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RESEARCH PAPER

Histological Examination of Various Organs of Asian Seabass, *Lates calcarifer* after an Oral Inactivated Vaccine against *Vibrio harveyi*

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الفحص النسيجي لأعضاء مختلفة من سمك الباراموندي *Lates calcarifer* بعد لقاح معطل عن طريق الفم ضد بكتيريا *Vibrio harveyi*

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Abstract. Disease outbreaks and consequential losses are a challenge to the aquaculture industry. Diseases in aquaculture are caused by pathogenic agencies, such as bacteria, viruses, and parasites. The use of vaccines is one approach for the control of infections in fish and in building immunity against them. The goal of the present study was to create an effective oral vaccination against *V. harveyi* in order to ensure the long-term viability of aquaculture operations in the Sultanate of Oman. At a commercial farm, the target bacterium was isolated from infected fish and identified from a pure strain. Bacteria were killed with formalin and cleaned with saline several times. The vaccine was mixed with commercial feed to provide an oral vaccination for fish. This vaccinated feed was given for four weeks, and the efficiency of vaccine was determined by a challenge test, which involved injecting live same species of bacteria into healthy fish. Histology samples were taken when the experiment was completed. Multivitamins and vaccination therapy helped the fish to develop faster and to survive for extended periods of time without any organ damages. The control fish, on the other hand, demonstrated an incapacity to resist bacteria and died as a result, with external and internal organ damage. Despite the positive findings of this study, more research is required.

Keywords: Oman, *Vibrio harveyi*, Vaccine, Histology

الخلاصة: يمثل تفشي الأمراض وما يترتب عليها من خسائر تحديا لصناعة تربية الأحياء المائية. بسبب تتسبب العوامل المسببة للأمراض في تربية الأحياء المائية مثل البكتيريا والفيروسات والطفيليات. يعد استخدام اللقاحات إحدى الاستراتيجيات في مكافحة العدوى في الأسماك وبناء المناعة ضدها. كان الهدف من هذا البحث هو إيجاد تطعيم فموي فعال ضد *V. harveyi* من أجل ضمان استمرارية عمليات الاستزراع المائي على المدى الطويل في سلطنة عمان. في مزرعة تجارية، تم عزل البكتيريا المستهدفة من الأسماك المصابة والتعرف عليها من سلالة نقية. تم تدمير البكتيريا وتنظيفها باستخدام الفورمالين. تم خلط اللقاح مع الطعام لتوفير تطعيم فموي للأسماك. تم إعطاء هذا العلف الملقح لمدة أربعة أسابيع، وتم تحديد كفاءة اللقاح من خلال اختبار التحدي، والذي تضمن حقن الجراثيم الحية في الأسماك السليمة. تم أخذ عينات الأنسجة عند اكتمال التجربة. ساعدت الفيتامينات المتعددة وعلاج التطعيم الأسماك على النمو بشكل أسرع والبقاء على قيد الحياة لفترات طويلة من الزمن دون أي تلف في الأعضاء. من ناحية أخرى، أظهرت أسماك التحكم عدم قدرتها على مقاومة البكتيريا وماتت نتيجة لذلك، مع تلف الأعضاء الخارجية والداخلية. على الرغم من النتائج الإيجابية لهذه الدراسة، هناك حاجة إلى مزيد من البحث

الكلمات الرئيسية: الاستزراع السمكي ، لقاح ، علم الأنسجة

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Introduction

Aquaculture is one of the fastest growing food production industries, with a 5.8% annual growth rate between 2008 and 2020 (FAO, 2021). Sustainability in aquaculture is limited due to environmental concerns. There are many environmental impacts from aquaculture inputs and resources in many aspects including energy, feed, and water (Waite et al., 2014). However, mass mortality is one of the main problems facing in aquaculture sector (Kjørsvik et al., 2011). The occurrence of high mortality varies, it may be due to the environment, diseases and water quality, but the main cause of death is diseases, and especially what causes the highest rate of death in aquaculture is diseases caused by bacterial infection (Vadstein et al., 2013).

Economic losses and losses of fish resources in aquaculture are the most prominent negative consequences of disease outbreaks (Arijo et al., 2005). Therefore, it is necessary to find ways to control diseases in the aquaculture sector, examples of these methods optimization of feed, good sanitation, introduction of specific pathogen free (SPF) brood stock, improvement husbandry techniques, water quality and development immune capacity of fish (Grisez et al., 2005). Strategies are needed to increase immunity in fish. These strategies designed to be on relation with etiological agent or what called by opportunistic bacteria and early immune logical response. A strategy is used that aims to increase the immunity of fish and increase resistance against pathogens known as immune stimulation (IE), and it is done by adding compounds or factors that contribute to increasing the immune response of the fish (Barman et al., 2013). Macrophages are increased by adding the immune stimulant agent (IA) that leads to an increase in the numbers of macrophages and the activation of their functions (Sakai, 1999). Vaccination is the most effective strategy to increase the immune response in fish (Gudding and Van Muiswinkel, 2013; Gudding and Goodrich, 2014). Many kinds of vaccines have been used in salmon fish farms in Norway, which has been observed in a decrease in the mortality rate of fish and a decrease in the use of antibiotics (Gudding, 2014). The first approach to developing the vaccine is to use completely inactivated cells and this method is considered more viable due to its safety and efficacy for cultured fish (Toranzo et al., 1997). The vaccine can be delivered by injection, by mixing the vaccine with the food, or by immersion, placing the fish in a bath of vaccine for a certain period (Grisez et al., 2005).

The present study was designed to develop an ineffective vaccine against *Vibrio harveyi* and study its efficacy on Asian seabass, *Lates calcarifer* for sustainable of aquaculture in the Sultanate of Oman. In this study, the immune response of host fish and its ability to survive after the challenge test was also investigated.

Materials and Methods

Sampling

More than 30 sick Asian seabass, *L. calcarifer* were sampled from aquaculture farm at Sultan Qaboos University, Oman and measured weight and length prior to diagnosis of fish.

Isolated Bacteria

Bacteria were isolated and cultured in TSA (Tryptone soya agar, Sigma). Then bacteria were identified by DNA sequence. A portion of pure colony of bacteria (500 mg) was transferred from plates to sample tubes. 3ml of CTAB extraction buffer was added and 5 ul protease k added and placed at 55 °C for 12 hours. The samples were cooled to RT and added 5 ul RNase (10mg/ml).

Then, the samples were incubated at 37 °C for 30 minutes. PCI (25:24:1) was added in equal volume and centrifuged at 12000 rpm for 10 minutes. Next, supernatant was transferred to a new Eppendorf tube. Equal volume of chilled isopropanol was added and centrifuged at 12000 rpm for 10 minutes. Discarded supernatant and washed pellet with 70% ethanol. Then, pellet was dried and re-suspended in 70 µl autoclaved distilled water. Finally, samples were placed at 4 °C for short term. The genome of all isolated bacteria was extracted. The polymerase chain reaction (PCR) was done for all samples by making a several copies of DNA segments. Then, the target 16S rRNA gene was amplified to determine the DNA sequence.

Vaccine Processing

When bacteria species was identified, a pure strain of bacteria was cultured to produce needed amount of bacteria. After that, cultured *V. harveyi* cells were collected from all petri dishes and placed in 10% formalin. The mixture of bacteria and formalin was located in a magnetic stirrer for 2 hours to fix bacteria and killed them at the same time. Then, the bacteria in formalin were centrifuged with 3000 rpm for 1-2 minutes. The two-layers were formed; the first layer was formalin and the second one was bacteria. The first layer was carefully removed. Saline water was added and again centrifuged with 3000 rpm for 60 seconds for washing bacteria. This process was repeated 3 times to remove formalin. To confirm that all bacteria were dead, 0.5 ml from the solution was taken and spread on a new petri dish. It was placed in an incubator for 24 hours. No colonies indicated that all bacteria were dead. The density of bacteria in the vaccine was measured by using a spectrophotometer in 620 nm wavelengths. The density of vaccine was maintained between 4×10^6 -5×10^6. After that the vaccine was placed in the freezer until used.

Preparing the Oral Vaccine

After the vaccine was prepared, we mixed it with feed to make the oral vaccine. 3 treatments were used in this experiment. The vaccine with feed was prepared by adding 5 ml of vaccine to 10 g of feed. The second treatment was made by mixing 10 grams of feed with 2.5ml of vaccine and 2.5ml of the multivitamin. A volume of 2.5 ml of the vaccine was mixed with 2.5 ml of frankincense solution and 10 g of feed to prepare treatment. Also, the feed for control was mixed with only PBS. After that, the feed was put to dry for 48 hours.

Histology

After the 2 weeks of feeding trial, samples were taken from the vaccinated fish and the control fish. Then, the fish was dissected and shapes of the organs were compared for all fishes. The organs were separated and they were placed in 10% buffered formalin for 24 hours to fix. After 24 hours, the organs were cut and the tissue was placed in cassettes. Next, the cassettes were placed in water/ alcohol mixture of different alcohol concentrations to dehydrate. Finally, the cassettes were placed in 100% alcohol and then in xylene. The dehydrated samples were placed into melted paraffin. The tissues were embedding into paraffin blocks and sectioned. The 5 micrometre sections of representative tissues were stained with hematoxylin and eosin. Finally, they were mounted in DPX mountant.

Results

The external appearance and condition of the vaccinated fish after feeding trial was significantly different from the control one. The scale and colour were natural in the vaccinated fish.

On the other hand, the scale and colour was lost in the control fish. In addition, the control fish had pale color compared to the vaccinated fish. The tails of the control fish were rusted and lost their natural colors. Whilst, the appearance of the tail of the fish from vaccinated group did not show any signs of abnormality (Figure 1).

The internal organs of the vaccinated and the control fishes were different in their color and shape. All the internal organs of the vaccinated fish were normal in appearance but those from the control, unvaccinated fish, were cloudy, bloody and difficult to separate. The brain of the vaccinated fish showed that clean, tight and one complete mass whilst, the brain from control fish had separated parts and there were some erosion and loss of parts (Figure 2). Figure 3 illustrates histology of the heart in the vaccinated fish. The heart in the vaccinated fish appears as a complete organ without any erosion. However, the heart of the control fish appeared with gaps and ruptures or erosion from the inside. Also, some of the outer parts were missing or corroded in the

Figure 1. External appearance of vaccinated and control fish after orally vaccinated. Vaccinated fish showed clean and clear body color (up) while controlled fish showed whitish and rusted tails (down).

Figure 2. The brain of the vaccinated fish displayed no damage and appeared in one mass (up), while histology of the brain from fish receiving the oral vaccinated appeared abnormal and was separating (down).

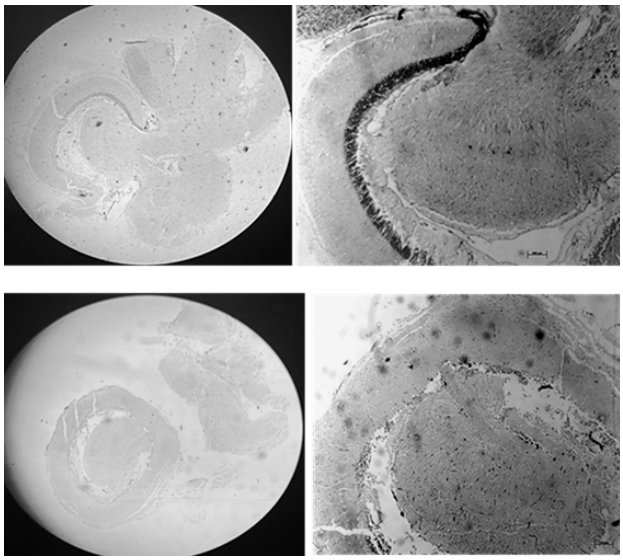
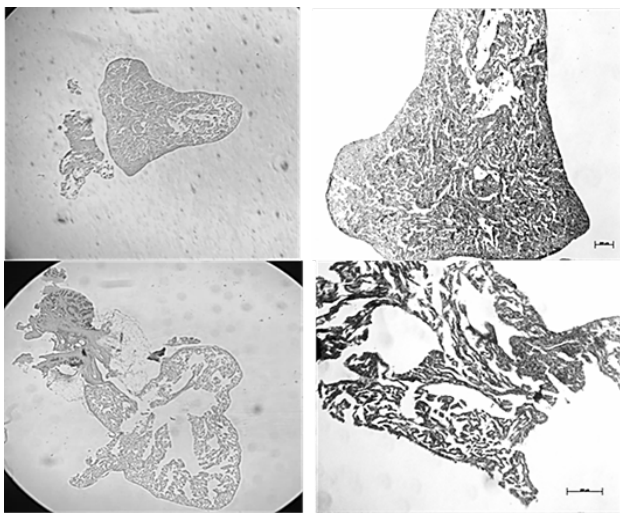
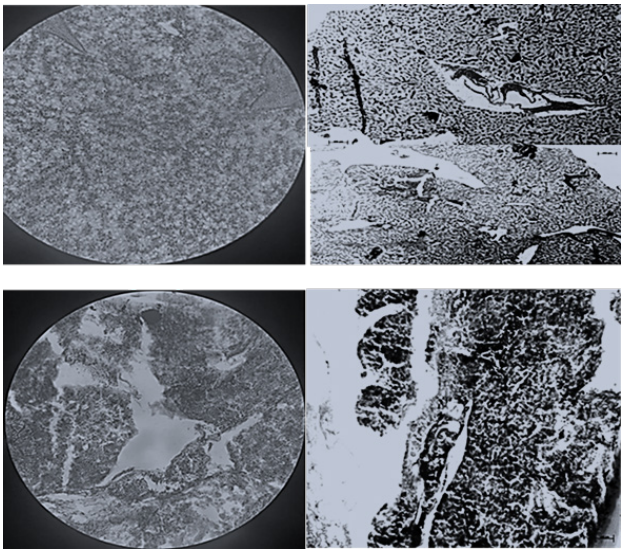


Figure 3. Histology of heart in vaccinated fish vaccinated and control fish after orally vaccinated (up). The heart of vaccinated fish appeared as a complete organ without any erosion but the histology of heart in control fish showed with gaps and ruptures (down).



core of the control fish. Figure 4 shows the liver of the vaccinated fish. In the vaccinated group, the liver appeared as one mass and there was no erosion or deficiency in any part of it. The tissue of the liver appears to be clear without any scratches or damages. However, the liver in the control fish had many parts that were missing. In addition, the erosion in the liver was very high and the color was dark comparing with the liver in the vaccinated fish.

Figure 4. The liver appeared of vaccinated fish as one mass and there is no erosion or deficiency in any part (up). However, there are many parts were missing in liver of control fish. The erosion of liver was very high and the color was dark (down).



Discussion

V. harveyi is one of the causative diseases in aquaculture organisms of vertebrates and invertebrates, it also infects freshwater fish. Infections with *V. harveyi* have been detected in farmed barramundi fish in Malaysia and the Philippines. According to the study, symptoms of *V. harveyi* infections varied from scale drop to muscle necrosis in farmed barramundi*, L. calcarifer* in Vietnam. The external symptoms of infection were the loss of scales in fish, erosion of fins, and the appearance of skin wounds, because the bacteria erodes the skin of the fish (Dong et al., 2017). Similar symptoms were found in our study.

The current study indicated a big difference between the tissues of the organs of the vaccinated fish and the control fish. After the challenge test, the vaccinated fish was able to overcome the bacterial infection due to the presence of immunity against the pathogen. This immunity was established after the fish were given an inactivated vaccine against *V. harveyi* bacteria, so the fish were able to resist and fight the bacteria, which reduced the damage to the internal organs, as well as the external appearance of the fish. Moreover, the vaccinated fish survived for a longer time after the challenge test. In contrast, the control fish showed the exact opposite, as after the challenge test the fish died after 24 hours and showed all symptoms of infection with *V. harveyi*. The brain in the control fish had many damages, including separation of parts, congestion of the blood. However, the brain appeared in a healthy condition in the vaccinated fish. This could be explained by the fact that he control fish could not fight the bacteria compared to the vaccinated fish after being infected with *V. harveyi*. Heart tissues in the control fish shows necrosis caused by *V. harveyi* attacking the fish's organs. The presence of immunity in the vaccinated fish means that the bacteria do not attack the fish organs. Thus, the heart of the vaccinated fish is healthy and without any necrosis or damage. As for the intestine, it was clear in all its parts and the villi were present in the vaccinated fish. Similar findings were obtained in the study of the villi in the uninfected fish (Stephens et al., 2006). As for the control fish, the intestine was greatly eroded to the extent that the villi were not present on its wall. This is due to the bacteria that attacked the intestine and led to the erosion and necrosis of the villi in the intestine. The liver in the fish infected with *V.*

harveyi after challenge test showed the presence of fluid congestion, and focal necrosis in the liver tissues. This is because the control fish could not resist the bacteria (Mohamad et al., 2019). In opposite, in the vaccinated fish, the liver did not suffer any necrosis.

Conclusion

Bacterial diseases are a major problem of aquaculture sector in the world. In order to solve this problem the immune system in fishes need to be improved. Vaccine is one of the strategies that is used to improve immune stimulation in fishes against bacterial diseases. The development of an ineffective vaccine against *V. harveyi* and its efficacy on barramundi fish used in aquaculture projects in the Sultanate of Oman was the objective of this study. Our data suggested that the immune response of fishes and their ability to survive after the challenge test against *V. harveyi* was improved by the vaccination. The study demonstrates high potential of vaccines to fight bacterial infections in aquaculture in the Sultanate of Oman.

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