International Journal of Zoological Research

ISSN 1811-9778

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS International Journal of Zoological Research

ISSN 1811-9778
DOI: 10.3923/ijzr.2018.14.20

Research Article
Detection and Molecular Characterization of Some Bacteria Causing Skin Ulceration in Cultured Nile Tilapia (*Oreochromis niloticus*) in Kafr El-Sheikh Governorate

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Abstract

Background and Objective: Cultured Nile tilapia [*Orechromis niloticus* (*O. niloticus*)] is so far the most widespread species in earthen ponds in Egypt. These cultured fish suffers from yearly bacterial mass mortalities with a characteristic external hemorrhages and ulcer formation. This study aimed to investigate some of these bacterial pathogens with emphasis on their specific anti-biogram profile. **Materials and Methods:** A number of 150 naturally infected *O. niloticus* were collected from different fish farms at Kafr El-Sheikh Governorate, Egypt where the skin ulcers were the most common clinical sign. The fish were bacteriologically examined and all bacterial isolates were identified phenotypically, serologically and by using VITEK 2 Compact System and finally by polymerase chain reaction. **Results:** The results revealed that, the isolated bacteria; were *Aeromonas* sp. (25.9%) [*A. hydrophila* (23.3%) and *A. caviae* [2.6%)] and *Pseudomonas* sp. (23.3%) [*P. fluorescens* (9.3%), *P. putida* (2%) and *P. aeruginosa* (12%)] in addition to *S. aureus* (7.3%) while mixed infections were (36.6%). Antimicrobial sensitivity test of *Aeromonas* spp. revealed their sensitivity to ciprofloxacin, while the sensitivity of *Pseudomonas* spp. were to chloramphenicol, ciprofloxacin, tetracycline and streptomycin, respectively. Finally *S. aureus* was sensitive to ciprofloxacin, ampicillin, gentamicin and amoxicillin. **Conclusion:** The results concluded that antimicrobials (if applicable) could be used as effective candidate for fish treatment, after performing sensitivity testing and maintaining good water quality in fish ponds fish could be recovered from such infections.

Key words: *Oreochromis niloticus*, skin ulcers, VITEK 2, polymerase chain reaction, bacterial isolates

Citation: Adel Mohamed El-Gamal, Mohammed Sayed El-Gohary and Alkhateib Yousry Gaafar , 2018. Detection and molecular characterization of some bacteria causing skin ulceration in cultured Nile tilapia (*Oreochromis niloticus*) in Kafr El-Sheikh Governorate. Int. J. Zool. Res., 14: 14-20.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Beside its role as the frontline barrier against external intruders, fish skin acts as a health status mirror for stocked fish during the act of aquaculture, since some pathogens attack and harm the skin during the invasion process[1]. An ulcer is defined as a breach of continuity of all the skin layers that fails to heal and is often accompanied by inflammation around its circumference[2]. Without treatment it progressively expands. Ulcers can be caused by damages to the skin due parasites or chemicals such as high/low pH or high levels of ammonia or nitrite or from trauma due to handling or breeding efforts. In most, but not in all cases, they are caused by opportunistic bacteria[3]. Presence of immuno-compromised fish or high organic loads in fish ponds favors opportunistic attacks by some waterborne bacteria such as *Pseudomonas* spp., *Aeromonas* spp., *Streptococcus* spp. and *Staphylococcus* spp. Among all these naturally occurring aquatic bacteria, *Aeromonas* and *Pseudomonas* represent the major widely distributed bacterial fish pathogens in nature[4,5]. Precise fish ulcerative syndrome causative agent is still not confirmed, however, organisms belonging to the potentially fish pathogenic genera *Aeromonas*, *Vibrio*, *Pseudomonas* and *Plesiomonas* were often incurred from the blood and lesions of infected fish[6], in such cases in *O. niloticus* the most isolated bacterial pathogens were *Aeromonas* sp., *Pseudomonas* sp., *Streptococcus* sp.[7,8], *Bacillus flexus*, *B. cereus*, *B. firmus*, *B. vietnamensis*, *Halomonas* sp., *Staphylococcus* sp.[9,10] and *Enterococcus faecalis*[11]. Some of these pathogens are of zoonotic importance such as *Aeromonas* sp., *Streptococcus iniae*[12,13]. Due to its ubiquitous presence in freshwater bodies, *Aeromonas* outbreaks commonly occur in aquaculture facilities, in between fish suffering from stress factors, such as poor sanitation and nutritional deficiencies[14] and are mostly associated with hemorrhagic skin ulcers, the most accused species are *Aeromonas salmonicida* and *A. hydrophila*[15]. Skin lesions caused by *Aeromonas* spp. often secondarily infected by opportunistic fungi or *columnaris* spp. When the skin lesion is the sole affection, despite the presence of severe ulceration, fish may continue to feed and survive for a while, this chronic form of the disease may be associated with low frequent mortalities which can increase over time[16]. *Aeromonas* spp. were recovered from fish ulcerative disease cases in the Indo-Pakistan region[17] and from 27% of fish with ulcerative symptom in Malaysia, Thailand and Bangladesh[18], also Maimona and Sabiel[19] isolated *Aeromonas* spp. from 25% of Nile tilapia with skin affection. While in Egypt, *Aeromonas hydrophila* was recovered from skin ulcers from 43.77% of *O. niloticus* cases[3]. In *O. niloticus* fish hatcheries, both *Aeromonas* spp. (*A. hydrophila*, *A. sobria* and *A. caviae*) and *Pseudomonas* spp. (*P. fluorescens*, *P. putida* and *P. aeruginosa*) were claimed in causing severe outbreaks especially during winter season in Egypt[20]. Hence the aim of study was the detection of some bacteria causing skin ulcers in cultured *O. niloticus* at Kafr El-Sheikh Governorate fish farms and the antimicrobials controlling of these bacteria.

MATERIALS AND METHODS

Fish sampling and examination: During summer months in 2017, a total number of 150 naturally infected *O. niloticus* showing skin ulceration were collected from various aquaculture facilities at Kafr El-Sheikh Governorate, Egypt. They were transferred in aerated tanks to the laboratory of Health Research Institute, Kafr El-Sheikh branch. Clinical signs and postmortem examination were carried out as described by Schaperclaus *et al.*[21]. All chemicals and reagents used in this study is ACS and reagent grade chemicals.

Bacteriological examination: Fish were dissected under aseptic conditions, where bacteriological sampling was performed from gills, posterior kidney, hepatopancreas, spleen and brain. External lesions were swabbed as their surroundings were disinfected using 70% ethanol[22]. Swabs for bacteriological examination were inoculated into nutrient broth and Tryptic Soy Broth (TSB) and incubated at 25°C for 24-48 h. Inocula were streaked on general and selective bacteriological media, such as nutrient agar, tryptic soy agar and brain heart infusion agar, selected to ensure the recovery of most of the accused bacteria and incubated at 25°C for further 48 h. After picking up the suspected purified colonies were streaked over specific medium for further purification; such as Rimler's-Shotts medium (R.S. medium), *Aeromonas* selective agar base with Ampicillin supplement, *Pseudomonas* selective agar base and Mannitol salt agar and then incubated at 25°C for 24 h. For detection the hemolytic activity, the picked up colonies were subcultured on blood agar and incubated at 25°C for 24 h. Each pure culture was inoculated on nutrient agar slant for further identification, another loop full was inoculated on semisolid nutrient agar for motility testing and preservation. Identification of the isolates was carried out using the routine study of the morphological character and biochemical reactions[21,23,24]. Selected strains were then preserved at -80°C in TSB (Bioxon) with 15% (v/v) glycerol[22].

Phenotypic characterization of bacterial isolates (biochemical identification): The purified isolates were further identified and confirmed using their biochemical and phenotypic characteristics, utilizing a growth-based technology through incorporated colorimetric reagent cards that are interpreted automatically after incubation (VITEK 2 Compact System, BioMerieux, France). Identification was carried out according to Austin *et al.*[25].

Serological identification (slide agglutination test): As described by Sorensen and Larsen[26] sera were obtained in rabbits using formalin killed bacterial cells as antigen. Whole-cell antisera against reference strains of different isolated bacteria were used. The assays were done against the heat stable O-antigen reacted with rabbit whole-cell antisera. After heating bacterial suspensions of each strain in phosphate buffered saline (PBS) at 100°C for 1 h, the O-antigens were obtained. A drop of each O-antigen suspension (opacity, McFarland standard No. 3) was mixed with a drop of 1/10 diluted rabbit antiserum on a multi-well glass slide. An immediate visible agglutination was identified as positive and no or only a weak latent agglutination resulting after 1-2 min was considered as negative[27].

Polymerase chain reaction (PCR) of the isolated bacteria: The PCR were performed in a volume of 50 µL containing 3 µL template DNA, 5 µL of 10×PCR buffer, 200 µM of each dNTPs, 2 mM of $MgCl_2$, 0.5 µM of each primer and 1.5 U of Taq polymerase (Fermentas, Lithuania). The amplification process was performed in a DNA thermal cycler (BioRad, Tgradient, USA). The PCR products were separated on 2% agarose gel, stained with (0.5 µg mL^{-1}) ethidium bromide where a 1 kb DNA ladder (Promega) was used as a molecular weight standard on gel, which was photographed by gel documentation system (Biometra Bio Analysis). The oligonucleotide sequences for all the bacteria are shown in Table 1[28-30].

Antibacterial susceptibility: The antimicrobial susceptibility was performed according to the limit given by Schaperclaus *et al.*[21] using the disc diffusion method on Muller's Hinton agar medium and the interpretations of the zones of inhibition.

RESULTS

Clinical and postmortem examination: Clinical signs of the collected fish specimens includes skin alterations as body color, exophthalmia, raised and detached scales, eroded opercula, reddening, ulcers, dropsy, clubbed and abraded gills, fins and tail rot were noticed (Fig. 1a, b). Necropsy revealed hemorrhages enlarged hepatopancreas (Fig. 1c), congested friable posterior kidney and splenomegaly.

Bacteriological and serological examination: Based on the colony morphology and serological tests, 3 types of bacteria were found, *Pseudomonas* spp. by 23.3% and *Aeromonas* spp. by 26% and *Staphylococcus aureus* by 7.3%, while mixed infections were 36.6%, the percentages of the identified bacteria infecting the fish in comparison to the total number of examined fish were presented in Table 2.

Table 1: Oligonucleotide sequence of selected genes for the different isolated bacteria

Bacteria	Product name	Sequence (5' to 3')	Product size (bp)	Reference
Pseudomonas aeruginosa	*PA-SS-F*	5′GGGGGATCTTCGGACCTCA 3′	956	Pirnay *et al.*[28]
	PA-SS-R	5′TCCTTAGAGTGCCCACCCG 3′		
Pseudomonas fluorescens	*16SPSEflu-F*	5′TGCATTCAAAACTGACTG 3′	850	Scarpellini *et al.*[29]
	16SPSE-R	5′AATCACACCGTGGTAACCG 3′		
Staphylococcus aureus	*Saa-442-F*	5′GTCGGTACACGATATTCTTCACG 3′	370	Martineau *et al.*[30]
	Saa442-R	5′CTCTCGTATGACCAGCTTCGGTAC 3′		

Table 2: Results of serological test of the isolated bacteria

		Infected fish (n=150)	
Bacterial group	Strains	+ve	%*
Pseudomonas spp.	*P. fluorescens*	14	9.30
	P. aeruginosa	18	12.00
	P. putida	3	2.00
Aeromonas spp.	*A. hydrophila*	35	23.30
	A. caviae	4	2.60
Staphylococcus spp.	*S. aureus*	11	7.30
Mixed infection		55	36.60

*Percentage calculated according to total number of examined fish

Fig. 1(a-c): Naturally infected *Oreochromis niloticus* showing (a, b) Detachment of scales and hemorrhagic ulceration of the skin and (c) Dissected fish showing congestion, hepatic enlargement (arrow)

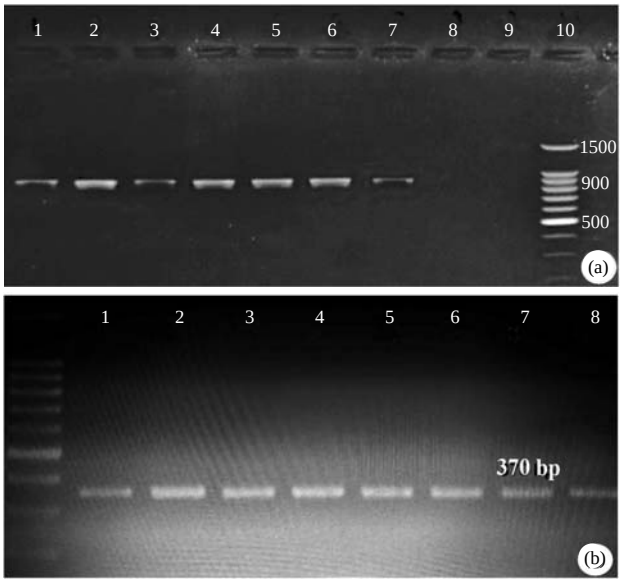

Fig. 2(a-b): PCR characterization of (a) *P. aeruginosa* showing positive amplification at 956 bp products at lanes 1-7, lane 8 and 9 are negative control and lane 10 is 100 bp DNA marker and (b) *S. aureus* showing positive amplification at 370 bp product at lanes 1-8, blank lane is 100 bp DNA marker

Biochemical examination: Biochemical identification of the isolated bacteria showed that *Pseudomonas* spp. were Gram negative motile rods. H_2S production, urease, nitrite, oxidase and catalase were positive while Indole test and methyl red-Voges Proskauer (MR-VP) were negative, ferment glucose, fructose, dextrose, galactose, sucrose and xylose. *Aeromonas* spp. were Gram negative motile rods, H_2S production, urease, nitrite, Indole test, MR-VP, oxidase and catalase were positive, they fermented glucose, fructose, dextrose, galactose, sucrose and xylose. *Staphylococcus* sp. was Gram positive, non-motile, non-capsulated cocci. Gelatin hydrolysis, coagulase, citrate, catalase, MR, nitrate reduction, urease, VP and lipase were positive, while oxidase, H_2S production, indole were negative. It fermented fructose, galactose, glucose, lactose, maltose, mannitol, sucrose and mannose and didn't ferment cellobiose, raffinose and xylose.

Polymerase chain reaction of the isolated bacteria: Results of PCR amplification for characterization of various isolated bacteria were shown in Fig. 2. For *Pseudomonas aeruginosa* the primers were designed to specially amplify the *toxR* gene of virulent strain of *P. aeruginosa* using the primer set PA-SS-F and PA-SS-R for amplification of a single DNA fragment of 956 bp. While, the primers for *Pseudomonas fluorescence* were designed to specially amplify the *16SPSE* gene of virulent

Table 3: Results of antibiogram sensitivity test for the isolated strains

Disc (code µg)*	CIP5	T30	C30	A10	S10	STX25	AMX25	CN10	E
A. hydrophila	S	S	S	R	S	S	S	S	R
A. caviae	S	S	S	R	S	S	S	S	R
S. aureus	S	R	R	S	R	R	S	S	R
P. putida	S	S	S	R	S	S	R	R	R
P. aeruginosa	S	S	S	R	S	S	R	R	R
P. fluorescens	S	S	S	R	S	S	R	R	R

CIP: *Ciprofloxacin, T: Tetracycline, C: Chloramphenicol, A: Ampicillin, S: Streptomycin, STX: Trimethoprim+sulfamethoxazole, AMX: Amoxicillin, CN: Gentamicin and E: Erythromycin

strain of *P. fluorescens* for amplification of a single DNA fragment of 850 bp. For the identification of *Staphylococcus* spp., PCR assays targeting the *Saa-442* gene sequence of a single 370 bp DNA fragment.

Antimicrobial sensitivity testing: Results of antibiogram sensitivity test of isolated bacteria showed that all isolated bacterial strains were sensitive to ciprofloxacin and resistant to erythromycin, the effects of other tested antimicrobials were variable as presented in Table 3.

DISCUSSION

Bacterial diseases of fish rise as one of the most important causes of losses in the aquaculture industry, affecting the economic development in that sector in many countries. The ability of bacteria to express their virulence factors outlines their ability to invade the host, produce pathological effects and evade host defenses, i.e. cause disease[19].

The disease problem in fish culture usually arises from interaction in-between the host, pathogen and their environment, the last one is the more critical factor[25].

Nile tilapia is susceptible to a wide range of bacterial pathogens. Many of these bacteria are not obligate pathogens in nature, but saprophytes. They turn into pathogens when fishes are immunocompromised or physiologically unbalanced due to nutritional deficiency or due to exposure to other stressors, i.e., poor water quality, overstocking, which allow opportunistic bacterial infections to proceed[14].

This study aimed for detection and outlining some bacterial pathogens causing skin ulceration in cultured Nile tilapia in Kafr El-Sheikh Governorate, Egypt. Observed clinical signs such as raised and detached scales, ulceration and hemorrhage of the skin (Fig. 1a, b) and postmortem examination; hemorrhagic and enlarged hepatopancreas (Fig. 1c), posterior kidney and spleen were common findings similar to those recorded by Abou El-Atta and El-Tantawy[3] and Austin *et al.*[25].

Based on the colony morphology, biochemical and serological tests, 3 types of bacteria were found (Table 2) many records reported similar ratios in their results[7,10,31,32].

From the previous findings it's clear that, *Pseudomonas* spp. and *Aeromonas* spp. could be considered of the most important bacterial fish pathogens responsible for ulcerative syndrome, this result agrees with Paniagua *et al.*[33] and Rahman *et al.*[34].

As shown in Table 2 *A. hydrophila* and *A. caviae* were the main isolated *Aeromonads*, while *P. fluorescens*, *P. putida* and *P. aeruginosa* were the main *Pseudomonas* spp. implicated in causing skin ulceration in *O. niloticus*, similar findings were recorded by El-Sayyad *et al.*[20].

As shown in Table 2 *Aeromonas* spp. has been isolated from ulcerated fish by 26.9%, this result is similar to McGarey *et al.*[18] and Maimona and Sabiel[19]. On contrary, concerning *P. fluorescence*, in previous study in Egypt it was isolated from *O. niloticus* with skin ulcers by 29.63%[3]. While Maimona and Sabiel[19] in Sudan, didn't find *Pseudomonas* spp. in similar cases. This difference may be attributed to difference in seasonal or temperature variations during which the samples were collected, as *Pseudomonads* prefer winter period[20]. From the same table *Pseudomonas* spp. were isolated by 23,3% (*P. fluorescens* 9.3% , *P. aeruginosa* by 12% and *P. putida* by 2%) these percentages were less than those recoded by Abou El-Atta and El-Tantawy[3].

Mixed infection (Table 2) had the highest record (36.6%), which may be attributed to the effect of high organic loads, which favors the growth of various types of the opportunistic bacteria, including *Pseudomonas* spp., *Aeromonas* spp. and *Staphylococcus* spp. found normally in aquatic nature[4, 5].

Biochemical identification of *Pseudomonas* sp., *Aeromonas* sp. and *Staphylococcus aureus* revealed nearly similar results to those recorded by El-Refaey[35]. PCR confirmation was done for *P. aeruginosa*, *P. fluorescens* and *Staphylococcus aureus*.

The antibiogram sensitivity testing profile of the isolated *Aeromonas* spp. and *Pseudomonas* spp. (Table 3) were nearly similar to those obtained by Attia[31] and Abou El-Atta and El-Tantawy[3]. The observed resistance of the isolated bacteria to various types of antibacterial agents denotes the gradual loss of the effective tools in competing bacterial fish diseases due to antimicrobial misuse during aquaculture practice; this necessitates the obligate application of antibacterial sensitivity

testing when dealing with clinical cases of bacterial fish diseases. Further research on the emerging bacterial fish pathogens concerning drug resistance mechanisms and the involved virulence genes is recommended in the near future.

CONCLUSION

In this study, identified bacteria causing skin ulcer in cultured Nile tilapia (*Oreochromis niloticus*) were mainly; *Aeromonas* spp. (*A. hydrophila* and *A. caviae*) and *Pseudomonas* spp. (*P. fluorescens*, *P. putida* and *P. aeruginosa*) in addition to *S. aureus*. The control and treatment of such infection through use of antimicrobial sensitivity testing together with decreasing overall stressors on fish during the process of aquaculture.

SIGNIFICANCE STATEMENT

This study revealed to some extent the most common bacterial pathogens accused for skin ulceration in cultured Nile tilapia (*Oreochromis niloticus*) earthen ponds in the largest freshwater fish farming area in Egypt (Kafr El-Sheikh Governorate). Also found that antimicrobial sensitivity testing is crucial to determine the candidate drug for their treatment with emphasis on the resulted antibiotics as primary candidates when performing this test with regards to the drug possible usability. Thus this study would help the researchers in determining and formulating the effective treatment against the pathogenic microorganisms.

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