Original Research Article

ISSN (Print) 2786-0272 ISSN (Online) 2786-0280

A novel bacterial infection in cultured Nile tilapia, Oreochromis niloticus in New Valley, Egypt

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Submitted 5/10/2021
Accepted 6/10/2021

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Abstract

Pseudomonas and micrococcus *(Pseudomonas fluorescens and M. luteus)*are an emerging opportunistic fish pathogens that results in considerable economic losses among the infected fish. In the present study, a total number of 150 cultured Nile tilapia, *Oreochromis niloticus* were collected from concrete ponds farms in Alkharga oases, New Valley governorate during the period from March to July 2019 and subject to clinical and bacteriological examination. including excessive black colouration of skin, scales loss, skin ulcer and focal hemorrhages, exophthalmia, fin congestion and rot. Internally, the diseased fish had congested internal organs including liver, spleen, and kidney., The prevalence of *Pseudomonas fluorescens* and *M. luteus* infection was 6.7 % withe a total number of 10 isolates and 11.3% withe a total number of 17 isolates respectively. Were recovered from infected fish. The suspected *Pseudomonas fluorescens* isolates were biochemically similar while The suspected *M. luteus* isolates were biochemically similar, except for Arginine hydrolysis, Vogus-Proskauer and Urease tests. The isolation and biochemical characterizations suggested that the isolates belong to the genus *Pseudomonas*. And genus *micrococcus*. The recovered *M. luteus* isolates were pathogenic to *O. niloticus*, this is the first report of *M. luteus* infection in *O. niloticus* in Egypt.

Keywords: Pseudomonas, *Micrococcus luteus*, , *Oreochromis niloticus*, Pathogenicity test, Egypt.

Introduction

Aquaculture has been the fastest growing animal food sector in the world during the past decades (FAO, 2012). Egypt was arranged as the seventh-largest aquaculture producer in the world and the largest in Africa with approximately one million tons per year (Feidi, 2018 and Shaalan *et al.*, 2018), also classified as the second-largest Nile tilapia, Oreochromis niloticus producer next to China (FAO, 2019). Aquaculture represents an important sector in the Egyptian national income structure (Abd El Tawab *et al.*, 2018). O. niloticus is the main cultured species in Egypt due to its economic value, palatability and easy cultivation (El-Gohary *et al.*, 2020) and its production contributes about 65.2% of Egyptian fish production (Elsheshtawy *et al*., 2019).

Fish bacterial diseases constitute one of the most important challenges confronting fish aquaculture (Hamouda *et al.*, 2019) and cause high economic losses to fish farms and sometimes they have zoonotic threats to fish consumers (Plant and LaPatra, 2011). The bacterial causative agents of these diseases were predominantly opportunistic pathogens and the Gram-negative bacteria still dominant pathogens while Gram-positive bacteria were observed in the recent years (Akayli *et al.*, 2020). The incidences of *Pseudomonas* spp. In the examined cultured fishes were 55.3% from *Oreochromis niloticus*, 36% from *Mugil cephalus*, 44% from *Cyprinus carpio* and 40% from *Hypophthalmichthys molitrix* (EL-Hady and Samy, 2011).

Genus *Micrococcus* belongs to family *Micrococcaceae*, order *Actinomycetales* and consists of Gram-positive cocci arranged in tetrads and in irregular clusters, non-motile and non-spore forming (Kocur *et al.*, 2006). They live in various environments, inhabit the soil, marine sediment, chicken meat, fresh water and mammals (Kim *et al*., 2004*). M. luteus* is a natural member of the aquatic environment and was isolated as a member of the intestinal microflora of various fish species (Akayli *et a*l., 2016 and Akayli *et al*., 2019). Also, it is isolated from the diseased fishes in some cases, especially under culture conditions and/or stress (Akayli *et al*., 2019 and Akayli et al., 2020). Pękala et al. (2018) described *M. luteus* as an emerging opportunistic pathogen and supposed that it will appear more often in the future

causing considerable commercial losses in fish farms. Infection with *M. luteus* causes excessive skin pigmentation, exophthalmia, gill damage, abdomen distention, pale, elongated spleen and kidney (Austin and Austin, 2007).

M. luteus infection was reported in some countries in rainbow trout, *Oncorhynchus mykiss*, (Aydin *et al*., 2005; Mousavi et al., 2010; Türk *et al*., 2013 and Pekala et al., 2018), common dentex, *Dentex dentex* (Akayli *et al.*, 2019), *gilthead seabream*, *Sparus aurata* (Çanak and Akayli, 2018*), O. niloticus* (Parra-Laca *et al.*, 2020 and Wanja *et al.*, 2020) and in sharpsnout seabream *Diplodus puntazzo* (Akayli and Yardimci, 2018 and Akayli *et al*., 2020).But, , The present study was conducted to address the *Pseudomonas fluorescens and M. luteus* infection among *O. niloticus* at New valley governorate, investigate prevalence of the microorganisms and explore its pathogenicity.

Materials and Methods

All the experimental, euthanasia and other procedures in this study were performed according to the Guide for the Care and Use of Laboratory Animals and the protocols were reviewed and approved by the research ethics committee of Faculty of Veterinary Medicine, Sohag University, Egypt.

Fish sampling:

150 hundred *O. niloticus* (40 – 100 gm), showing abnormal signs, were collected from concrete pond farms at Alkharga at New Valley Governorate during the period from March to July 2019. The collected fish included live and freshly dead fish. Fish were transported to the laboratory of Animal Medicine Department, Faculty of Veterinary Medicine, New valley University.

Clinical and post-mortem examination:

Fish samples were subject to clinical and post-mortem examination according to Noga (2010) for reporting the external and PM lesions.

Bacterial isolation and identification:

The fish were anesthetized by tricaine methanosulfonate (MS222 - Sigma Alderich) before dissection. Bacteriological samples were obtained from the liver, kidney and spleen under aseptic condition. The samples were immediately inoculated into tryptone soya broth (TSB; Oxoid, England) and incubated under aerobic condition at 22°C for 24 hours then streaked onto Tryptone Soya Agar (TSA; Oxoid, England) and incubated at 22°C for 48 hours as was previously described by Akayli et al. (2020). The isolates were preserved at -80°C in TSB supplemented with 15% glycerol till further identification (Pękala et al., 2018). The suspected isolates were identified using the standard laboratory methods described by Holt et al. (1994) and API 20E system (BioMerieux, France) according to manufacturer's instructions. The isolates were characterized according to criteria of Buller (2004) and Austin and Austin (2016).

Pathogenicity test:

The pathogenicity of recovered isolates (M. luteus) for O. niloticus was investigated using 20 apparently healthy O. niloticus (76 ± 5g). These fish were reared in fiberglass tanks supplied with chlorine-free tap water & and continuous aeration and kept at 25 ± 2°C. Fish were acclimatized one week before the experiments and fed on commercial fish pellet of 30% protein, at a rate of 3% of fish body weight/day. Fish were divided into 2 equal groups that were anesthetized by MS222 and the first group was injected intraperitoneally with 0.1 ml of sterile bacterial suspension containing 3× 107 cfu/ml, while the 2nd group was used as control group and injected with 0.1ml of sterile phosphate buffer saline/fish. All groups were observed daily for two weeks and the clinical signs, PM lesions and mortalities were recorded. Furthermore, isolation and identification of M. luteus from the internal organs of the experimentally infected fish were performed. The experiment was conducted in triplicate

Results

The naturally infected O. niloticus showed one or more clinical signs including excessive skin pigmentation or depigmentation, scales loss, skin hemorrhages, body ulcers, fin congestion and rot, slight to severe corneal opacity and exophthalmia (Figures 1, 2, 3 and 4). Internally, there were congested and sometimes enlarged liver, spleen, and kidney in addition to distended gall bladder (Figure 5).

Clinical signs and PM lesions in the naturally-infected *O. niloticus* by *Pseudomonas fluorescens* and *M. luteus*

Fig. (1): *O. niloticus* naturally infected with *M Luteus* showing excessive skin pigmentation.

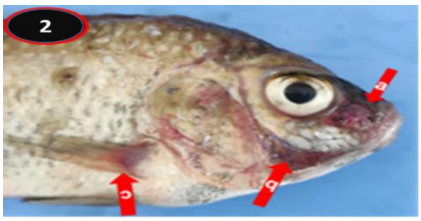

Fig. (2): O. niloticus naturally infected with M Luteus showing skin depigmentation and extensive hemorrhages.

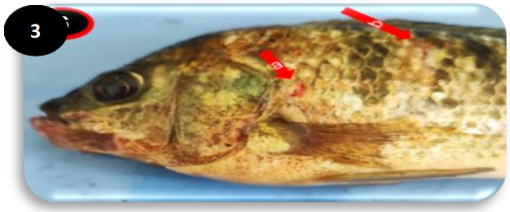

Fig. (3): *O. niloticus* naturally infected with *M Luteus* showing caudal fin rot, body ulcer and scales loss.

Fig. (4): *O. niloticus* naturally infected with *M Luteus* showing corneal ~~or eye~~ opacity,

Fig. (5): *O. niloticus naturally-infected with Pseudomonas fluorescens showing congested internal organs including liver, spleen, and kidney*

Prevalence of *P. fluorescens and M. luteus* infection

The results showed that the prevalence of *P. fluorescens and M. luteus* infection among cultured *O. niloticus* was 6.7% and 10.3%, respectively.

Table (1): The prevalence of *P. fluorescens and M. luteus* infection among *O. niloticus* in relation to total examined fish.

Locality	No. examined fish	*P. fluorescens*		*Micrococcus luteus*	
		No.	%	No.	%

Alkharga	150	10	6.7	17	10.3

Bacterial isolation and phenotypic identification:

Ten bacterial isolates bacterial isolates collected from the clinically diseased and apparently healthy O. niloticus were identical and identified as *Pseudomonas fluorescens* by the morphological characters and biochemical tests table (2a). Seventeen bacterial isolates were recovered from kidney, liver and spleen of the examined fish. They were identified as *M. luteus* according to their morphological and biochemical characteristics, illustrated in the table (2b) where all the isolates were Gram-positive cocci arranged in tetrads, produced small, spherical, smooth, and bright yellow colonies on TSA (Figure 6) and exhibited the same biochemical characteristics except for Arginine hydrolysis, and Urease tests where the results were variable.

Table (2a): Morphological and biochemical characteristics of *P. fluorescens isolated* from cultured *O. niloticus*.

Characteristic	Result	Characteristic	Result
Gram staining	-	ONPG	+
Motility	-	ADH	+
Cytochrome oxidase	+	LDC	+
Catalase	+	ODC	-
GEL	+	CIT	-
GLU	+	H2S	-
MAN	+	URE	+
INO	-	TDA	-
SOR	-	IND	-
RHA	-	VP	-
SAC	+	ONPG	+
MEL	-	ADH	+
AMY	-	LDC	+
ARA	-	ODC	-

O. niloticus groups	Fish number	Number of dead fish/days										Total number of dead fish	Mortality rate
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th - 14th			
Challenged groups	10	0	1	1	0	2	2	0	1	0		7	70 %
Control group	10	0	0	0	0	0	0	0	0	0		0	0 %

Table (2b): Morphological and biochemical characteristics of *M. luteus* isolated from cultured O. niloticus.

Characteristic	Result	Characteristic	Result
Gram staining	+	Urease	V
Motility	-	Tryptophane deaminase	-
Cytochrome oxidase	+	Indole production	-
Catalase	+	Vogus-proskauer	V
GLU	O	Gelatinase	+
lactose	-	Mannitol	-
Methyl red	-	Inositol	-
ß-galactosidase	-	Sorbitol	-
ADH	V	Rhaminose	-
LDC	-	Sucrose	+
ODC	-	Melibiose	-
Citrate	-	Amygdaline	-
H_2S	-	Arabinose	-

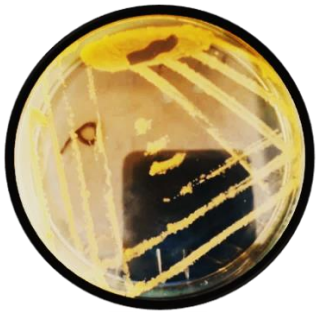

Figure (6): M. luteus colonies on TSA; small, spherical, bright yellow colonies

Pathogenicity of M. luteus isolates in O. niloticus:

Seventy percent mortalities occurred in the challenged fish groups starting from the 2nd till the 8th day post infection (table 5). The clinical signs and PM lesions observed in the infected fish were similar to that present in the naturally infected fish. Many challenged fish showed excessive black coloration, lethargy, inappetence, congestion and hemorrhage on body and at fin bases, fin rot, light to severe corneal opacity, skin ulceration and sometimes eye congestion. Internally, there were liver and spleen congestion (Figures 8, 9, 10 & 11). These findings proved that our M. luteus isolates were pathogenic to O. niloticus. M. luteus was also re-isolated and identified from the internal organs of the challenged fish.

Table (3): Mortality rate in the experimentally infected O. niloticus by M. luteus

Clinical signs and PM lesions in the experimentally infected *O. niloticus* by *M. luteus*

Fig. (7): Experimentally infected *O. niloticus* by *M. luteus* showing excessive black coloration on the skin and fins.

Fig. (8): Experimentally infected *O. niloticus* by *M. luteus* showing corneal opacity.

Fig. (9): Experimentally infected *O. niloticus* by *M. luteus* showing scale loss and skin ulceration.

Fig. (10): Experimentally infected *O. niloticus* by *M. luteus* showing fin rot.

Discussion

Bacterial pathogens are naturally present in fish environment and under some stressful conditions they become the etiological agents of the most important diseases in aquaculture (Olsson et al., 1998).

M. luteus is considered as a potential bacterial fish pathogen **(Aydin *et al*. 2005)** and supposed to appear more often in the future causing considerable commercial losses in fish farms (Pękala *et al*. 2018**)**. Information about prevalence of *M. luteus* infection among *O. niloticus* and investigation of the pathogen characters is essential to determine its significance and take the appropriate measures to prevent and control it. For our knowledge, there are no studies on *M. luteus* infection in Egypt, this is the first report proved that *M. luteus* infects *O. niloticus* in Egypt. In this study, it was found that prevalence of *Pseudomonas fluorescens* was 6.7% Pseudomonas fluorescens infections in cultured O. niloticus at New Valley province. Our results came near to El-Barbary and Hal (2017) who registered 13.8 % Pseudomonas infections among O. niloticus in El-Serw fish farm, Damietta province along the harvest season and differed with Hassan et al., (2020) who recorded 23.08% Pseudomonas infection in cultured tilapia in El-Abassa Fish Farm, Sharkia Governorate and Eissa *et al*., (2010) reported 30.8% pseudomonas infection among O. niloticus at El-Fayum province. The prevalence differences may be attributed to the difference in locality, water quality and temperature .Also the prevalence of *M. luteus* among the examined *O. niloticus* was 11.3 % This finding agreed with results of **Wanja *et al*. (2020)** who reported that incidence of *M. luteus* among the diseased cultured *O. niloticus* in Central Kenya was 10%. **Parra-Laca *et al.* (2020)** reported higher incidence of *M. luteus* (33%) in the diseased cultured *O. niloticus* at Morelos in Mexico central zone. This difference in prevalence may be attributed to the difference in water temperature, stocking density and water quality.

M. luteus has been described as a natural member of the aquatic environment and the intestinal microflora of various fish species (Akayli *et al.*, 2016 and Akayli *et al*., 2019) and as an emerging opportunistic pathogen (Pękala ***et al*. 2018)**. So, its isolation from the diseased *O. niloticus* in this study may be related to fish immunity which decreased by the effect of different stressors present in the farms. Furthermore, *M. luteus* may be a secondary pathogen in some infected *O. niloticus* where *Aeromonas hydrophila* were isolated from these samples together with *M. luteus* (data not shown). However, more studies are necessary to verify this role within the ecosystem.

Clinically diseased *O. niloticus* infected with *Pseudomonas fluorescens* showed external clinical signs including excessive black colouration of skin, scales loss, skin ulcer and focal hemorrhages, exophthalmia, fin's congestion and rot. Internally, the diseased fish had congested liver, spleen, and kidney. The recorded clinical signs agreed with clinical

signs reported by Eissa *et al*., (2010) in O. niloticus in Wadi El-Rayan Lake and El Barbary and Hal (2016) in cultured O. niloticus in Damieta province, who reported excessive mucus secretions on skin and gills, ascites with slightly protruded congested vent, hemorrhages all over the body surface, frayed and rotten fins especially tail fin. The hemorrhages all over the body may be due to the elastase enzyme secreted by the isolated bacteria damaged the blood vessels which mainly composed of elastic and collagenous fibers and hemolysin factor which has been contributed the hemorrhagic septicemia (Zhang and Austin 2005). Also, the clinical sings and postmortem lesions may be attributed to the isolated septicemic bacteria and their virulence genes where, the pathogenic bacteria might secrete extracellular products as haemolysin, cytotoxic toxins, extracellular enzymes as proteases, lipases, hyaluronidase that involved in the clinical signs or lesions development (Takahashi et al., 2014)

In this study, the infected fish showed excessive skin pigmentation or depigmentation, scales loss, skin hemorrhages, body ulcers, fins congestion and rot, slight to severe corneal opacity and exophthalmia. Internally, there were congested and sometimes enlarged liver, spleen, and kidney in addition to distended gall bladder. Most of these clinical signs and post-mortem findings were observed in *M. luteus* infections in rainbow trout (Pękala *et al*., 2018), common dentex (Akayli *et al*., 2019), gilthead seabream (Çanak and Akayli 2018) and sharpsnout seabream (Akayli *et al*., 2020**)** but skin depigmentation, congestion and erosion of the fins, corneal opacity and gall bladder distention weren't observed in these studies and this may be attributed to the differences in fish species and environmental conditions which determine infection severity.

Phenotyping is used in conjunction with genotyping to identify the bacterial pathogens **(Coquet *et al*., 2002)**. Biochemical characterizations have proved to be a valuable method for typing and differentiation of bacterial fish pathogens (Austin *et al*., 1997**)**. The findings of the present study showed that Pseudomonas was Gram-negative, motile rods with positive reaction to cytochrome oxidase, catalase, CIT, URE, MR, GEL and glucose and it was negative for OPNG, ADH, LDC, ODC H2S, IND, TDA, VP and not grow at 4 & 40^0C. Our results augmented by the results recorded by Eissa *et al.*, (2010), and El-Barbary and Hal (2016). In this study *M. luteus* isolates were Gram-positive, non-motile cocci arranged in tetrads. They produced small, smooth, spherical and bright yellow colonies on TSA. Biochemically, the isolates were homogeneous except for Arginine dehydrolase, Vogus-Proskauer and Urease tests that were variable and there were positive reactions in cytochrome oxidase, catalase, gelatine liquefaction and sucrose fermentation tests. These findings did not show any significant differences with the morphological and biochemical characteristics of *M. luteus* strains previously isolated form diseased fishes (**Aydin *et al*., 2005 and Akayli *et al*., 2020)**. The variation in any biochemical characteristic may be attributed to presence or absence of plasmid (s) that controls its metabolic trait. Results of pathogenicity test in this study proved that our *M. luteus* isolates were pathogenic to *O. niloticus*. Similar clinical signs and PM lesions were observed in naturally- and experimentally infected fish with 70.0% mortalities in the experimentally infected group. Koch's postulates were also fulfilled in this experiment where *M. luteus* was isolated and identified from the experimentally infected fish. These findings agreed with those of Pękala *et al.* (2018) who observed identical pathological symptoms in *M. luteus* infection outbreaks in farmed rainbow and brown trout, as well as in the experimentally infected ones with isolation of *M. luteus* from them. Our findings also agreed with Aydin *et al.* **(2005)** who observed similar clinical signs in naturally-and experimentally infected rainbow trout with mortality rate up to 100%.

Conclusion

This study is the first report for *M. luteus* infection in *O. niloticus* in Egypt. This bacterium was isolated from cultured *O. niloticus* at New Valley governorate, Egypt and it was identified by morphological characters, and biochemical tests. Results of this study will be a starting point for prevention and control plans of *M. luteus* infection in Egypt and others research are necessary**.**

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Cite this article.

Omnia Hamad Saleh, Mahmoud Hashiem Mohamed, Mohamed Abd El-Aziz Ahmed Abd El-Galil, Ahmed A. Elkamel and Haitham Helmy Sayed (2021). A novel bacterial infection in cultured Nile tilapia, Oreochromis niloticus in New Valley, Egypt. New Valley Veterinary Journal. 1(1): 1-7.