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# To Reduce Mortality of Fry Fish (*Oncorhynchus mykiss*) Caused with Viral Infection (IPNV and VHSV) by Water Treatment with Chloramin-T as Disinfectant

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## Abstract

In winter of 2015 we observed gross mortality of fry fish in some tanks of a hatchery in Iran. They had dissonant swimming, spiral swimming, skin darkness, abdominal distension, and anorexia. At the beginning, mortality was low but it increased more and more during the several days. The fry were in fiberglass tanks with 1000 litres of water. It was about 20000 fry in each tank at the beginning. Two activities did synchronously while mortality observation. At the first, some fry sampled from each tanks and sent to laboratory for pathogen detection. The next, 9 tanks selected and grouped as 3 treatments (control, treatment-1 and treatment-2). Control treatment was consisting of 3 tanks that they had not mortality. Treatment-1 and Treatment-2 have the highest mortality and each of them was consisting of 3 tanks. Then, 10 ppm chloramin-T as disinfectant compound added to each tanks of treatment-1 during 1 hour in 3 continuous days (3 times). The tanks of treatment-2 added no drug. After 7 days mortality of fry in each tank estimated and compared with each other. Survival in tanks of treatment-1 was about 76% while survival in tanks of treatment-2 was about 27% while survival in control tanks was about 98%. One month later, results of laboratory tests reported. We found that fish of control tanks were safe (without pathogen) while fish of treatment-1 and treatment-2 were infected with IPN-virus and VHS-virus based on RT-PCR test. Totally 36 fry had been examined by RT-PCR. We founded that 10 fry were IPN+ and 2 of them were VHS+. Clearly; results showed that chloramin-T is able to control the viral infection of *Oncorhynchus mykiss* ($p<0.05$) in statistical comparison, it confirmed with SPSS software by using Anova-test. Chloramine-T increased surveillance from 27% to 76% while viral contamination had been confirmed.

**Keywords:** Chloramin-T; *Oncorhynchus mykiss*; Virus; Control; Infection

## Introduction

Aquaculture is developing in our era. Totally aquaculture productions have been increased from 41 million tons in 2004 to 55.1 million tons in 2009, so it increased 2.63 million tons per year. There are conflicts over aquaculture development. Aquaculture is going to produce more food for the human while disease out breaks in aquatic animals that makes food losing and economic loss [1]. Infectious diseases in farmed fish such as viral diseases are a significant economic problem for aquaculture producers. There are several important viral diseases in rainbow trout fish (*Oncorhynchus mykiss*). IPNV (Infectious Pancreatic Necrosis Virus) and VHSV (Viral Haemorrhagic Septicemia Virus) are major viral pathogen for the fish which causes high mortality especially in small fish. They reported from Iran [2-4].

VHS is pathogen for a broad range of aquatic animals. It causes disease at 48 fish species for which there is conclusive evidence of susceptibility with VHSV. VHSV belongs to the genus *Novirhabdovirus*, within the family Rhabdoviridae. Virions are bullet-shaped (approximately 70×180 nm in size), contain a negative-sense, single-stranded RNA genome of approximately 11,000 nucleotides. Diseased fish may display nonspecific clinical signs in the early stages of infection, including rapid onset of mortality (which can reach up to 100% in fry), lethargy, darkening of the skin, exophthalmia, anaemia (pale gills), haemorrhages at the base of the fins, gills, eyes and skin, and a distended abdomen due to oedema in the peritoneal cavity. VHS can also occur in a nervous form, characterized by severe abnormal swimming behaviour, such as constant flashing and/or spiralling. Mortality varies, depending on many environmental and physiological conditions, most of which have not been fully determined. In generally, the disease is a cool or cold water disease with highest mortality at temperatures around 9-12°C. Small rainbow trout fry (0.3-3 g) are most susceptible with the virus (genotype Ia) while mortalities close to 100%, but all sizes of rainbow trout can be affected with mortalities ranging from 5 to 90%. Although research on vaccine development for VHS has been continued for more than three decades, a commercial vaccine is not yet available. No therapies are currently available. Several immunostimulants, such as yeast-derived beta-glucans, IL-1β-derived peptides, and probiotics have been assessed for enhancing protection against VHS. Several authors reported positive effects, but no immune-stimulant directed specifically at enhanced resistance to VHS is available [5,6].

IPNV is a highly contagious viral disease of young fish of salmonid species held under intensive rearing conditions. The disease most characteristically occurs in rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), and several Pacific salmon species (*Oncorhynchus* spp.). Fish susceptibility generally decreases with age increasing. Clinical signs include darkening pigmentation, a pronounced distended abdomen and a corkscrewing-spiral swimming

motion. Cumulative mortalities may vary from less than 10% to more than 90% depending on the combination of several factors, such as virus strain and quantity, host and environmental conditions. The disease is transmitted both horizontally via the water route and vertically the egg. The causative agent, IPNV, is a bi-segmented double-stranded RNA virus belonging to the family Birnaviridae. Control methods currently rely on the implementation of control policies and hygiene practices in salmonid husbandry, through the avoidance of the introduction in fertilized eggs originating from IPNV-carrier brood stock, and the use of a protected water supply. In outbreaks, in the population density a reduction may help to reduce the overall mortality. No treatment or entirely effective vaccine is available at present [7].

Chloramin-T is a disinfectant compound which applying to inactivate some pathogens. Linear formula of chloramin-T is $C_7H_7C_lNO_2S.Na$ ($3H_2O$) and its IUPAC name is: *N*-chloro 4-methylbenzenesulfonamide, sodium salt. Chloramin-T is white powder. Its solubility in water is 150 g/L (25°). It is well known antimicrobial agent especially as antiviral agent for sanitation.

However, there are several strategies to control of disease in aquaculture such as sanitation, antibiotic and drug recommendation, vaccination, probiotics recommendation and use of disinfectant compounds. We use of a disinfectant to control mortality in fry fish successfully. It was Chloramin-T.

## Material and Methods

### Sampling

About 6 fry fish gathered from each tank. Moribund fry which showed sign of disease (bad swimming) gathered from tanks which have the highest mortality rate. The fry of other tanks (Control) had not sign of disease. Each samples (each fry) immersed in a sterile-plastic tube which contained about 5 ml VTM (Virus transport medium). The tubes accommodated in a special-transport tank (nitrogen tank) that which semi filled with liquid nitrogen. It transferred to a laboratory to do viral detection.

### Virus detection method

It consists of two steps. At the first step, samples cultured on cell lines. Second step was PCR-test which done based on RT-PCR (Reverse Transcriptase-PCR) method.

**Culture on cell line:** EPC and BF-2 cell lines had growth at EMEM (Eagle's minimum essential media) 24 hours earlier. Then, Each sample (fry fish) homogenized with about 2 ml (PBS, pH: 7) and filtrated with 0.45 µ membrane filter. About 500 µl of filtrate added to EPC cell line as much as BF-2 cell line. Cell culture plates incubate at 15°C for 7 days and evaluated for CPE effect of virus. Samples which had been CPE positive selected and they evaluated with RT-PCR (reverse transcriptase-PCR) method by means of viral detection [5,8].

**RNA extraction and PCR method:** One-step RT-PCR method done. Qiagen OneStep RT-PCR System applied according to the manufacturer's instructions.

**Primers for PCR:**

- Primer which handled for VHS-virus detection [8].
- VHS3: CGGCCAGCTCAACTCAGGTGTCC,
- VHS4: CCAGGTCGGTCCTGATCCATTCTGTC. Primer which handled for IPN-virus detection [8].
- WB1:CCGCAACTTACTTGAGATCCATTATGC, and
- WB2: TCTGGTTCAGATTCCACCTGTAGTG.

**Antiseptic agent:** It was chloramin®-T. It was made by BOCHEMIE Company.

### Method of antiseptic application

10 grams of chloramin-T dissolved in 20 litres of water and added to each tank of treatment-1 slowly (During 20 minutes). A plastic barrel with a tap used to do it. Water of the each tank was about 1000 L and its draining was about 30 L/min. However, 10 ppm of chloramin-T added to each tank while water flow of each tank was about 30 L/min, thus water of each tank was refreshing during 33 minutes. Drug concentration increased slowly during of 20 minutes then it decreased slowly by water flow of tanks. So, during the day, concentration of antiviral agent had a dynamic rule. The procedure was done for 3 days frequently and 30 grams drug used totally for a 1000 L tank. The tanks were in shade not under the sunlight. They were in a hall.

### Statistical method

One-way Anova test used as statistical method and SPSS (ver: 21) software applied to do it [9].

## Results

In this study, there were three treatments; each of them had been 3 replications. Fry fish of control treatment had very low mortality. They were active without sign of disease such as abnormal swimming, so we believe they were free of pathogen. It confirmed by laboratory test. Fry fish of control tanks show surveillance 97.36% to 97.77% at the end of study (Table 1). It was acceptable as a normal condition. Fry fish of other tanks show mortality which increased during the time. It believed that an exotic agent caused the mortality. Pattern of mortality was as like as infectious disease. Some fry fish show sign such as lethargy, skin darkness, exophthalmia, abdomen distended and abnormal swimming. They were swimming without harmony with flock of fish. At the next days they settled at bottom of tank and were deceased a few hours later. Some moribund fry fish of the tanks gathered and send to laboratory for diagnostic test such as virological tests by PCR method. The tests were time consuming and needed to 7-10 days for reporting. On the other hand; mortality was increased, so it was necessary to do something rapidly. We decided to do research on it. Tanks with mortality arranged in two treatments, each of them with 3 replication and called treatment-1 and treatment-2. We use chloramin-T as disinfectant for inactivation of unknown infectious agent while did nothing to control of mortality for tanks of treatment-2. Chloramin-T applied for 3 days by tanks of treatment-1. After 3 days we found rapturous results. Fry fish of tanks of treatment-1 show mortality less than treatment-2. They show decreasing of mortality during the next days. Mortality was controlled in tanks of treatment-1. Fry fish of treatment-2 show increasing in mortality unlike of treatment-1. They show mortality more and more, it continued after the study. One week after that chloramin-T recommendation, surveillance of fry fish of each tank estimated (Table 1). It analysed by SPSS software with ANOVA-test. Total surveillance of fry fish of Control was 97.56% while surveillance of Treatment-1 and Treatment-2 were 76.47% and 26.66% orderly. Difference between surveillance of treatments with control confirmed by statistical test

(P<0.05) (Tables 2 and 3). On the other hand, difference between surveillance of treatment-1 with treatment-2 confirmed by statistical test (P<0.05). Laboratory results show that fry fish of treatment-1 and treatment-2 were infected by IPN-Virus or VHS-Virus. Frequency of each viral infection mentioned in Table 4.

| Treatment | Drug application | Tank[1] number | No of sampled fry | Laboratory test result (Detected virus)[2] | Population of fry at stacking time (Estimated) | | Population of fry 7 days after drug recommendation while signs of disease had started (Estimated) | | Surveillance (%): (7 days after drug recommendation) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | No | 1 | 6 (Fry fish) | Negative (Virus didn't detect). VHS-and IPN- | 19000 | Mean: 20500 | 18500 | Mean: 20000 | 97.36 | Mean: 97.56% =100(20000/20500) |
| | | 2 | 6 | VHS- and IPN- | 22500 | | 22000 | | 97.77 | |
| | | 3 | 6 | VHS- and IPN- | 20000 | | 19500 | | 97.50 | |
| Treatment-1 | Yes (10 ppm of Chloramin-T used once a day for 3 days repeatedly). | 4 | 6 | IPN+(2 of 6 sample) VHS-(0 of 6 sample) | 19500 | Mean: 19833 | 14500 | Mean: 15166 | 74.35 | Mean: 76.47% |
| | | 5 | 6 | IPN+(1 of 6 sample) VHS-(0 of 6 sample) | 22000 | | 17500 | | 79.54 | |
| | | 6 | 6 | IPN-(0 of 6 sample) VHS+ (1 of 6 sample) | 18000 | | 13500 | | 75.00 | |
| Treatment-2 | NO | 7 | 6 | IPN+(1 of 6 sample) VHS-(0 of 6 sample) | 18500 | Mean: 20000 | 6000 | Mean: 5333 | 32.43 | Mean: 26.66% |
| | | 8 | 6 | IPN+(1 of 6 sample) VHS-(0 of 6 sample) | 20500 | | 4500 | | 21.95 | |
| | | 9 | 6 | IPN-(0 of 6 sample) VHS+(1 of 6 sample) | 21000 | | 5500 | | 26.19 | |

[1]-Tank wall was made from a part of a large pipe which made of fiberglass and plastic while its bottom was made of concrete. Each tank contains 1000 liters of fresh water. Fresh water added to each tank with a valve by 30 L/min and drained by the same rate.

[2]-Viral detection done by RT-PCR method following cell culturing.

**Table 1:** The properties of tanks and treatments.

| Group (Treatment) | Mean of surveillance | Std. error of mean | F | P |
|---|---|---|---|---|
| Control | 20000 | 1040.83 | 61.56 | 0.000 |
| Treatment-1 | 15166 | 1201.85 | | |
| Treatment-2 | 5333 | 440.95 | | |

**Table 2:** Variance analysis.

| Group | Group | Mean difference | P |
|---|---|---|---|
| Control | Treatment-1 | 4833 | 0.032 |
| | Treatment-2 | 14666 | 0.000 |
| Treatment-1 | Control | -4833 | 0.032 |
| | Treatment-2 | 9833 | 0.001 |
| Treatment-2 | Control | -14666 | 0.000 |
| | Treatment-1 | -9833 | 0.001 |

**Table 3:** ANOVA result and multiple comparisons by scheffe-test.

| Treatment | Tank number | Frequency of infected fry with IPN-virus (%) | Frequency of infected fry with VHS-virus (%) | Total frequency of infected fry with viruses (%) |
|---|---|---|---|---|
| Control | 1 | 0 | 0 | 0 |
| | 2 | 0 | 0 | |
| | 3 | 0 | 0 | |
| Treatment-1 | 4 | 33.33 | 0 | 22.22 |
| | 5 | 16.66 | 0 | |
| | 6 | 0 | 16.66 | |
| Treatment-2 | 7 | 16.66 | 0 | 16.66 |
| | 8 | 16.66 | 0 | |
| | 9 | 0 | 16.66 | |

**Table 4:** Frequncy (%) of infected fry fish in each tank based on laboratory test.

## Discussion

Surveillance of fry fish of 3 groups (Control, Treatment-1 and Treatment-2) was difference based on statistical test ($P<0.05$). Fry fish of control were safe so they have high surveillance. Fry fish of Treatment-1 and Treatment-2 were infected with the pathogens so their surveillance could be equal but statistical test show difference between them. Total frequency of infected fry of Treatment-1 was 22.22% while it was 16.66% for Treatment-2 (Table 4). Therefore, fry fish of Treatment-1 were more illness than Treatment-2. Based on laboratory test predicted surveillance of Treatment-2 was more than Treatment-1; it not happened. Surveillance of fry fish of Treatment-1 was 49.81% more than Treatment-2. As a result, the surveillance of fry fish of Treatment-1 not only didn't less than Treatment-2 but also it was more. We ascribe to antiviral effect of chloramin-T the higher surveillance rate of Treatment-1 compare with Treatment-2. Chloramin-T recommended only for tanks of Treatment-1. Based on this study, chloramin-T inhibit mortality of fry fish which caused by IPN-virus and VHS-virus. If chloramin-T didn't recommended, mortality will be about 73.33% while it decreased as much as 23.53% by application of chloramin-T.

## Conclusion

While mortality is going to start, it is important that a farmer be able to control of disease before $10^{th}$ day, otherwise mortality increase day to day and cause high financial damage. Laboratory tests are time consuming; they need 3-10 days to get a result. But, sometimes, you must choice the best activity before getting the laboratory results and use an antiseptic. Chloramin-T is economic, because 30 grams of chloramin-T is sufficient for 20000 fry fish to be safe. So, a breeder can apply it while think a viral disease is going to emerge. Samples (for laboratory) must gather before applying of antiseptics or antibiotics, otherwise laboratory can't detect pathogenic agents.

Chloramin-T isn't an antiviral drug so you can't use it as food additive for antiviral therapy of fry fish. I think that chloramin-T inhibit virus transfer in water so it controlled the mortality. On the other hand, chloramin-T deactivated virus in water and blocked its spreading rule. Therefore, some fry fish survive.

In future; it is require a study to evaluate antiviral effect of Chloramin-T against VHS-virus and IPN-virus on molecular level in an equipped laboratory.

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