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Experimental *Vagococcuc salmoninarum* infection in Rainbow Trout (*Oncorhynchus mkiss*) by İntraperitoneal İnjection

Mikail Ozcan[1,]Unal Ispır[2], Yasemin Yılmaz[1]
[1]*Kahramanmaras Sutcu Imam University, Faculty of Agriculture, Department of Fisheries Kahramanmaras-TURKEY*
[2]*Inonu University, Fisheries Faculty Battalgazi-Malatya- TURKEY*

Abstract:*Experimental Coldwater 'streptococcosis', caused by Vagococcus salmoninarum, was induced in rainbow trout (Oncorhynchus mykiss) using intraperitoneal injection challenge model. V. salmoninarum at a concentration of $1.6x10^9$, $9.6x10^9$, $1.55x10^{10}$, $7.22x10^{10}$ and $3.7x10^{11}$CFU/ml was intraperitoneally injected. Phosphate buffered saline (PBS) was intraperitoneally injected to control group. The signs of disease was observed in experimental groups. After post injection, fish were monitored and mortality was recorded for 14 days. Fish mortality rate increased with the increase of bacterial concentrations of V. salmoninarum. The first mortalities were observed after 48 hours, although the signs of the bacterial infection were already observed at the 7 days after the injection. The results indicated that the 14 days LD_{50} value of V. salmoninarum was 1.09 × 10^9CFU ml^{-1}.*

Keywords:*Oncorhynchus mykiss, Vagococcuc salmoninarum, injection*

I. Introduction

Vagococcus salmoninarum is the causative agent of coldwater streptococcosis. An infectious disease which produces high mortalities and severe economic losses on fish farms, particularly in rainbow trout and atlantic salmon (Ghittino *et al*., 2004; Schmidtke & Carson, 1994). *V. salmoninarum* is a Gram positivecoccobacilli which is first described as a Lactobacillus strain in 1968 (Austin & Austin, 2012). This strain were defined as *V.salmoninarum* in later years. However, in the last two decades the disease has become established in fish farmed in many countries in Europe and the world (Schmidtke & Carson, 1994; Michel *et al*., 1997; Ghittino *et al*., 2004; Ruiz-Zarzuela *et al*., 2005; Salogni *et al*., 2007; Didinen *et al*., 2011).Mortality rates ranging up to 20-50% per year were (Nougayrède *et al*., 1995; Michel *et al*., 1997; Ruiz-Zarzuela *et al*., 2005). Thestreptococcosis caused by *Lactococcus garvieae*, *Streptococcus iniae, Streptococcus agalactiaeStreptococcus parauberis* and *V. salmoninarum.* There are detailed informations about the other four strains.But, for *V. salmoninarum* there are limited information available. There arelittle information in previous studies lethal doses related *V. salmoninarum*have been reported.Ghittino *et al* (2004) reported that lethal dose of *V. salmoninarum* to rainbow trout of 1×10^9 cfu/fish. The aim is this study was to investigate of lethal dose of *V. salmoninarum* afterinjection of five different bacteria dosess in rainbow trout.

II. Materials and Methods

1. Fish

Rainbow trout for the experiments (weight~71g) were obtained from a commercial fish farm in Kahramanmaras (Turkey) without any previous history of infection with *V.salmoninarum*. The fish were kept in a 225 L fiberglass tank. Prior to each experiment the fish were transferred to aquaria containing aerated well water and acclimatised for a minimum of 2 weeks. Fish were fed *ad libitum* with a commercial (Ecobio) feed throughout the experiments. To verify the *V. salmoninarum*-free status the rainbow trout, samples were obtained for bacterial culture from kidney. The samples were incubated at 22 ^oC for 72h. *V. salmoninarum* was not isolated from selected rainbow trout. The use of the fish and the experimental protocol were approved by the Animal Experimentation Ethics Committee of the Agriculture Faculty, Sutcu Imam University (Kahramanmaras, Turkey).

2. Bacteria

V.salmoninarum, originally isolated from a rainbow trout farm suffering from this bacteria in Turkey, was used for experiments. The strain were stored in tryptone soy broth (TSB) supplemented with 15% (v/v) sterile glycerol at –80°C. Isolate of *V.salmoninarum* was then confirmed by standard biochemical tests as described in Bergey's Manual of Determinative Bacteriology (Holt *etal*.,1994) as well as PCR analysis according to Diler *et al*., (2011). For each experiment the bacteria was inoculated on tryptone soy agar, and several colonies were transferred into TSB and incubated for 48hour at 22°C with shaking and then pellet was

collected by centrifuged. The supernatant fluid was removed and the bacterial pellet was reuspended in 0.9% sterile NaCl as the stock bacterial suspension for experiments.

3. Experimental design

The inocula were prepared from the bacterial suspension by a dilution series with sterile PBS. The diluted inocula were plated on TSA and incubated for 48 h at 22°C. The doses of intraperitoneal injection were calculated as $1.6x10^9$, $9.6x10^9$, $1.55x10^{10}$, $7.22x10^{10}$ and $3.7x10^{11}$CFU/ml. Control injections were prepared by sterile TSB. Experimental fish were injected with 100µl of live *V. salmoninarum*. Then, fish were monitored and mortality was recorded twice daily for 14 days following injection and dead fish removed. Dead fish were removed twice a day and bacterial samples were obtained aseptically from the anterior kidney, and liver of and dead fish to confirm the presence of *V. salmoninarum*. Samples were cultured ontoTSAplates and incubated at 22°C for 72h. The extrapolated median lethal concentration (LD_{50}) of *V. salmoninarum* by immersion was calculated by the arithmetical method of Karber (Turner, 1965).

III. Results and Discussion

In experimental groups the fish in the 48 h began to die after post challenge and mortalities continued for the next 5 to 14 d(Figure 1).

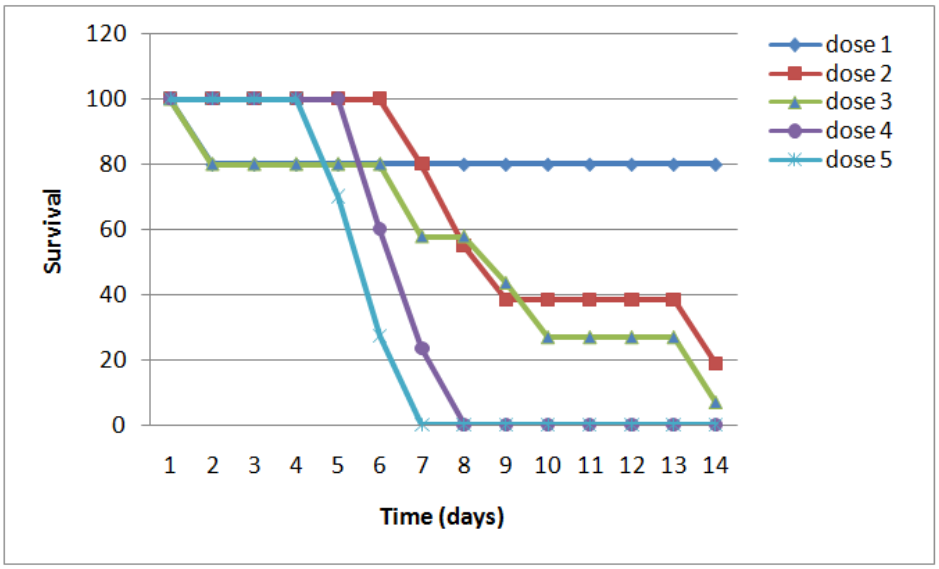
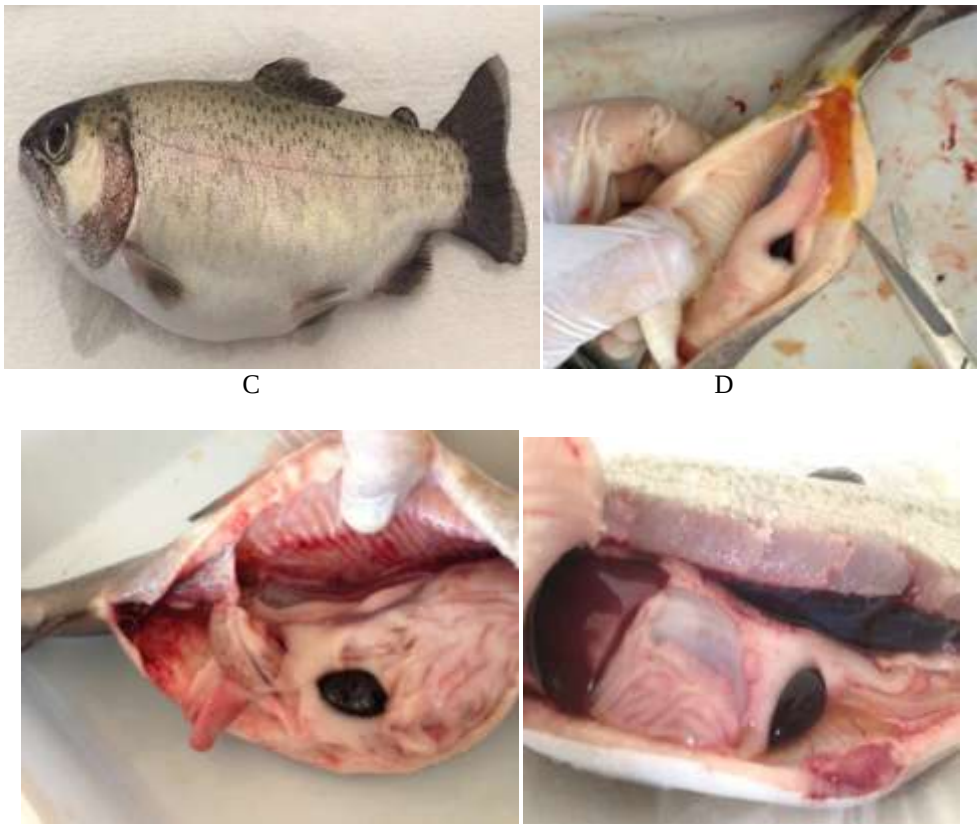


Figure 1: In experimental groups the fishsurvival by day

All dead fish in these groups showed the following typical signs of disease (Figure 2): erratic swimming; darknes on skin; dermal melanosis; exophthalmia (mono and/or bileteral); eyeball disruption; redness of anus; bloody ascitic fluid in the body cavity; palaness in liver; discoloration and growth in splen and kidney; petechial hemorrhages in muscles, gonads and air bladder. Bloodly and yellowish exudate accumulation in the intestines; spleen, and liver and hemorrhage in the viscera. However, in control groups, disease and internal signs were not observed.

Figure 2: Clinical signs of infection by *V. Salmoninarum*

A B



C D E F

Figure 2: Clinical signs of infection by *V. Salmoninarum*

A: Dark in skin
B: Bilateral exophthalmos in eyes
C: Severe distention in the abdomen.
D: Exudates filled the peritoneal cavity.
E: Hemorrhage in peritoneal cavity and Splenic enlargement
F: Haemorrhagic liver and swollen kidney of fish.

The result revealed that first mortality was observed in $1.6x10^9$ and $1.55x10^{10}$ cfu/ml injected group after 48 hours. However, the external symptoms of bacterial infection, such as erratic swimming; darknes on skin; exophthalmia, were observed at 168 hours after the inoculation. Fish signs of infection and death rate increased as the increase of bacterial concentration. Levels of mortality in the control group and experimental fish group are given in Tables 1.Two mortality was noted in fish injected with1.6×10^9 CFU/mL at 96 h post infection. No fish died following exposure to 9.6×10^9 CFU/mL at 48 h post infection, and six fishdied at end of experiment.The mortality in the 1.55×10^{10} CFU/mL injected group was one at 48 h post infection, but six fish died in total at end of experiment. All fish injected with 7.22×10^{10} and 3.7×10^{11} CFU/mL was died at end of experiment. Not mortality was noted in the negative control group. *V. salmoninarum*were isolated in all died and moribud fish. The cumulative mortality was 0, 20, 55, 65, 100 and 100.0% at CFU/mL concentrations, respectively, of experimental groups (Table 1). The extrapolated LD_{50} dose was 1.09×10^9 CFU/mL.

Table 1 Mortality observed in rainbow troutinfected with *V. salmoninarum* by intraperitoneal injection

			Cumulative mortality hours after post infection					
Dose (CFU/mL)	No. fish/replicate	0	24h	48h	72h	96h	At the end of study	Mean percent mortality (%) for replicate
Control TSB	10/2	-	-	-	-	-	-	0.00%
$1.6x10^9$	10/2	-	-	2	-	-	2/2	20
$9.6x10^9$	10/2	-	-	-	-	-	6/5	55
$1.55x10^{10}$	10/2	-	-	1	-	-	6/7	65
$7.22x10^{10}$	10/2	-	-	-	-	-	10/10	100
$3.7\ x10^{11}$	10/2	-	-	-	-	-	10/10	100

Experiments in rainbow trout demonstrated by *V. salmoninarum* infection, the mortality was high. The disease was easily initiated by intraperitoneal injection and were observed. The clinical signs of infection and lesions observed following the present study. These results, including the severity of external and internal organs, and the persistence of the infection in rainbow trout, provided sufficient information to demonstrate *V.salmoninarum* as a fish pathogen. Rainbow trout *Oncorhynchus mykiss* naturallyand experimentaly infected of *V.salmoninarum* in usually show clinical signs such as swimming difficulty,lethargic activity, darkening skin coloration; anorexia, external hemorrhages, furuncles, exophthalmia, petechial haemorrhages of gills, paleness on the gills, ascites(Michel *et al*.,1997; Ghittino*et al*., 2004; Salogni *et al*., 2007; Didinen *et al*., 2011).At present study, dead fish exhibited the lesions commonly seen in experimental groups, especially exophthalmia. Clinical signs and gross findings in *V.salmoninarum* infection in rainbow trout described here are similar to those reported in rainbow trout and other fish with other Gram positive bacteria such as *Lactococcus garvieae*, *Streptococcus iniae*and *Streptococcus agalactiae*(Erdar & Ghittino, 1999; Klesius *et al*., 2000; Abdullah *et al*., 2013).Ghittino*et al*., (2004) reported LC_{50} values of 10^9 cfu of virulent *V. salmoninarum*/fishusing a 50g rainbow trout infected by intraperitoneal injection challenge. The LD_{50} in this study was 3.4×10^7 CFU/ mL in uninjured 40g rainbow trout. This difference in the LD_{50} values between those experiments was attributed differences in innate immune mechanisms in the fish populations. While it is possible the fish were stressed during the challenge may be observed difference in lethal doses Streptococcosis stands for a common name of a disease caused by different genera and species. *Lactococcus garvieae*, *Streptococcus iniae*, *Streptococcus parauberis* and *Streptococcus agalactiae*are the etiological agents of warmwater streptococcosis. Coldwater streptococcosis, however, is caused by *Vagococcus salmoninarum* (Ozturk & Altinok, 2014). *Streptococci* spp. have been shown to gain entry into fish by ingestion of moribund fish or contaminated feed (Shoemaker *et al*., 2000; Bromage & Owens, 2002), injured skin from contaminated water (Rasheed & Plumb, 1984), and through intraperitoneal and intramuscular injection for experimental purposes (Shoemaker *et al*. 2000, 2001; Bromage & Owens, 2002). Disease progression is variable and dependent upon the virulence of the isolate, the host species affected, route of infection, fish age, and other environmental and water quality factors(Eldar & Ghittino, 1999).There are differences with regard to in the lethal doses between Streptococcus species and its strains. The reported LD_{50} for intraperitoneal injected *Streptococcus iniae* and *Lactococcus garvieae* in rainbow trout has 2.5 X 10^5 and 1.25 X 10^1cfu/fish, respectively (Erdar & Ghittino, 1999).Bromage *et al*., (1999) investigated that LD_{50} value of *S. iniae* on *Lates calcarifer* and they reported that the LD_{50} value estimated to be 2.5 x 10^5 cfu and 3.2 x 10^4 cfu. Pasnik *et al*. (2011) reported to have lethal dose of *S. ictaluri* to catfish of 3.8×10^7 cfu/fish. According to these results, it was suggested that the LD_{50} for *V. salmoninarum*by injectioncould be diverse for rainbow trout than other fish species. This differences in the virulence of the *V. salmoninarum* isolates, sensitivity against infection, high immunity of experimental fish, or difference of isolates and experimental design may be elucidations for diversity in results between studies.

IV. Conclusion

In experimental groups the fish in the 48 h began to die after post challenge and mortalities continued for the next 5 to 14 d. The extrapolated LD_{50} dose was 1.09×10^9 CFU/mL.

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UI participated in study design, data collection, laboratory analyses, organization of the study and data interpretation and preparation of the manuscript. MO participated in study design, laboratory analyses and data interpretation and wrote the first draft of the manuscript. YY participated in data collection and laboratory analyses and assisted in every aspect of the study.

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