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## Occurrence of Multidrug-Resistant Enterococci in Fresh Water Fishes

Mohamed Enany<sup>1</sup>, Yasmine H. Tartor<sup>2</sup>, Rania M. Kishk<sup>3</sup>, and Eman M. Ali<sup>4</sup>

<sup>1</sup>*Bacteriology, Immunology and Mycology Department, Faculty of Veterinary Medicine, Suez Canal University 41522, Ismailia, Egypt*

<sup>2</sup>*Microbiology Department, Faculty of Veterinary Medicine, Zagazig University 44511, Zagazig, Egypt*

<sup>3</sup>*Microbiology and Immunology Department, Faculty of Medicine, Suez Canal University 41522, Ismailia, Egypt*

<sup>4</sup>*Suez Canal University Hospital, Ismailia, Egypt*

### Abstract

The imprudent use of antibiotics in therapeutics and as growth promoters in aquaculture farms has led to the increasing prevalence of multidrug resistance worldwide. This study investigated the occurrence and resistance phenotype of enterococci from freshwater fishes. A total of 120 fishes including (70 Nile tilapia and 50 catfish) which showed signs of septicemia (opaque eye, hemorrhage on operculum and around mouth, distended abdomen, as well as hemorrhage and ulceration of the skin) were collected from freshwater farms. *Enterococcus faecalis* 54/72 (75%) was the predominant when compared to *E. faecium* 18/72 (25%). Antibiotic sensitivity testing as a way to control *Enterococcus* species infection revealed that the recovered isolates were resistant to erythromycin, azithromycin, chloramphenicol (100% each), tetracycline (91.6%) nitrofurantoin (86.1%), and meropenem (44.4%). Meanwhile, all isolates (100%) were sensitive to linezolid and ampicillin (81.9%). In conclusion, the high occurrence of *Enterococcus* species in fishes, its antibiotic resistance is diversified, indicating a higher risk of infection and raising worries about its public health significance. Antibiotic stewardship in the fish business is now required to avert massive losses in aquaculture owing to MDR enterococci.

**Key word:** Fish, tilapia, isolation, fresh, sensitivity

## Introduction

Over the last few decades, food fish supply is one of the most important food production industries has grown at an annual pace of 8.3 percent. (FAO, 2014). Disease is a major problem that affects the productivity of farmed Tilapia, as it is in any other fish culture enterprise. Despite the fact that Tilapia is a tough fish with a high disease resistance, it is susceptible to bacterial illnesses caused by *Streptococcus* and *Enterococcus* species (Chen et al., 2015; Shen et al., 2016). Enterococci is a Gram-positive, diplococcal commensal found in fish, shellfish, and other aquatic animals (Chajęcka-Wierzchowska et al., 2016; Paganelli et al., 2017).

They are naturally resistant and tolerant to a wide range of commercial antibiotics, and they can acquire drug resistance through plasmid transfer or transposing genetic sequences that confer resistance in other bacteria (Hammerum et al., 2010).

$\beta$ -lactam antibiotics, cephalosporines, aminoglycosides, lincosamides, and streptogramins are the most common antibiotics against which enterococci develop resistance. Glycopeptides (vancomycin), macrolides, tetracyclines, and phenicols all have acquired resistance (Hollenbeck and Rice,

2012). Obtaining the genes responsible for vancomycin resistance is one of the most important aspects of Enterococcus genus adaptation (Ch'ng et al., 2019). Resistance to glycopeptides is of particular concern because these antibiotics are commonly used to treat MDR enterococci and other Gram-positive bacteria (Hollenbeck and Rice, 2012).

This study describes the isolation, identification, antimicrobial sensitivity patterns of Enterococci produced from apparently healthy and diseased *Oreochromis niloticus* and *Clarias gariepinus*.

## Materials and Methods

### Samples

A total of 120 apparently healthy and diseased fishes (70 *Oreochromis niloticus* and 50 *Clarias gariepinus*) from freshwater farms were collected as random samples and transferred to the laboratory within 2 hours for macroscopically examination of gills, fins and skin and postmortem inspection of internal organs (intestine, liver, spleen, and kidney) as previously described (Austin and Austin 2007). Samples were taken from livers, kidneys, spleen, and ascetic fluid under aseptic conditions. Inoculum from internal organs was enriched in trypticase soya

broth (TSB) with adding 6.5% sodium chloride.

#### **Isolation and Identification of *Enterococcus* species**

The surface of examined organ was sterilized by using hot spatula and aseptically a loopful was taken deeply from these organs (liver, spleen, kidney, and ascetic fluid) and immediately was inoculated into trypticase soya broth (TSB) with adding 6.5% sodium chloride for enrichment. Then a loopful was taken from the broth and directly inoculated on M-Enterococcus agar medium (APHA, Washington). Presumptive colonies were subcultured on blood agar (OXOID, UK) and bile esculin agar medium (Himedia, India) then incubation at 35 °C for 48 hrs.

All purified samples were identified by studying colony growth characteristics, Gram's staining for morphology examination, and also motility of each isolate was tested. The bacterial isolation was identified according to (*Holt et al., 1994*) and sugar fermentation for sorbitol, arabinose, and mannitol (*MacFaddin, 2000*).

#### **Heat tolerance test (*Kagkli et al., 2007*).**

Approximately 1 mL of 24 hrs. culture was incubated in a 60 °C, a loopful was streaked on blood-agar after 15, 30, 45, and 60 min.

#### **Sodium chloride tolerance test (*Shewmaker et al., 2003*).**

Separate colonies of 24 hrs. culture plate were inoculated into brain heart infusion broth with adding 6.5% sodium chloride. Then incubated at 37°C for 1-3 days, to detect the ability of bacterial growth that causes turbidity.

#### **Antibiotic Sensitivity test**

Enterococcal isolates were tested for their susceptibility to different antibiotics by disc diffusion assay (*CLSI, 2021*). The antimicrobial discs (**Oxoid, UK**) were linezolid (LNZ, 5µg), Meropenem (MRP, 10 µg), Nitrofurantoin (F, 20µg), Ampicillin (AMP, 10 µg), Tetracycline (TE, 30µg), Chloramphenicol (CLM, 20µg), Azithromycin (AZT, 15µg).

The antibiotic resistance was determined according to (*CLSI, 2021*).

#### **Results**

The clinical signs and post mortem examination of the diseased fishes was unilateral or bilateral opaque eyes, removing scales, congested and ulcerated skin, or dark pigmentation, congested fins, abdomen distension, and protrusion of anus (Figures 1,2).

Post mortem examination showed congested muscles, darkened kidney, friable/ congested liver, enlarged gall bladder, darkened

spleen, and redness gills (Figure 3).

Bacteriological examination of tested samples revealed the isolation of *Enterococcus* species on M-Enterococcus agar which appeared smooth, pink or red colonies. On blood agar media: small translucent dew drops colonies with  $\gamma$  hemolysis. On bile esculin agar (BEA) medium *Enterococcus* species produced black zone around colonies. All isolates were negative for oxidase and catalase tests.

*E. faecalis* ferment mannitol and sorbitol while *E. faecium* ferments mannitol and arabinose.

The obtained results of bacteriological examination of fish samples revealed 41/70 isolates of *Enterococcus* with percentage of 58.5%, and 31/50 isolates with percentage of 62% from Nile tilapia and catfish, respectively.

The tested fish's isolates were sensitive to linezolid (100%), and ampicillin (81.9%) as revealed in Table 1. The resistance rate to meropenem, erythromycin, azithromycin, chloramphenicol, nitrofurantoin, tetracycline was (100%).

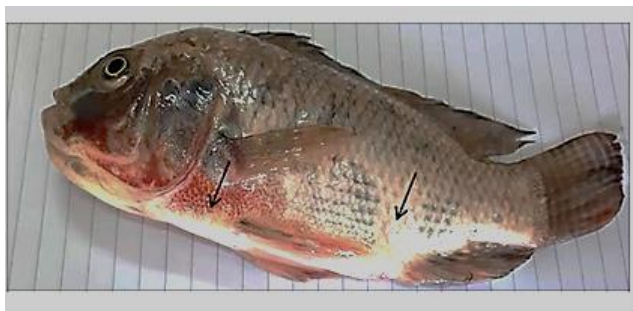


Figure (1): Nile tilapia showing hemorrhage on the skin and detached scales.



Figure (2): Catfish showing hemorrhage on body surface and ulceration of the skin

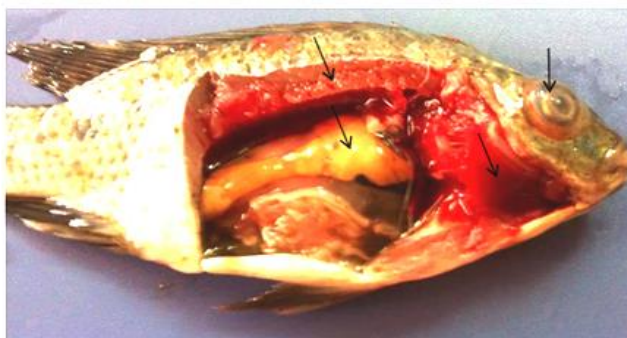


Figure (3): Nile tilapia showing redness of muscle, congested gills, friable and pale liver, and eye opacity.

Table (1): Antibiogram of fish's isolates

Antimicrobial disc	Concentration	Sensitive						Intermediate		Resistant	
		Number	%		Number	%		Number	%		
Meropenem	10	30	(41.6)		10	(13.8)		32	(44.4)		
Ampicillin	15	59	(81.9)		0	(0)		57	(79.1)		
Erythromycin	20	0	(0)		0	(0)		72	(100)		
Linzolid	15	72	(100)		0	(0)		0	(0)		
Azithromycin	30	0	(0)		0	(0)		72	(100)		
Chloramphenicol	20	0	(0)		0	(0)		72	(100)		
Nitrofurantoin	20	0	(0)		10	(13.8)		62	(86.1)		
Tetracycline	30	6	(8.3)		0	(0)		66	(91.6)		

**Discussion**

Opportunistic bacterial fish infections have been recognized as the cause of catastrophic outbreaks in aquaculture operations in recent years. *Enterococcus* species has emerged as one of the most important fish infections, having a significant impact on aquaculture techniques around the world (Paganelli et al., 2017).

In this study, bacteriological examination of 120 diseased fishes revealed the occurrence of Enterococci among 58.5% and 62% of Nile tilapia and catfish respectively. These results were higher than those obtained by Khafagy et al. (2009) who isolated enterococci from 23.76% of *Oreochromis niloticus*.

Only two species of Enterococci, *E. faecium* and *E. faecalis* were

isolated in the present study. The most common species found in fish's samples were *E. faecalis* 54/72 (75%) followed by *E. faecium* 18/72 (25%). These results corroborate with other studies (*Palanisamy et al., 2013; Tripathi et al., 2016; Novais et al., 2018*) that reported *E. faecalis* (44.3%) and *E. faecium* (13.9%) from Nile tilapia. Moreover, *Walaa et al. (2014)* detected *E. faecalis* from 45, 65% of Nile tilapia. However, much lower percentage (5%) was reported by *El-Sayed and Abou El-Gheit (2005)* from farmed and wild tilapia. Moreover, *El-Kader and Mousa-Balabel (2017)* isolated *E. faecalis* from 2.7% of Nile tilapia. It was found in this study that pure culture of enterococci was isolated on M-Enterococcus agar medium and gave characteristic type of smooth, pink or red colonies while on bile esculin agar medium *Enterococcus* is able to grow in 4% bile esculin, it tolerates 6.5% NaCl and heat 45°C which agree with the findings of other studies (*Ch'ng et al., 2019; Khafagy et al., 2009*). In this study, the antibiogram of *Enterococcus* species from fish's samples revealed that nearly all tested isolates were MDR but sensitive to linezolid (100%). This result is in harmony with previous researches (*Arias and Murray, 2012; Chen et al., 2015*) that

linezolid used to treat serious invasive infections caused by MDR enterococci.

The isolates were sensitive to ampicillin (81.9%), and meropenem (41.6%), while, (100%) resistance was found for tetracycline, nitrofurantoin, chloramphenicol, erythromycin, azithromycin, and tetracycline. These go hand in hand with *Mezalira et al. (2019)* who reported that the resistance of Enterococci against cephalosporins, aminoglycosides, erythromycin, sulphonamides, and clindamycins. On the contrary, *Rahman et al. (2017)* found that *Enterococcus* from fishes in Bangladesh was resistant to ampicillin, erythromycin, and penicillin, intermediate against ciprofloxacin, erythromycin, Gentamicin, tetracycline, and maximum resistance were noted to penicillin-G (100%). These findings disagree with those of *Araújo et al. (2020)* who reported sensitivity of *Enterococcus* species isolated from carp and Nile tilapia to ampicillin (98.7%), ciprofloxacin (64.6%), chloramphenicol (97.4%), erythromycin (63.3%), gentamycin (100%), tetracycline (78.5%), linezolid (100%), and nitrofurantoin (89.9%).

In conclusion, the high occurrence of *Enterococcus* species in fishes, its antibiotic resistance is

diversified, indicating a higher risk of infection and raising worries about its public health significance.

Antibiotic stewardship in the fish business is now required to avert massive losses in aquaculture owing to MDR enterococci.

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**حدوث الانتيروكوكس المقاومة للأدوية المتعددة في أسماك المياه العذبة**  
**محمد السيد عثاني، ياسمين حسنين ابراهيم طرطور، رانيا محمد عوض الله كشك، ايمان محمد**  
**علي علي**  
**الملخص العربي**

أدى الاستخدام الغيرحكيم للمضادات الحيوية في العلاجات وكمحفزات للنمو في مزارع تربية الأحياء المائية إلى زيادة انتشار مقاومة الأدوية المتعددة في جميع أنحاء العالم. استقصت هذه الدراسة تواجد ومقاومة المكورات المعوية المعزولة من أسماك المياه العذبة للمضادات الحيوية. تم تجميع 120 سمكة حية منها (70 بلطي نيلي و 50 سمكة قرموط) والتي ظهرت عليها علامات تسمم الدم (عين معتمة ، نزيف على الخيشوم وحول الفم ، انتفاخ البطن نزيف جلدي). لاحظنا أن انتيروكوكس فيكالييس هي العزلة السائدة 72/54 (75%) عند مقارنتها بانتيروكوكس فاشيوم 72/18 (25%). أظهر اختبار الحساسية للمضادات الحيوية كوسيلة للسيطرة على عدوى أنواع الانتيروكوكس أن العزلات كانت مقاومة للإريثروميسين ، أزيثروميسين ، كلورامفينيكول (100%) ، التتراسيكلين (91.6%) نتروفورانوين (86.1%) ، وميروبيديم (44.4%). في حين أن جميع العزلات (100%) كانت حساسة للينزوليد والأمبيسلين (81.9%). في الختام ، فإن الانتشار الكبير لأنواع المكورات المعوية في الأسماك ، ومقاومتها المتنوعة لمضادات الميكروبات تشير إلى زيادة مخاطر هذه العدوى وتثير مخاوف بشأن أهميتها على الصحة العامة. أصبح الاستخدام الحكيم للمضادات الحيوية في صناعة الأسماك إلزاميًا الآن لتجنب الخسائر الكبيرة في تربية الأحياء المائية بسبب المكورات المعوية المقاومة للمضادات الحيوية.