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Fish diseases

Isolation and Characterization of *Micrococcus luteus* from *Oreochromis niloticus* in Egypt

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ABSTRACT

Micrococcus luteus (*M. luteus*) is an emerging opportunistic fish pathogen. This study was carried out to investigate incidence of *M. luteus* infection among *Oreochromis niloticus* (*O. niloticus*) farms at Al Dakhla city, New Valley Governorate, Egypt through clinical and bacteriological examinations and to determine antimicrobial susceptibility of *M. luteus* isolates and the minimum inhibitory concentration (MIC) of silver nanoparticles (AgNPs) and zinc oxide nanoparticles (ZnONPs) against *M. luteus* isolates. *O. niloticus* samples (n=150) were collected from several farms at Al Dakhla city from March to October 2019 and they were clinically and bacteriologically examined. Suspected isolates were identified as *M. luteus* by the morphological and biochemical characteristics then by 16S rRNA gene sequencing and phylogenetic analysis where 14 isolates were identified as *M. luteus* from the examined *O. niloticus* with prevalence of 9.3%. *M. luteus* isolates were biochemically identical except in arginine dehydrolase, Vogus-Proskauer and urease tests results. *O. niloticus* infected with *M. luteus* showed excessive skin pigmentation, loss of scales, hemorrhages and ulcers on the body, congestion and rot of fins, corneal opacity, exophthalmia and congested enlarged liver, spleen and kidney. Antimicrobial susceptibility of *M. luteus* isolates revealed that they were sensitive to penicillin, ampicillin/sulbactam, amoxicillin/clavulanic acid, norfloxacin, chloramphenicol and tetracycline, while were resisantant to cefotaxime, amikacin, tobramycin, erythromycin and ciprofloxacin. Furthermore, it was found that MIC of AgNPs against *M. luteus* isolates was 100µg/ml while *M. lutues* isolates were resistant to all ZnONPs concentrations used in this study. This study reported fish infection with *M. luteus* for the first time in Egypt and its results will be a starting point for the further studies to investigate prevalence and distribution of *M. luteus* infection among fishes in Egypt and the plan of its prevention.

Keywords: Characterization, Egypt, Isolation, *Micrococcus luteus, Oreochromis niloticus*.

INTRODUCTION

Aquaculture represents an important sector of the Egyptian national income structure (Abd El Tawab *et al*., 2018) and food security in providing a cheap source of protein for the Egyptian people (Macfadyen, 2011). Nile tilapia or *O. niloticus* is the main cultured fish species in Egypt due to its economical price, palatability and easy cultivation in the rivers, ponds or dams (El-Gohary *et al*., 2020).

Bacterial fish diseases are one of the most important challenges facing aquaculture (Hamouda *et al*., 2019). They may be the most important cause for the economic losses in fish

farms and they have zoonotic threats for fish consumers sometimes (Plant and LaPatra, 2011). During the rapid growth of aquaculture sector, serious economic losses occur due to the diseases which occur because of the stress factors and changes the environmental conditions (Arda *et al*., 2005). The causative agents of these diseases are predominantly opportunistic pathogens (Akayli *et al*., 2020).

Although Gram-negative bacteria still the dominant fish pathogens, Gram-positive bacteria have been observed in the recent years (Akayli *et al*., 2020). *Micrococcus* spp. consists of Gram-positive cocci includes nine species and belongs to family *Micrococcaceae*, order *Actinomycetales* (Kocur *et al*., 2006).

Micrococcus luteus is naturally found in the aquatic environment and was isolated from the intestinal microflora of various fish species (Akayli *et al*., 2016 and Akayli *et al*., 2019). It is also isolated from the diseased fishes in some cases especially those related with stress and/or culture conditions (Akayli *et al*., 2019 and Akayli *et al*., 2020). It is supposed that *M. luteus* infection will appear more often in the future causing large economic losses in the farms (Pękalaa *et al*. 2018). *M. luteus* infected fishes exhibited enhanced skin pigmentation, damage of gills, exophthalmia, distention of abdomen and filling the abdominal cavity with ascitic fluid, pale, elongated/swollen spleen and swollen and/or watery kidney (Austin and Austin, 2016).

Data about the possible pathogenicity of *Micrococcus* spp. for human are very poor and controversial, however, they may be considered as opportunistic pathogens particularly with increasing number of the immunocompromised patients (Kocur *et al*., 2006).

Antimicrobial resistance problem is increasingly present and requires discovery of new antimicrobial agents (Talapko *et al*., 2020). Nanomaterials become increasingly investigated as an alternative for traditional antimicrobials (Seil and Webster, 2012).

Several researchers reported infection of some fish species with *M. luteus* in some countries, but to the best of our knowledge there are no previous studies on *M. luteus* infection of fishes in Egypt. Data about incidence of *M. luteus* infection among the cultured *O. niloticus* is essential to determine its importance and take the suitable measures for its prevention and control. Therefore, the present study was carried out to investigate incidence of *M. luteus* infection among the cultured *O. niloticus* at Al Dakhla city, New Valley Governorate, Egypt through clinical and bacteriological examinations and to determine the antimicrobial susceptibility of *M. luteus* isolates and MIC of AgNPs and ZnONPs against *M. luteus* isolates.

MATERIALS AND METHODS

Study area and fish sampling:

A 150 *O. niloticus* samples (38-98g in weight) showing signs of disease were collected from several concrete pond farms at Al Dakhla city, New Valley Governorate, Egypt from March to October 2019. Alive fish were collected in small tanks containing suitable volume of pond water with continuous aeration while dead fish were collected in insulated ice box containing ice. The collected fish were immediately transported to the laboratory for clinical, post-mortem and bacteriological examinations.

Clinical and post-mortem examination:

The fish collected were subjected to clinical and post-mortem examination according to Noga (2010) for detection and reporting the clinical abnormalities present externally and PM lesions. Live fish were euthanized by tricaine methanesulfonate (Sigma-Aldrich) prior to the dissection according to American Veterinary Medical Association guidelines on euthanasia (2007).

Bacterial isolation and phenotypic identification:

Under aseptic conditions, samples were taken from liver, kidney and spleen of fish by a sterile bacteriological loop and inoculated into tryptone soya broth (TSB, Oxoid, England) and incubated at 25°C for 24 hours. Inocula from this broth were streaked onto tryptone soya agar (TSA) (Oxoid, England) and incubated at 25°C for 48 hours (Akayli *et al*., 2020). The isolates recovered were preserved in TSB supplemented with 15% glycerol at -80°C (Pękalaa *et al*., 2018). The suspected isolates were identified through their morphological characteristics, Gram-staining, motility test, oxidase test and catalase test (Holt *et al*. 1994) and through API20E system (bioMerieux, France) according to the manufacturer instructions and based on criteria of Buller (2004).

16S rRNA gene sequence analysis:

Total bacterial DNA was extracted from an overnight subculture on TSA using QIAamp DNA Mini Kit (QIAGEN Inc., USA) according

to manufacturer's protocol, its concentration was measured by Nanodrop™ spectrometer (NanoDrop Technologies, Inc., USA) then it was preserved at -20°C till be used.

PCR was conducted to amplify bacterial 16S rRNA gene (1500 bp) using universal primers; F27; 5`AGAGTTTGATCCTGGCTCAG 3` & R1492; 5` TACGGCTACCTTG TTACGACTT 3`) (Weisburg *et al*., 1991) and EmeraldAmp® GT PCR mastermix (Takara, Japan). The reaction mixture was prepared in 25 µl mixture according to mastermix manufacturer's instructions where it contained mastermix (12.5 µl), primers (1 µl from each of forward and reverse), extracted DNA (3 µl) and nuclease-free water (7.5 µl). PCR was conducted in a thermocycler (Biometra, Germany) under the conditions previously described by **Pękalaa *et al*. (2018);** initial denaturation at 94°C for 2 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 1 minute followed by final extension step at 72°C for 10 minutes.

Amplicons were purified and sequenced in Eurofins Scientific (Germany). Isolates sequences were analyzed by MEGA 7.0 software and were compared with 16S rRNA genes sequence of *M. luteus* isolates available on GenBank database. Evolutionary distances were computed using the maximum likelihood method (Tamura *et al*., 2004). Phylogenetic tree based on 16S rRNA gene sequences was constructed by the neighbor-joining method (Kumar *et al.*, 2016).

Antimicrobial Susceptibility Testing:

Antimicrobial susceptibility of *M. luteus* isolates was determined by Kirby-Bauer disc diffusion method using (11) different antibiotics namely penicillin (P) (10µg), amoxicillin/clavulanic acid (AMC) (30µg), ampicillin/sulbactam (Sam) (20µg), cefotaxime (CTX) (5µg), amikacin (AK) (30µg), tobramycin (Tob) (30µg), erythromycin (E) (15µg) ciprofloxacin (Cip) (5µg), norfloxacin (Nor) (10µg), tetracycline (T) (30µg) and chloramphenicol (C) (30µg) (Oxoid, UK). Each isolate was streaked onto Mueller-Hinton agar (Oxoid, UK), then the antibiotic disks were dispensed on the plate and the inoculated plate was incubated at 25°C for 48 hours. Later, diameters of inhibition zones were measured, and results were interpreted according to the Clinical and Laboratory Standards Institute (2006).

Determination of MIC of AgNPs and ZnONPs against M. luteus isolates:

Antibacterial effect of AgNPs (20nm) (Sigma Aldreich) and ZnONPs (less than 100nm) (Sigma Aldreich) on *M. luteus* isolates were tested at various concentrations of 10, 20, 50, 100, 150, 200, 250 and 300µg/ml. These concentrations of AgNPs and ZnONPs were prepared in sterile test tubes containing 5 ml of TSB broth with using one test tube as control then they were inoculated with equal volumes (200 µl) from freshly prepared bacterial suspension of *M. luteus* diluted to 0.5 optical density (OD) and incubated at 25 °C for 24 hours. Later, the absorbances were measured at 600 nm by spectrophotometer (PG T80, UK) and a graph was plotted against OD and nanoparticles concentration. The concentration which gave the lowest OD corresponds the MIC of the used nanoparticles (Rautela *et al*., 2019).

RESULTS

Clinical and post-mortem examination: -

The infected *O. niloticus* exhibited excessive skin pigmentation, loss of scales, hemorrhages and ulcers on the body, congestion and rot of fins, corneal opacity, exophthalmia. Internally, there were congestion and sometimes enlargement of liver, spleen and kidney and distendion of gall bladder (Figs. 1, 2 and 3).

Fig. (1): *O. niloticus* naturally infected by *M. luteus* showing (a) caudal fin rot (b) body ulcer (c) scales loss.

Fig. (2): *O. niloticus* naturally infected by *M. luteus* showing exophthalmia, mild corneal opacity and hemorrhages on body.

Fig. (3): *O. niloticus* naturally infected by *M. luteus* showing congested and enlarged liver.

Prevalence of M. luteus infection among O. niloticus:

14 *M. luteus* isolates were isolated and phenotypically identified from kidney, liver and spleen of the examined *O. niloticus* with prevalence of 9.3% (14/150). Morphological and biochemical characteristics of *M. luteus* isolates were summarized in Table (1).

16S rRNA gene sequence analysis:

Analysis data of 16S rRNA gene sequencing of our *M. luteus* isolate was illustrated in Table (2) and Fig. (4). By 16S rRNA gene sequencing and phylogenetic analysis, our isolate was genotypically identified as *M. luteus* and was deposited into NCBI as *M. luteus* H210201

(GenBank accession no. MZ156875.1). As illustrated in Table (2), 16S rRNA gene sequence of *M. luteus* H210201 isolate showed similarity of 99.32%, 99.09%, 99.09% and 99.09% with 16S rRNA genes sequence of *M. luteus* DSM 20030T (GenBank accession no. LN681571.1), *M. luteus* ATCC4698 (GenBank accession no. CP035298.1), *M. luteus* NCTC2665 (GenBank accession no. MN075406.1) and *M. luteus* NCTC2665 (GenBank accession no. NR075062.2) respectively.

Antimicrobial Susceptibility Testing:

Antimicrobial susceptibility testing for *M. luteus* isolates revealed that they were sensitive to penicillin, ampicillin/sulbactam, amoxicillin/clavulanic acid, norfloxacin, chloramphenicol and tetracycline while they were resisantant to cefotaxime, amikacin, tobramycin, erythromycin and ciprofloxacin.

MICs of AgNPs and ZnONPs against M. luteus isolates:

It was found that MIC of AgNPs for *M. luteus* isolates was 100µg/ml while *M. lutues* isolates were resistant to all ZnONPs concentrations used in this study.

Table (1): Morphological and biochemical characteristics of *M. luteus* isolates isolated from the examined *O. niloticus*.

Characteristics	Result	Characteristics	Result
Colonies on TSA	Small, spherical, smooth, bright yellow	Tryptophane deaminase	-
Gram staining	+	Indole production	-
Morphology	Cocci arranded in tetrads	Vogus-proskauer	-/+
Motility	-	Gelatinase	+
Cytochrome oxidase	+	O/F of glucose	-
Catalase	+	O/F of mannitol	-
ß-galactosidase	-	O/F of inositol	-
Arginine dehydrolase	-/+	O/F of sorbitol	-
Lysine decarboxylase	-	O/F of rhaminose	-
Ornithine decarboxylase	-	O/F of sucrose	+
Citrate utilization	-	O/F of melibiose	-
H_2S production	-	O/F of amygdaline	-
Urease	-/+	O/F of arabinose	-

Table (2): Similarity between 16S rRNA gene sequence of *M. luteus* H210201and 16S rRNA genes sequence of the other maximum identical related strains.

Isolate	GenBank accession no	Identity percentage with *M. luteus* H210201
M. luteus DSM 20030T	LN681571.1	99.32%
M. luteus ATCC4698	CP035298.1	99.09%
M. luteus NCTC2665	MN075406.1	99.09%
M. luteus NCTC2665	NR075062.2	99.09%

Fig. (1): *O. niloticus* naturally infected with *M. luteus* showing (a) caudal fin rot (b) body ulcer (c) scales loss.

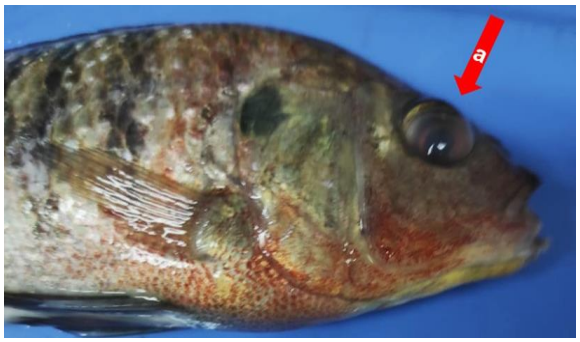

Fig. (2): *O. niloticus* naturally infected with *M. luteus* showing exophthalmia, mild corneal opacity and hemorrhages on body.

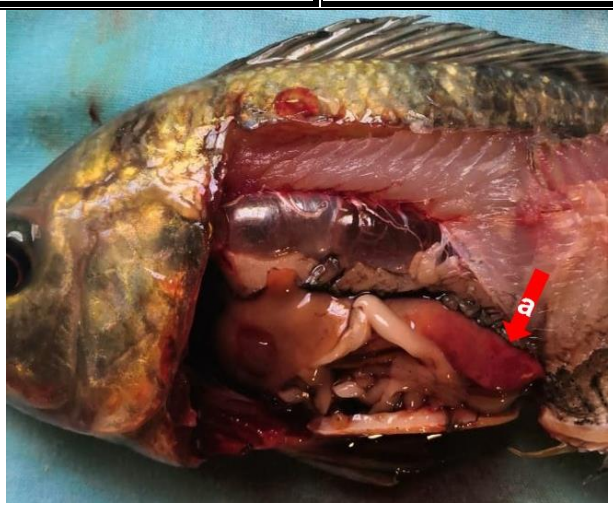

Fig. (3): *O. niloticus* naturally infected with *M. luteus* showing congested and enlarged liver.

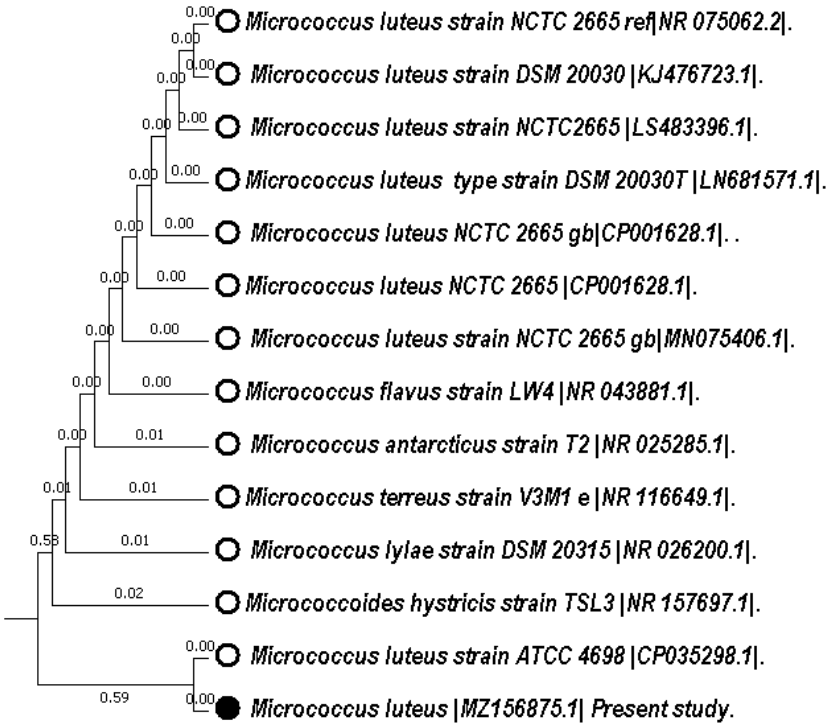

Fig. (4): Phylogenetic tree showing evolutionary relationship of *M. luteus* strain H210201 with the other maximum identical related strains on basis of 16S rRNA genes sequence evolutionary distance.

DISCUSSION

Micrococcus luteus should be considered as a potential fish pathogen (Aydin *et al*. 2005) and it is supposed that the infection will appear more often in the future causing large economic losses in the farms (Pękalaa *et al*. 2018). To the best of our knowledge, there are no previous studies on *M. luteus* infection of fishes in Egypt and this is the first report about *M. luteus* infection among fishes in Egypt. In this study, the prevalence of *M. luteus* infection in *O. niloticus* (9.3%) is almost similar to the finding of Wanja *et al*. (2020) who reported that prevalence of *M. luteus* infection among the cultured *O.* niloticus in Kenya was 10%. On the other hand, Parra-Laca *et al.* (2020) reported that prevalence of *M. luteus* infection among the cultured *O. niloticus* at Morelos in Mexico was 33%. This difference may be attributed to the differences in locality, sampling season, water quality, water temperature and/or stocking density.

In this study, the infected *O. niloticus* exhibited clinical signs and PM lesions similar to those reported by Çanak and Akayli (2018), Pękalaa *et al*. (2018), Akayli *et al*. (2019) and Akayli *et al*. (2020) in *M. luteus* infections in rainbow trout, common dentex, gilthead seabream and sharpsnout seabream respectively.

Accuracy of bacterial diseases diagnosis plays a great role in their successful control and subsequently protecting fish industry from the high economic losses and possible human health hazards (Buller, 2004) Phenotyping is used alongside serology and genotyping to identify the bacterial pathogens (El-Seedy *et al*., 2015). 16S rRNA gene sequence is an important tool for identification of microbes when used alongside the biochemical tests (Buller, 2004) and it gives a quick and accurate identification (Tringe and Hugenholtz, 2008). In this study, *M. luteus* isolates were phenotypically identified based on their morphological and biochemical characteristics and confirmed genotypically by 16S rRNA gene sequence and phylogenetic analysis.

According to data illustrated in Table (1), *M. luteus* showed morphological characters and biochemical activities similar to those reported by Austin and Stobie (1992), Aydin *et al*. (2005) and Akayli *et al*. (2020) for *M. luteus* isolated from the diseased fishes.

As shown in Table (2), on comparing 16S rRNA gene sequence of our *M. lutus* isolate H210201 with the known 16S rRNA genes sequence of *Micrococcus* spp. isolates stored in GenBank databases, it showed similarity of 99.32%, 99.09%, 99.09% and 99.09% with 16S rRNA genes sequence of *M. luteus* DSM 20030T (GenBank accession no. LN681571.1), *M. luteus* ATCC4698 (GenBank accession no. CP035298.1), *M. luteus* NCTC2665 (GenBank accession no. MN075406.1) and *M. luteus* NCTC2665 (GenBank accession no. NR075062.2) respectively. Our H210201 strain was genotypically identified as *M. luteus* based on this homology with these *M. luteus* isolates and according to Schleifer (2009) who stated that the bacteria of similarity less than 98.7% in 16S rRNA gene sequence considered to be different species.

According to results of this study, *M. luteus* isolates were sensitive to penicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, norfloxacin, chloramphenicol and tetracycline while they were resistant to cefotaxime, amikacin, tobramycin, erythromycin and ciprofloxacin. Our results agreed with findings of Çanak and Akayli (2018) who found that *M. luteus* isolates were sensitive to ampicillin and tetracycline while resistant to ciprofloxacin and also with findings of Akayli *et al*. (2020) who found that *M. luteus* was sensitive to chloramphenicol and tetracycline while resistant to ciprofloxacin while in contrast to our results, Aydin *et al*. (2005) found that *M. luteus* was sensitive to cefotaxime, tobramycin and erythromycin while resistant to oxytetracycline and chloramphenicol. These different results may be attributed to the different usage of these antibiotics in aquaculture in these areas and to difference of *M. luteus* strains.

Several reports revealed the antibacterial activity of AgNPs and ZnONPs against broad range of microorganisms (Padmavathy and Vijayaraghavan, 2008 and Dakal *et al.*, 2016). According to the available literature data, there are no previous studies on the antibacterial activity of AgNPs and ZnONPs against *M. luteus* which was investigated in this study. In this study, it was found that MIC of AgNPs for *M. luteus* was 100µg/ml while *M. lutues* isolates were resistant to all ZnONPs concentrations used in this study.

CONCLUSION

This study reported fish infection with *M. luteus* for the first time in Egypt where *M.*

luteus was isolated from the diseased cultured *O. niloticus* at New Valley Governorate, Egypt. In this study, *M. luteus* was identified phenotypically through the morphological and biochemical characteristics and genotypically through 16S rRNA gene sequence and phylogenetic analysis. *M. luteus* isolates were sensitive to penicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, norfloxacin, chloramphenicol and tetracycline. Furthermore, MIC of AgNPs for *M. luteus* was 100 µg/ml. Results of the current study will be a starting point for the further studies to investigate prevalence and distribution of *M. luteus* infection among fishes in Egypt and the plan of its prevention.

Author's contribution

All the authors contributed equally in this work. They read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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