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Isolation and Identification of Bacteria Associated With Farm-Cultured Tilapia (*Oreochromis niloticus*) in Sultanate of Oman

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Abstract

Aquaculture is essential to meet the growing demand of seafood worldwide. In the Sultanate of Oman, can indeed be considered a sustainable industry. Although aquaculture is on the rise, there is a potential threat to the health and safety of aquatic animal production. To ensure the quality of seafood, it becomes necessary to ensure these threats so that appropriate action can be taken. Physical properties of pond water, microorganisms associated with pond water and fish, bacterial identification and microbial resistance. The bacterial colonies ranged from $8.9 \pm 7.2 \times 10^3$ to $2.5 \pm 1.9 \times 10^4$ cfu / mL and $4.6 \pm 1.7 \times 10^5$ to $5.1 \pm 2.5 \times 10^6$ cfu / g and $3.6 \pm 2.4 \times 10^{10}$ to $4.6 \pm 1.8 \times 10^{11}$ cfu / g in the ponds water, gills and intestines, respectively. In total, three types of bacteria were identified: *Aeromonas sobria, Shewanella putrefaciens* and *Streptococcus agalactiae*; among them *A. sobria* were the most prevalent (71.4% prevalence). Antibiotics resistance was tested antibiotic concentrations, ranging from 0.7-1.8 cm, 1.0-4.2 cm and 1.0-3.5 cm for *A. sobria*, *S. putrefaciens* and *S. agalactiae*, respectively. All isolates were multidrug resistant, although only *A. sobria* was resistant to ampicillin, a certified aquaculture property in Oman.

Keywords: Oman; Aquaculture; Tilapia; *Oreochromis niloticus*

Introduction

Seafood sector, if tapped properly, can increase the food supply globally and hence, the source of animal protein. In the time to come, aquaculture of tilapia and shrimp is expected to show the fastest growth, particularly in India, Southeast Asia, Latin America and the Caribbean[1]. The burgeoning demand for seafood provides an opportunity to the global fisheries and aquaculture industry to improve their management leading to the achievement of sustainable seafood economy. The trial production of *Penaeus monodon* (giant tiger prawn) in 1986 conducted in Al-Sharqiyah South Region, Sur marked the beginning of aquaculture in Oman. The technical difficulties faced in this effort prompted the Government to establish the Aquaculture Laboratory at the Marine Sciences and Fisheries Centre (MSFC) with an objective to upgrade the utilization of the country's natural fishery resources and providing employment at the same time. Since the commercial viability of a project is dependent on the site for aquaculture, the MSFC conducted two projects[2,3] for the determination of these sites along the Omani coast. The widespread consumer appeal of tilapia and their effortless breeding and culturing has led to the commercialization of their production worldwide[4]. The advantages include rapid growth on feeds irrespective of the protein percentage, tolerance to higher carbohydrate levels, resistance to poor water quality and diseases. The over-reproduction of tilapia in ponds is due to the male monosex populations.

While freshwater resources are limited throughout the Persian Gulf region, small-scale tilapia culture has been successfully introduced in Kuwait and Saudi Arabia. Tilapia

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has been widely used in malaria-control programs and its culture is being assessed as a potentially new activity on the traditional farms in Oman. Following high demands, particularly from the non-natives, not only the freshwater aquaculture of the Nile tilapia, *Oreochromis niloticus* has become very popular in Oman but also the import of tilapia fry from Egypt and Thailand has increased. There are 10 integrated farms, each with a production unit of 1200 m^2 that consists of fifteen cement ponds for nursery and fattening[5] and expected to produce 36 tons/year of tilapia. This produce is majorly consumed locally and valued at USD 6/ kg. The water quality, the physiological status and the post-harvest quality of fish is influenced by aquatic microorganisms. Since aquaculture holds a promising sustainable industry and is in its initial stages of development in Oman, it is the responsibility of the Government to acquire baseline data on the existence and prevalence of aquatic diseases and pathogens, so as to make policy decisions to develop health management regimes[6]. However, the Government database lacks information in this regard. As mentioned earlier, tilapias are relatively resistant to diseases in comparison to most other cultured fin fishes but they get infected by some pathogenic organisms such as several species of *Vibrio*, the bacteria that inhabit marine and estuarine environments and cause food poisoning[7]. The bacterial content of the fish organs should be monitored as the quality of fish determines the storage life and the quality of the fishery products[8]. It is of paramount importance to collect detailed information about the type and amount of bacterial load in the internal organs of apparently healthy fish, to predict possible disease outbreaks and also to design preventative management actions.

The current study investigates the bacterial population in pond water, and in the aquaculture tilapia organs including gills and intestine, both qualitatively and quantitatively.

Materials and Methods

Experimental pond conditions:

The study was conducted on tilapia in a farm located in Al-Musannah, a town in the Al Batinah region of northern Oman. This farm was dependent on recirculation aquaculture system (RAS), an advanced and unique way to farm fish where the rearing of fish is carried out at high densities within indoor tanks under controlled environment. The recirculating system filters the used water and clean water is recycled through the tanks. Limited vegetation such as cabbage, lettuce and strawberry also existed in the tanks owing to the nitrogen supply provided by aquaculture, a technique known as aquaponics. In this study, the cultured tilapia fish were collected from two cement ponds that contained tilapia in different stages. Animal feed containing 32% protein was imported from the Kingdom of Saudi Arabia (KSA).

Physiochemical Analysis of Pond water

Water parameters, such as temperature, pH (Jenwey portable pH meter, model 3020), dissolved oxygen and total hardness (DR/890 Portable Colorimeter, HACH) were determined. Water samples were collected in sterilized containers and transported to the laboratory to check alkalinity and total ammonia according to the method proposed by American Public Health Association[9].

Bacteriological Sampling and Analysis

In order to investigate the bacterial populations, pond water and the cultured tilapia's organs (gills and intestine) were collected from the two ponds.

Analysis of pond water

Water samples were collected in a sterile 250 ml glass bottle from the two ponds and from 15-20 cm below the surface and analyzed individually. Water samples were transported to the fishery Quality Control Center (FQCC) laboratory in a cool box contains ice bags and analyzed within 24 hours. Briefly, 1 ml of each sample was added to 9 ml of sterile physiological saline (0.85% NaCl), mixed properly and then serially diluted from 10^{-1} to 10^{-4}. Then 0.1 ml of each dilution was spread on the surface of tryptone soy agar (TSA) plates supplemented with 1.5% NaCl and incubated at 30°C for 48 h.

Analysis of the Gills and Intestine of fish

Bacteriological analysis was carried out after collecting fish samples from the ponds. In this study a total of six tilapia fish were collected (three from each pond). Fish were transported to the laboratory in a cool box contains pond water and analyzed within 24 hours and under a septic environment. Briefly, fish were killed by physical destruction of the brain. The skin was sterilized by wiping with 70% ethanol prior dissection, the gills and intestine were taken and homogenized separately in a mortar under aseptic conditions. 1 g of these homogenates was then suspended in 25 ml of sterile physiological saline solution (0.85% NaCl). 1 ml of the sample was added to 9 ml of sterile physiological saline mixed properly and then serially diluted from 10^{-1} to 10^{-7}. Then 0.1 ml of each dilution was spread on the surface of TSAplates supplemented with 1.5% NaCl and incubated at 30°C for 48 h.

Isolation of bacteria

After incubation, the bacterial colonies grown on the first culture of the saline TSA were described based on their shape, color, size, elevation, surface texture, etc., selected and further sub-cultured in new saline TSA to produce a purity plate that contain a single type bacteria each. Six colonies from water samples and twenty colonies from fish samples were picked from different plates and sub-cultured on TSA. The isolated bacteria were divided into different groups according to their shape, size, color and surface texture.

Biochemical Identification of the Isolated bacteria

For biochemical identification of bacteria, the isolated colonies were subjected to the following primary tests; oxidase test to identify bacteria that produce cytochrome c oxidase (an enzyme of the electron transport chain), Gram stain using both, the conventional Gram stain to differentiate between the Gram positive and Gram negative bacteria based on the composition of the bacterial cell wall (reference) and the rapid potassium hydroxide (KOH) test as a second method for Gram stain determination[10,11] and the catalase test that differentiates between the staphylococci bacteria (catalase-positive) from the streptococci bacteria (catalase-negative). For further biochemical testing, VITEK-2 Compact automated system (bioMérieux) were used according to the manufacturer's instruction. Briefly, a suspen-

sion of each single bacteria was prepared with different densities depending on the Gram type and a specific VITEK-2 cards were used accordingly for a proper identification.

Antibiotic Sensitivity Test

The antibiotic sensitivity of all the identified bacterial isolates was determined by the Disk Diffusion Method[12]. The antibiotics tested have been authorized for use in aquaculture and they were ampicillin AMP 10 and tetracycline TE 10. Other antibiotics including amikacin AK 30, ceftazidime CAZ 30, gentamycin N 10, ciprofloxacin CIP 10, oxacillin OX 1, penicillin GP 10, cephalothin KF 30, imipenem IPM 10, vancomycin VA 30, piperacillin PRL, 100 and clindamycin DA 10 were considered since they found to have an effect on *A. sobria*, *S. putrefaciens*, and *S. agalactiae*. Out of the 13 antibiotics mentioned above 9 antibiotic disks were used in this study for each isolate). *In vitro* antimicrobial activity was screened by using Mueller Hinton agar (MHA) obtained from Himedia (Mumbai, India). The MHA plates were prepared and 0.1% inoculum suspension of isolated bacteria including *Aeromonas sobria*, *Shewanella putrefaciens, Sphingomonas paucimobillis, Staphylococcus lentus,* and *Streptococcus agalactiae* was swabbed uniformly and allowed to dry for 5 min. The standard antibiotics disk was placed on the surface of medium (3 antibiotics's disks per plate) and the plates were kept for incubation at 37°C for 24 h. The test was performed in triplicate. At the end of incubation, inhibition zones formed around the disk were measured with transparent ruler in millimeter.

Molecular identification

DNA Extraction

Genomic DNA was extracted from the pure cultures of the bacteria isolated from the ponds's water and tilapia fish samples using DNeasy Kit (Qiagen) according to the manufacturer's instruction. The integrity of the extracted DNA was evaluated using gel electrophoresis and DNA was run in 0.8% agarose gel containing ethidium Bromide and visualized under UV light using a Gel documentation system (BIO-RAD Gel Doc. 2000 system).

Polymerse chain reaction (PCR)

PCR amplification was performed usingthe universal bacterial 16s RNA gene in the biochemicallyidentified isolates, a forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC-3') [13] in athermal cycler (Eppendorf Mastercycler Pro S). Each reaction consisted a 25 µl volume reaction (0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 1X PCR buffer, 1.5 mM MgCl2, 0.5 mM of each primer, and 2 µl of DNA, 0.5 U Taq polymerase usingillustrate PuReTaq Ready-To-Go PCR Beads (GE Healthcare). The temperature profile was as the follow: 30 cycles consisting of 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min followed by a final extension step of 5 min at 72°C. The template-free reaction was included in the PCR setup as negative controls.The amplified product was loaded in 1.5% ethidium bromide containing agarose gel in 1X Tris-boric acid-EDTA buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3) and run at 120 volts for 60 minutes. After electrophoresis, amplification products were visualized by the Gel-Doc UVtrans-illuminator (Bio-Rad Gel Doc 2000, USA).

Results

Physicochemical parameters

The temperature of pond water was 25°C and the pH was slightly alkaline (7.8±0.084). The amount of dissolved oxygen was 5.7±0.084. Mg/L and the total ammonia level was 1.19±0.0084 ppm. Total alkalinity, the measurement of all bases (bicarbonates, carbonates, phosphates, hydroxides, etc) in water that buffer and resist pH changes, was found to be 196.60±S.D mg/L and total hardness or general hardness, which is the measurement of divalent cations (calcium, magnesium, iron, etc.) in water, was found to be 137.89±S.D mg/L.

Quantitative data

Quantitative estimation of bacteria in pond water and gills, and intestine of farm cultured tilapia revealed that the bacterial load in the intestine of tilapia was comparatively high than in the gills and pond water. Bacterial load in pond water ranged from $8.9\times10^{3}\pm$ 7.2 to 2.5×10^{4} ± 1.9 cfu/mL; in gills $4.6\times10^{5}\pm$ 1.7 to 5.1×10^{6} ± 2.5 cfu/g and in intestine 3.6×10^{10} ± 2.4 to 4.6×10^{11} ± 1.8 cfu/g. Each count was the mean value of viable colonies grown on agar plates made per individual sample.

Qualitative data

The primary and the biochemical tests carried out on the water samples and gills and intestines of tilapia using VITEK led to the identification of 3 species of bacteria, *A.sobria, S. putrefaciens* and *S.agalactiae*. Out of the 3 species, 2 were isolated from the intestines (*A. sobria, S. putrefaciens*) and 1 each from the gills *(S. agalactiae*) and pond water (*A. sobria*). *A. sobria* was found to be the most common bacteria with a prevalence of 71.4% and was abundantly present in the intestine.

Table 1: Identification of bacteria inhabiting the pond water and the gills and intestine of tilapia

Bacteria	Water		Gills		Intestine	
	No.	%	No.	%	No.	%
Aeromonas sobria	1	100			4	80
Shewanella putrefaciens					1	20
Streptococcus agalactiae			1	100		

Antibiotic Sensitivity Test

Antibiotic sensitivity test using 13 antibiotics was carried out with the 3 isolated bacterial species where each bacterium was tested against 9 antibiotics. The resistance and susceptibility (zone of inhibition) of the isolates is depicted in Table 2. *Aeromonassobria* and *Streptococcus agalactiae* were esistant to 4 antibiotics while *Shewanella putrefaciens* was resistant to 2 antibiotics. All the 3 bacterial species were resistant to cephalothin KF 30 while resistance to penicillin was observed in *A. sobria* and *S. agalactiae*.

Table 2: Antibiotic sensitivity test

Bacteria	Antibiotics used	Zone of inhibition (cm)
Aeromonas sobria	Amikacin AK 30	1.7
	Ampicillin AMP 10	R
	Cephalothin KF 30	R
	Clindamycin DA 10	1
	Gentamycin CN 10	1.8
	Oxacillin OX 1	R
	Penicillin G P 10	R
	Tetracyclin TE 10	0.7
	Vancomycin VA 30	0.8
Shewanella putrefaciens	Amikacin AK 30	2.4
	Ampicillin AMP 10	2.3
	Ceftazidime CAZ 30	1.5
	Cephalothin KF 30	R
	Ciprofloxacin CIP 10	4.2
	Imipenem IPM 10	2.7
	Oxacillin OX 1	R
	Penicillin G P 10	1
	Piperacillin PRL 100	3
Streptococcus agalactiae	Amikacin AK 30	1
	Ampicillin AMP 10	1.3
	Cephalothin KF 30	R
	Ciprofloxacin CIP 10	3.5
	Clindamycin DA 10	R
	Gentamycin CN 10	1.2
	Penicillin G P 10	R
	Tetracyclin TE 10	3
	Vancomycin VA 30	R

Analysis of Molecular Identification

Analysis of PCR for universal primer

The 16S rRNA (universal bacterial primer) was amplified in all 6 bacterial isolates along with positive control which contained extracted DNA for *vibrio parahemogyticus*.

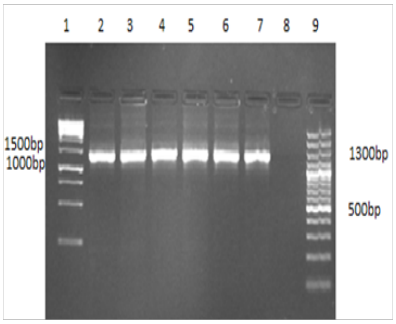

Figure 1: Ethidium bromide 1.5% agarose gel electrophoresis for universal Bacterial 16S rRNA showing amplified PCR products (1300 bp) of six bacterial isolates

Lane 1: GeneRuler 1 kb DNA Ladder (0.1 µg/µL), thermos Scientific, Lane2: *Aeromonas sobria*, lane3: *Streptococcus agalactiae*, lane4: *Leuconostoc pseudomesent*, lane5: *Shewanella putrefaciens*, lane6: *Sphingomonas paucimobilli*, lane7: *Staphylococcus lentus*, lane 8: negative control of no DNA template, lane 9:GeneRuler 100 bp Plus DNA Ladder (0.5 µg/µL),thermos Scientific.

Discussion

In this study, we cultured tilapia (*Oreochromis niloticus*) in two cement ponds followed by sampling of the pond water and gills and intestine of tilapia to enumerate the bacterial load. The bacteria were isolated, identified and their response to anti-microbial agents was established.

The quality of water is very important for aquaculture[14]. Tilapia are known to withstand a wide range of environmental conditions such as high temperature, salinity and ammonia concentrations and low oxygen levels making them suitable for aquaculture[15] of the studied physico-chemical parameters, the temperature of the pond water was optimal for growth and reproduction while pH, concentration of ammonia, dissolved oxygen and total alkalinity and total hardness were within acceptable levels. The total alkalinity concentration should not be lower than 20 mg/L[16]. The major source of ammonia in the culture pond is the excreta of tilapia. Here, the total ammonia nitrogen (TAN) comprising the un-ionized ammonia (NH_3) and the ionized form (NH_4^+) was measured. The ratio of NH_3: NH_4^+ is dependent on the temperature and pH of the water; higher these parameters,higher is the toxicity as the percentage of unionized form of ammonia increases. Though tilapia can tolerate high concentrations of ammonia, increasing concentration is proportional to the stress induced leading to depressed feeding and mortality.

The bacterial load was high in all the samples, possibly due to the high water temperature[17], optimal for the growth of many mesophilic bacteria of the ecosystem[18]. The bacterial load in pond water was $7.2 \pm 8.9 \times 10^3$ to $1.9 \pm 2.5 \times 10^4$ cfu/mL, similar to that found in another study[19] where the load was 1.6×10^3 to 1.6×10^4 cfu/mL and ambient temperature was 27 - 28°C. Similarly, the bacterial load of the tilapia gills $1.7 \pm 4.6 \times 10^5$ to $2.5 \pm 5.1 \times 10^6$ cfu/g was comparable to those found in turbot gills (7.0×10^5 cfu/g)[20]. In the present study, the highest bacterial load was found in the tilapia intestine ($2.4 \pm 3.6 \times 10^{10}$ to $1.8 \pm 4.6 \times 10^{11}$ cfu/g), a result similar to a study carried out with hybrid tilapia[21]. High temperature leads to an increased feeding rate which in turn increases the metabolic activity of the fish, and this could be a reason for the high bacterial load in the intestine and gills of tilapia. It is therefore seen that the bacterial load is different in different fishes depending on the optimal physico-chemical parameters[21,22].

In agreement with earlier studies[21,22], *Aeromonas spp*. was found to be most prevalent pathogen. *Aeromonas sobria* was isolated from the pond water; this has been reported previously[22]. The presence of *Shewanella putrefaciens* in the intestine and *Streptococcus spp*. in the gills has also been reported earlier. Aquaculture and related quality control regulations reported by the ministry of agriculture and fisheries wealth, 5 showed that ampicillin, erythromycin, and tetracyclin have an effect on bacteria associated with cultured fish, but the isolated bacteria *Aeromonas sobria* showed resistance to ampicillin in this study. The sensitivity to antibiotics demonstrated by *Aeromonas sobria* (Table 2) was similar to a previous report[23].

Primers 63f and 1387r have been successfully used to

amplify 16S rRNA gene from a variety of microorganisms[24]. The same primers were used for the isolated bacterial species of this study. *Aeromonas sobria* was confirmed by amplification of 16s rRNA using the primers reported by Morandi, et al 2005[25].

Conclusion

On the basis of the results obtained in this work, it can be concluded that 3 bacterial species *Aeromonas sobria, Shewanella putrefaciens* and *Streptococcus agalactiae* were identified from pond water, gills, and intestine of Tilapia (*Oreonchromis spp*.) Members representing with *Aeromonas sobria* being the most dominant, covering around one-third (71.4%) of the total bacterial populations.

All the isolates showed multi-drug resistance and susceptibility to various antibiotics with varying zone of inhibition range.

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