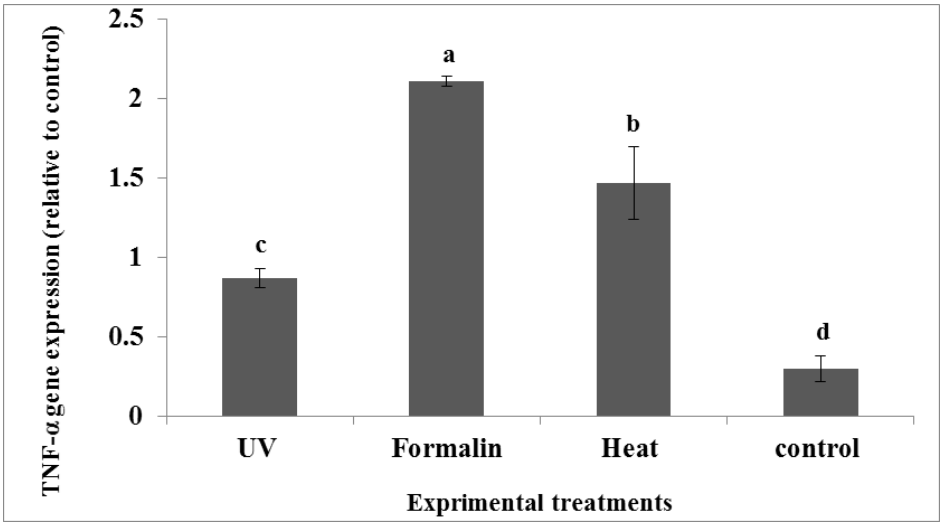

Fig. 3. The effect of different methods of killing *A. hydrophila* on TNF-α gene expression of rainbow trout (n = 15; mean ± SD). Different letters characterize significant difference ($p \leq 0.05$).

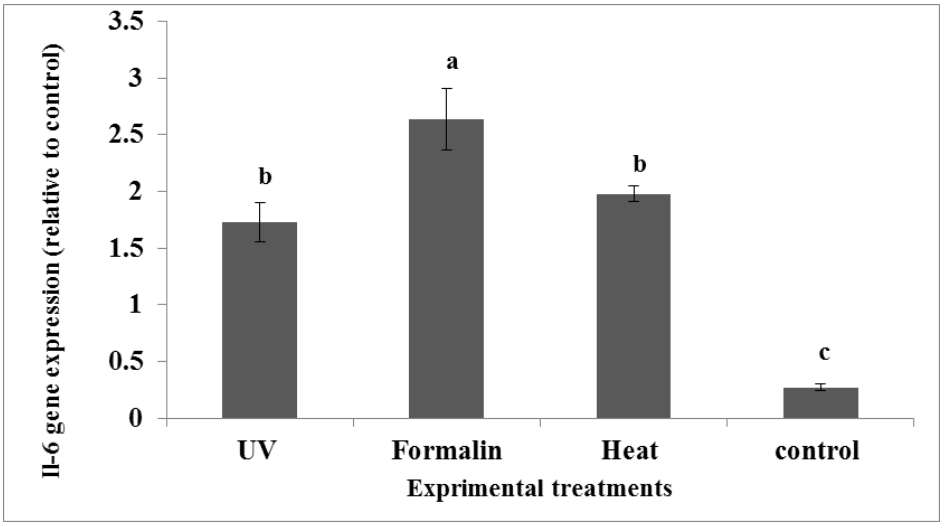

Fig. 4. The effect of different methods of killing *A. hydrophila* on IL-6 gene expression of rainbow trout (n = 15; mean ± SD). Different letters characterize significant difference ($p \leq 0.05$).

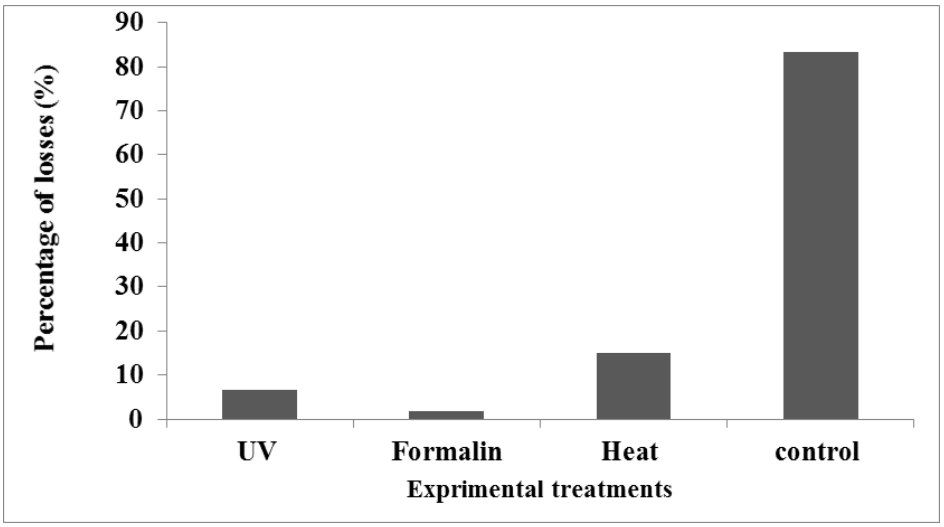

Fig. 5. Percentage of mortality in different experimental groups up to twenty days after injection of *A. hydrophila* into rainbow trout.

Alanine aminotransferase and aspartate aminotransferase enzymes are present in the mitochondria of aquatic animals and their activity level is an important indicator for diagnosing fish liver function (Liu *et al*. 2016). The enzymes alanine aminotransferase and aspartate aminotransferase, which are present in fish, are members of the transaminase family. These enzymes are concentrated in liver tissue. The levels of these enzymes are increased in acute necrotic liver disease due to exposure to liver toxins (Ahmad 2012; Karmakar *et al.* 2016; Rahbar *et al.* 2021). According to the results, the total protein content in the treatments exposed to formalin and UV-inactivated bacteria was at higher levels than the other groups. However, no statistically significant change was observed among the groups in the case of albumin. Similar results have been reported by Loghothetis (1994). According to the researchers, the effect of the bacteria killed by the formalin method on the blood protein was greater than those obtained from the heat method. Lamers & Muiswinkel (1986) suggested that formalin may change *A. hydrophila* antigens in a way that is detectable to macrophages, while the heat method will break down the bacteria. There is a similar report in the study of Loghothetis (1994). The results of the current study indicate a significant elevation in the expression of TNF-α and IL-6 genes in formalin treatment compared to the other treatments and the control group. However, no significant difference was observed in IL-6 gene expression in heat and UV treatments. Interleukin-6 (IL-6) is an anti-inflammatory cytokine that activates lymphocytes, macrophages, and antibodies against pathogens (Lauriano *et al*. 2016; Moustafa *et al*. 2020). TNF-α itself also causes the secretion of other cytokines, including IL-6 (Striz *et al.* 2014; Lauriano *et al*. 2016; Moustafa *et al*. 2020) in which activated phagocytic cells find foreign invaders faster than unstimulated condition. According to Dash *et al.* (2014) and Lamers & Muiswinkel (1986), heat and ultraviolet (UV) radiation damage cellular structures, impairing the immune system's ability to detect and process antigenic material. The expression induction of these genes by the killed *A. hydrophila* bacteria stimulates the immune system and neutralizes any toxins caused by these bacteria. Inactivated *A. hydrophilia* antigens may increase the immune response of aquatic organisms by activating lymphocytes. Studies by Irianto & Austin (2002) confirm the theory that cellular stimulation (i.e., increased white blood cell lymphocytes and total macrophages and increased phagocytes) is more important than humoral safety. Brunt & Austin (2005) also indicated that probiotic-treated macrophages were more susceptible to phagocyte than control groups. Irianto & Austin (2002) used probiotics to control the symptoms of furunculosis in rainbow trout, the number of white blood cells increased in treatments fed by probiotics. According to the findings, a significant effect of formalin treatment on various indicators of immunity, biochemistry, and gene expression was observed. The results related to the expression of immune genes (TNF-α and IL-6) in different treatments showed the highest expression of TNF-α and IL6 genes in formalin treatment. Moreover, the lowest expression of immunogenic genes was in the control group. This superiority in increasing gene expression is in line with other immunity and blood factors and showed an increased level compared to the other factors. However, based on the findings, the expression of the TNF-α gene has not been always in relation to the immune system, which means that simply increasing gene expression in a treatment does not require an increase in the immunity system. The results of the current study indicate that the inactivation of *A. hydrophila* with formalin is more effective in improving the immunity system of rainbow trout compared to the heat and UV methods.

CONCLUSION

By investigating the fish losses after the challenge with live bacteria, with a significant difference, the highest losses were observed in the control treatment, while no significant difference was observed in the formalin and UV treatments. In most cases, it was found that the amount of total blood serum protein in the formalin and UV treatments is significantly different from the other two treatments. This is while the amount of albumin does not show any variation in this context. Only the control treatment exhibited a significant difference compared to the other three treatments. The results of liver enzyme tests showed that the level of AST was the highest in formalin treatment, but no significant difference was observed in different treatments. Also, no significant difference was observed in ALT values and the highest value was observed in UV treatment. The expression of immune genes TNF-α and IL-6 showed significant changes in different treatments. The level of expression of TNF-α gene in formalin treatment was significantly higher than in the other treatments and the lowest level was observed in the control treatment. Also, similar results were obtained in the level of IL-6 gene expression, and the highest level with a significant difference was found in the formalin treatment and the lowest level with a significant difference was seen in the control treatment.

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