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RESEARCH PAPER

Bacterial infection in farmed barramundi juveniles, *Lates calcarifer*

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دراسة العدوى البكتيرية لأسماك البراموندي المستزرعة

بثينة الخزيري وأزهار البوسعيدي وعادل السليماني ونجلاء المنذري وجيلها يوني

Abstract. In 2016, Barramundi, *Lates calcaifer* juveniles imported from Thailand to the aquaculture station at Sultan Qaboos University exhibited remarkable external clinical signs of losing scales, severe muscle necrosis, and ascites. Fin rot was also observed in the diseased fish. The mortality reached more than 90% and it was assumed that some bacterial species were associated with the diseased fish. The objective of the present study was to investigate the infection route of disease, revealing the causative agent and finding the most effective antibiotic treatment. The suspected pathogen vehicle was mosquito larvae in the outlet of the culture tank as bacterium was isolated inside the mosquito larvae. Primary, traditional phenotypic tests and the *vitek* test confirmed that the bacteria were pathogenic *Aeromonas sobria* and *Lactococcus garvieae*. Eight of the most commonly used antibiotics in the aquaculture industry was used for antibiotic susceptibility test. It showed that that Gentamycin was the most effective antibiotic while the most effective environmentally friendly source was effort of henna, *Lawsonia inermis*, at a concentration of 10%.

Keywords: Barramundi, *Lates calcaifer*, mortality, antibiotic, *Aeromonas sobria*, *Lactococcus garvieae*, Henna.

المستخلص: عام ٢٠١٦، أظهرت أسماك البراموندي المستوردة من تايلاند إلى محطة الإستزراع السمكي في جامعة السلطان قابوس علامات سريرية خارجية ملحوظة كفقدان الزعانف، نخر العضلات الحاد، والاستسقاء. كما لوحظ تآكل الزعانف في الأسماك المريضة. بلغت نسبة الوفيات أكثر من ٩٠٪ وقد أفترض أن عدوى بكتيرية كانت السبب في هذه الوفيات. الهدف من هذه الدراسة هو البحث في طريقة العدوى البكتيرية، كشف العامل المسبب لها، وإيجاد المضاد الحيوي الأكثر فعالية. الناقل المشتبه لهذه العدوى هي يرقات البعوض الموجودة في خزان تفريغ المياه من أحواض الإستزراع السمكي، حيث تم عزل البكتيريا من داخل اليرقات. أكدت التحليلات التقليدية الأولية، وتحليل vitek أن البكتيريا المسببة للمرض هي *Aeromonas sobria* و*Lactococcus garvieae* . تم استخدام ثمانية أنواع من المضادات الحيوية الأكثر استخداما في الاستزراع السمكي في اختبار الحساسية للمضادات الحيوية. أظهرت الدراسة أن *Gentamycin* كان المضاد الحيوي الأكثر فعالية ضد البكتيريا المستخلصة، في حين أن أوراق الحناء *Lawsonia inermis* هي المضاد الحيوي الصديق للبيئة بتركيز ١٠٪.

الكلمات المفتاحية: البراموندي، ليتس كالكريفر، المضادات الحيوية، إيروموناس سوبريا، لاكتوكوكوس غارفيي، الحناء.

Introduction

Barramundi, *Lates calcarifer* is a catadromous fish of the family Latidae and the order of Perciformes. This species is widely distributed from the Indo-West Pacific region to Papua New Guinea and Northern Australia. Barramundi has been one of the most important cultured finfish in Asian countries. This fish has become the second highest potential species for freshwater aquaculture after tilapia In the Sultanate of Oman. Due to the recent introduction of Barramundi aquaculture in Oman, there has been an interest to study this species. However, as any other cultured fish species; a significant economic damage has been reported in farmed barramundi due to various infectious pathogenic bacteria around the world.

Bacterial infections of several species are being identified as the major cause of losses in the aquaculture industry (Bromage et al. 1999). A Gram-positive coccus of the species *Lactococcus garvieae* has been recorded as a zoonotic pathogen that has been identified as one of the major causative agents of aquaculture pathogenic problems worldwide (Chen et al. 2001). The Gram-negative *Aeromonas sobria* has also been identified as a primary pathogenic causative agent of many fish species (JurajMajtán et al. 2012).

Many of modern and effective drugs and antibiotic are synthesised from traditional folk of medicine (Natarajan et al., 2003). Therapeutic efficacy of many different plants has been discovered in the traditional medicine practices (Al Maqbool et al.1985). The healing attribution of henna leaves has been known for centuries and it is a subject of scientific studies (Azaizeh et al. 2003). This plant is scientifically known as *Lawsonia inermis* that belongs to the family Lythraceae (Abdulmoneim 2007). Henna leaves contain lawsone (2-hydroxy-1, 4-Napthoquinone) components that are red orange in colour. (Abdulmoneim 2007). The chemical data of the henna leaves prompted to investigate its antibacterial

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Table 1. The eight antibiotics used and their average inhibition zone for the *Aeromonas sobria* species.

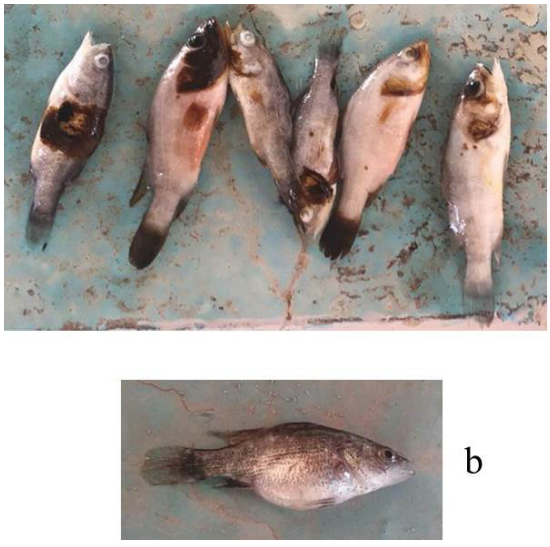
ANTIBIOTICS	Disk potency (µm)	Diameter of zone of inhibition (mm)			Average inhabited zone (mm)
		Susceptible	Intermediate	Resistant	
Oxytetracycline (OT 30)	30	≥ 23	19-22	≤ 18	24 ± 0.1
Ciprofloxacin (CIP 5)	5	≥ 21	16-20	≤ 15	24 ± 0.1
Erythromycin (E 15)	15	≥ 21	16-20	≤ 15	25 ± 0.2
Chloramphenicol (C 30)	30	≥ 21	18-20	≤ 17	21 ± 0.1
Gentamycin (CN 10)	10	≥ 15	13-14	≤ 12	22 ± 0.2
Tetracyclin (TE 30)	30	≥ 28	25-27	≤ 24	25 ± 0.2
Nitrofurantoin (F 300)	300	≥ 17	15-16	≤ 14	21 ± 0.1
Ampicillin (A 10)	10	-	-	-	0

activity.

As disease prevention and control is fundamental to the success of aquaculture, therefore, the present study aimed to investigate the infection route of diseased Barramundi, to reveal the causative agent that causes mass mortality and to find the most effective antibiotic treatment and finally to screen environmentally-friendly treatment methods derived from Omani herbs.

Materials and Methods

Different tests like primary, traditional phenotypic and experimental challenge tests were applied to identify the pathogenic vehicles that cause such a mass Barramundi mortality. An antibiotic susceptibility test was also applied to find the most effective treatment.



a

b

Figure 1. Remarkable external clinical signs ofthe infected fish: scale loss, severe muscle necrosis ad fin rot.

Fish history of samples

In August 2016, Barramundi juveniles were imported from Thailand to the aquaculture station at Sultan Qaboos University. There was an observed increase in the morbidity and mortality of the fish, where more than 90% of fish died in that period. The fish showed remarkable external clinical signs of scales losing, severe muscle necrosis, ascites, and fin rot (Figure 1, a and b).

Isolation of bacteria

The suspected pathogen vehicle (mosquito larvae) were collected from the outlet of the culture tank and grouped according to their different life stages. The bacteria were isolated from each stage and cultured in Tryptone Soya Agar (TSA).

The cultured bacteria were incubated at 37ºC for 24 hours. Each colony of the cultured bacteria was cultured again in separated TSA plates. The plates were incubated at 37ºC for 24 hours.

Identification of bacteria

Primary test

Gram Staining was used for the isolated bacteria to identify the group of bacteria; whether they were gram-positive or negative.

Secondary test

A 3% KOH Solution was used for a primary test to divide the groups of bacteria into gram positive and gram negative. A Densichek Plus was used to measure the density of the bacteria used: a saline solution was added to the samples to make the density between 0.03 to 0.5 mg/ml. An identification machine (Vitec Ltd.) was used to identify the bacterial species, within 24 hours automated.

Table 2. The eight antibiotics used and their average inhibition zone for the *Lactococcus garvieae* species.

ANTIBIOTICS	Disk Potency µm	Diameter of zone of inhibition (mm)			Average inhabited zone (mm)
		Susceptible	Intermediate	Resistant	
Oxytetracycline (OT 30)	30	≥ 23	19-22	≤ 18	25 ± 0.1
Ciprofloxacin (CIP 5)	5	≥ 21	16-20	≤ 15	23 ± 0.1
Erythromycin (E 15)	15	≥ 21	16-20	≤ 15	26 ± 0.1
Chloramphenicol (C 30)	30	≥ 21	18-20	≤ 17	21 ± 0.2
Gentamycin (CN 10)	10	≥ 15	13-14	≤ 12	24 ± 0.3
Tetracyclin (TE 30)	30	≥ 28	25-27	≤ 24	27 ± 0.2
Nitrofurantoin (F 300)	300	≥ 17	15-16	≤ 14	20 ± 0.1
Ampicillin (A 10)	10	-	-	-	0

Experimental challenge test

To identify the causative agent of the mass mortality, an experimental challenge test was performed by infecting healthy Barramundi juveniles by the suspected bacteria extracted from the mosquito larvae. The fish were kept and treated under the same conditions as the cultured ones, where the control fish were treated in a similar manner without bacteria. The infected fish were examined for the clinical signs of disease.

Treatments

Susceptibility test of commercial antibiotics

The antibiotic susceptibility test was carried out using eight of the most commonly used antibiotic in the aquaculture industry (Oxytetracycline, Ciprofloxacin, Erythromycin, Chloramphenicol, Gentamycin, Tetracyclin, Nitrofurantoin, and Ampicillin). ISO-Sensitest Agar (Oxoid) was used in this test. The diameter of the inhibition zones of the antibiotics were measured (mm) and compared with the antibiotic standard table from Oxoid.

Environmentally-friendly treatment

Alcohol based extraction was applied for two different Omani herbs (henna, *Lawsonia inermis* and Zamuta). The henna and Zamuta leaves were grounded and mixed with 100% ethanol for about 24 hours. The extracts were filtered to separate any solids and mixed with ISO agar plates with 5 different concentrations of 10%, 5%, 2.5% 1% and 0.5%, respectively. A pure culture of the bacteria was diluted with PBS (phosphate- buffered saline) and inoculated in each plate. The plates were incubated at 37ºC for 24 hours. The same concentrations of the henna extract were tested for their antibacterial activity using the spectrophotometer device.

Results

Identification of bacteria

The isolated pathogenic bacteria were A*eromonas sobria* and *Lactococcus garvieae*. These bacteria have been reported to cause loss of scales, severe muscle necrosis, and ascites or to cause fish mortalities in some cases.

Experimental challenge test

The challenge test was applied to fulfil the causative agent of the mortality and compare the clinical signs of the killed fish. The fish died within 24 hours after infection, and they displayed darkened, and with some losing scales, severe muscle necrosis, and fin rots. In the control fish tank, no mortality occurred.

Antibiotic test

Commercial antibiotics

Tables 1 and 2 show the results of the eight different antibiotics used for the two species of bacteria (*Aeromonas sobria*, and *Lactococcus garvieae*). The most effective antibiotic for the *Lactococcus garvieae* and *Aeromonas sobria* was Gentamycin (CN 10) with an average inhabited zone of 22 ±0.2 mm and 24 ± 0.3 mm respectively. Generally, both *Lactococcus garvieae* and *Aeromonas sobria* were resistant against Ampicillin (A 10). Henna extract showed more bio-activity against bacteria in at the concentrations of 10% and 5%, while it shows low bio-activity at the concentration of 2.5% and no activity against bacteria at the lower concentrations. For the Zamuta extracts, the bio-activity was low at the concentration of 10%, while it shows no activity at the lower concentrations (Fig. 2).

Discussion

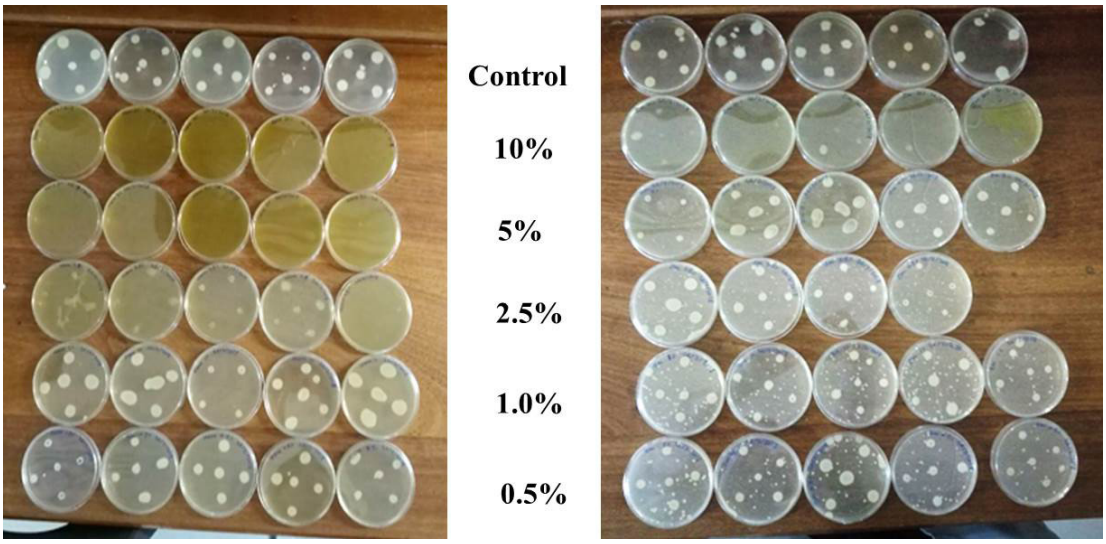


Figure 2. The bio-activity for both a henna, (*Lawsonia inermis)* and b (zamuta) extracts against bacteria at different concentrations.

Bacterial identification and experimental challenge test

Both pathogenic *Aeromonas sobria* and *Lactococcus garvieae* have been reported to infect and kill fish in the aquaculture industry (Chen et al. 2001, Majtán et al. 2012). In both cases the infected fish from the farm and from the challenge test, the clinical signs are similar. It also assumed that the pathological findings are similar within the visceral cavity. *Lactococcus garvieae* infection in fish causes lesions in the vascular endothelium, hemorrhages, swollen abdomens, and accumulation of ascitic fluid that mainly affect the spleen, liver, brain, gut, kidney, and heart (Vendrell et al. 2006). The pathogenic *Aeromonas sobria* has been reported to cause hemorrhages and bleeding of skin lesions (Majtán et al. 2012).

Aeromonas sobria and *Lactococcus garvieae* may act as a primary pathogen and a potential risk factor for Barramundi with mosquito larvae acting as potential reservoir for the pathogens.

Antibiotic test

Commercial antibiotics

Both of the isolated bacteria only showed resistance against ampicillin, whilst Gentamycin was the best at killing or inhibiting the growth of these bacteria.

Environmentally-friendly Alcoholic based extraction

Henna plant has been known for their healing attribute that considered as an active agent against bacteria and fungi (Abdulmoneim 2007). Since henna shows a high biological antimicrobial activity, it can be used for any microbial infection without any side effects or bacterial resistance (Abdulmoneim 2007). Henna leaves contain glycosidic compounds like Lawsone (2-hydroxy-1,4-naphthoquinone) which also known as hennotannic acid, gallic acid, α-d-Glucose and tannic acid (Ostovari 2009). These compounds have been also proved for their effective antimicrobial activity against human bacteria (Abdulmoneim 2007). However, Zamota leaves did not show high antimicrobial activity against the isolated bacteria.

Conclusion

It is suggested that *Aeromonas sobria* and *Lactococcus garvieae* can act as a primary pathogen for Barramundi that cause a risk of mass mortality. The clinical signs for both bacterial species were lesions in the vascular endothelium, hemorrhages, swollen abdomens, accumulation of ascitic fluid, and bleeding from skin lesions. The most effective antibiotic against these pathogenic bacteria was Gentamycin while henna extract from screened herbs proved to be also highly effective against these bacteria.

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