

Prevalence and Resistance Pattern of *Enterococcus faecalis* in Cultured *Oreochromis niloticus* at Ismailia Governorate, Egypt.

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Abstract

This study aims to examine the prevalence and antimicrobial activity of *E. faecalis* isolated from 100 *O. niloticus* that showed opaque eyes, hemorrhage, dark pigmentation in the whole body, distended abdomen, hemorrhage, and ulceration of the skin. Gills, skin, kidney, and spleen from each fish were collected for bacteriological findings. The samples were collected randomly from May to October 2022 from private fish farms located in the Ismailia government. The obtained pinpoint white colonies were confirmed biochemically and tested against different antimicrobial agents using disc diffusion assays. Isolates were examined for *16SrRNA* sequence by PCR. The prevalence of *E. faecalis* in all examined organs was 69/100 (69%). The highest prevalence rate of infection was found in the gills among the other collected organs, followed by the liver, kidney, and spleen with a percentage of 28.9%, 26.7%, 24.1%, and 20.3%, respectively. The obtained isolates showed resistance to ampicillin, tetracycline, and erythromycin. It was sensitive to linezolid, imipenem, and levofloxacin.

Keywords: *E. faecalis*, *O. niloticus*, Antimicrobial resistance.

Introduction

One of the rapidly growing industries in the world is aquaculture, which plays a

significant role in the food production sector. Giant intensification and growth are taken into consideration to fulfill an

elevated call for fish production. (Swaminathan, 2012). It represents the fastest rising agricultural industry in many countries, with freshwater aquaculture predominating the total aquaculture production. This global picture is inverted in Africa, as a central role in many local and national economies and generation of income for farming and fishing households is played by the supply of high-quality food at low cost to millions of people. (Ghanem and Haggag, 2015). Wastewater can be used to overcome the limited renewable assets of water in Egypt (Elbana et al., 2014).

Wastewater may wastewater may contain bacteria that are harmful to human and fish health. (Bixio et al., 2008). Many bacterial pathogens resorted to sewage pollution, including *E.faecalis*, in general, at hotter temperatures in the summertime (Elnaggar and El-Alfy, 2016).

According to the excessive stage of virulence in infecting *Enterococcus* bacteria, the clinical signs of *E.faecalis* are diverse. They include lethargy, swimming abnormalities, opacity and hemorrhagic in the eye, hemorrhagic patches on the operculum, around the mouth, anus, and fins, as well as dark pigmentation in the whole body (Siti-Zahrah et al., 2008; Anshary et al., 2014). Congested or faded gills and a faded friable liver, but in different instances, it became congested; there was also common

posterior kidney congestion and an enlarged gall bladder, which were reported as signs of a post-mortem examination of freshly dead fish. *E.faecalis* induced better mortalities, up to 80% in fish. (Zahran et al., 2019).

Isolates of *E.faecalis* infected fish sent to the bacteriological examinations showed non-motile, Gram-positive bacilli present in short chains. Using sheep blood agar, the bacteria were non-hemolytic and exhibited characteristic dewdrop-like colonies on Streptococcus KF agar. The colonies were sent for biochemical tests and found negative for catalase, oxidase, indole, and H₂S production, whilst they reacted positively to nitrate reduction and esculin hydrolysis tests. Also, colonies formed in the presence of 6.5% NaCl and 40% bile salts at a wide range of temperatures (25, 37, and 45 °C), but they never grew at 10 °C. (Hassan et al., 2022).

The evolution and implementation of molecular techniques were of excellent benefit and gain by facilitating precise identification, categorization of streptococci, and time savings. Consequently, the use of PCR in the species-specific analysis of the *16SrRNA* gene was of great help in the recent investigation, and it was proven that all of them are adjacent to the *Enterococcus* species. (Osman et al., 2017).

Numerous studies have reported that in aquaculture systems,

microbes are suffering from the development of multiple antibiotic resistance, which has recently increased (*Uma and Ronald, 2016*). Research findings indicated that certain strains of *E. Faecalis* have developed resistance to commonly used antimicrobials. Predominantly penicillins, tetracyclines, aminoglycosides, phenicols, cephalosporins, penicillins, and macrolides (*Ahmed and Baptiste, 2018*).

Materials and methods

Fish Sampling.

This study included different organs from 100 *O. niloticus* that showed signs of septicemia in the examined fish, including ulcers and hemorrhages on the skin, corneal opacity, detached scales, dark pigmentation in the whole body, hemorrhages at the base of fins, and abdominal distention. The samples were collected randomly from May to October 2022 from private fish farms located in the Ismailia government. Fish were transferred freshly in aerated, insulated iceboxes to the laboratory (Department of Microbiology, Immunology, and Mycology), Faculty of Veterinary Medicine, Suez Canal University, for further clinical, postmortem, and bacteriological examinations.

Isolation and identification of *Enterococci*.

Using a hot spatula, the surfaces of the examined organs were sterilized, and a loop was taken deeply from these organs (liver,

spleen, kidney, and gills) and immediately aseptically streaked on Streptococcus selective agar. The plates were then incubated to confirm the presence of *Enterococcus* strain at ± 37 °c for 48 hrs, according to *Quinn et al.2011*)

Molecular confirmation of *E. faecalis*:

Ten selected colonies were extracted using a QIAamp DNA Mini Kit (Cat. no. 51304). The primers used for *16 SrRNA* were obtained from the Midland Certified Reagent Company (Oilgos, USA). The amplified product was detected at 337 bp using the forward primer 5'

ATCAGAGGGGGATAACACTT 3' and reverse primer 5' ACTTCATCCTTGTTCTTCTC 3' (*Matsuda et al. 2009*). About one μ l of forward and reverse primers, 6 μ L of extracted DNA, and 12.5 μ l of Emerald Amp GT PCR Master Mix 2X (Code No. RR310A) were used in the reaction at a final volume of 25 μ L. The cycling conditions of the primers were 35 cycles of 94 °C for 5 minutes, 94 °C for 30 seconds, 50 °C for 40 seconds, 72 °C for 40 seconds, and a final extension at 72 °C for 10 minutes. The molecular weight of the PCR products was measured using agarose gel electrophoresis. The gel was photographed using a gel documentation system.

Antimicrobial sensitivity of *E. faecalis*.

A few colonies were transferred to a tube containing 5 ml

of sterile saline until the turbidity reached 0.5 McFarland standard tube. Dipping a sterile cotton swab into the suspension and streaking on the entire surface of a Muller Hinton agar plate (*Fingold and Martin, 1982*). The used antimicrobial drugs were: ampicillin (AMP, 10µg), vancomycin (VAN, 30µg), levofloxacin (LEV, 5µg), clindamycin (CLN, 20µg), erythromycin (ERT, 15µg), streptomycin (STP, 20µg), linezolid (LNZ, 30µg), tetracyclin (TE, 30µg), and imipenem (IPM, 10µg). After incubation, the degree of sensitivity was determined by the diameter of the inhibition zone (*CLSI, 2020*).

Results

The bacteriological examination of the recovered colonies revealed pinpoint white colonies on Streptococcus selective agar, while on blood agar colonies appeared small translucent dew drop colonies with no hemolysis and smooth, non-transparent, white spherical colonies on nutrient agar media. By Gram stain, colonies appeared to be gram-positive cocci

arranged in short chain, non-motile. The suspected colonies can grow at 6.5% NaCl, grow at 45 °C, and be negative to oxidase and catalase tests. The prevalence of *E.faecalis* in all examined organs was 69/100 (69%). In regard to the ferocity of *E.faecalis* in infected organs of *O. niloticus*, the highest prevalence rate of infection was found in the gills among the other collected organs, followed by the liver, kidney, and spleen, with a percentage of 28.9%, 26.7%, 24.1%, and 20.3%, respectively, as shown in **Table (1)**. 10 presumptively selected *E.faecalis* isolates were carried to molecular confirmation using the *16SrRNA* gene at 337 bp. as shown in (**Figure 1**). *E.faecalis* isolates susceptibility to different antimicrobial discs shown in **table (2)**. The tested isolates were sensitive to linezolid (100%), imipenem (90%), levofloxacin (84%), and clindamycin (60%) streptomycin (60%) and vancomycin (50%) other isolates were resistance to ampicillin (90%), tetracycline (80%) and erythromycin (64%).

Table (1) Prevalence of *Enterococcus* species in infected organs of *O.niloticus*:

Type of organs	No.of isolates	Percentage %
Gills	60	28.9%
Spleen	55	26.7%
Liver	50	24.1%
Kidney	42	20.3%
Total	207	100%

Table (2) Antimicrobial sensitivity results for 50 tested isolates:

Antimicrobials	Sensitive		Intermediate		Resistance	
	No.	%	No.	%	No.	%
Streptomycin	30	60	5	10	15	30
Ampicillin	5	10	0	0	45	90
Erythromycin	16	32	2	4	32	64
Clindamycin	30	60	0	0	20	40
Levofloxacin	42	84	3	6	5	10
Vancomycin	25	50	5	10	20	40
Linezolid	50	100	0	0	0	0
Tetracycline	0	0	10	20	40	80
Imipenem	45	90	2	4	3	6

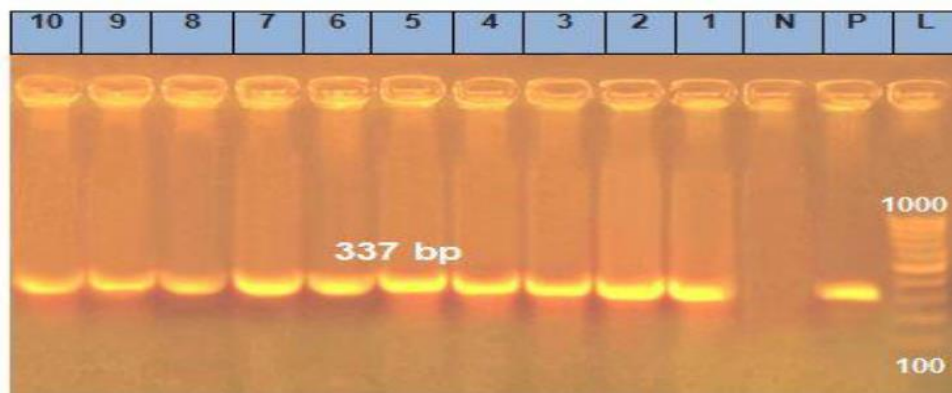


Figure (1): Agarose gel electrophoresis for 16SrRNA gene in selected 10 *E.faecalis* isolates. Lane (L): 100 bp molecular size marker, Lanes (1-10): 337 bp amplicons of *E.faecalis*. P= positive control (Kindly given by bacteriological unit, Animal Health Research Institute, Dokki). N= negative control (reaction without DNA template)

Discussion.

Recently, opportunistic bacterial fish pathogens have been identified as the main culprits behind severe outbreaks in aquaculture facilities. Among these, *Enterococcus* species have emerged as critical fish pathogens that affect aquaculture practices worldwide

(Paganelli et al., 2017). Due to the ongoing issue of sewage pollution in Egyptian fish farms, *E.Faecalis* has been linked to severe health hazards for both animals and humans. (Wang et al., 2014; Rahman et al., 2017).

The most isolated bacteria from Egyptian aquaculture was

E.faecalis (El-Refaey, 2013). The clinical signs of the examined *O.niloticus* including ulcers and hemorrhages on the skin, opacity eyes, detached scales, hemorrhages at the base of fins, and abdominal distention, nearly corresponded with those recorded by (Zeid, 2004) who reported in naturally and experimentally infected *O.niloticus* with *Enterococcus* species comparable lesions. Comparable findings were also recorded, which generalized to hemorrhage, scale detachment, and skin darkening (Elgohary et al., 2021). Severely congested liver and kidney, signs of enteric septicemia, pale necrotic gills, engorged spleen, congested kidney, inflamed heart, and distended intestine are lesions observed as postmortem lesions. These results are consistent with those of (Ikpi and Offem, 2011; Araújo et al., 2020). These variations in signs between fish may be due to the amount of *Enterococcus* that affects fish, the immune system decline in fish and the water quality of aquaculture (Wamala et al., 2018).

In agreement with a previous study (Osman et al., 2017a), the present investigation revealed the presence of pinpoint-white colonies in pure culture, showing the ability to grow at 37 °C. Moreover, regarding the morphological characters of *E.faecalis* were the same as prior data obtained with (Araújo et al., 2020) and (Rizkiantino et al., 2023). *E.faecalis*

isolates results in biochemical characterization are the same as those recorded with (Arumugam et al., 2017). *O. niloticus* infected with *E.faecalis* prevalence rate was 69%. This rate was nearly similar to prevalence rate of infection with (Hassan et al., 2022) who recorded a diffusion rate of (59%) of *E.faecalis* in *O. niloticus*. (Osman et al., 2017) reported a lower diffusion rate of *E.faecalis* (21.25%) which differs with our results and with (Elgohary et al., 2021) These differences may result from different agricultural practices, such as chicken manure added to the sample, wastewater containing animal and human waste, consumption of chicken intestines, and the use of agricultural water as a major water source, In addition, sampling period and time, environmental conditions, and the number of isolates studied cannot be ignored as factors affecting the variation of different studies (Ali, 2022).

The results shown in Table (1) reveal that the ferocity of *E.faecalis* in infected organs of *O. niloticus*, The higher prevalence rate of infection founded in the gills among the other collected organs,, then the liver, kidney, and spleen (28.9%, 26.7%, 24.1%, and 20.3%, respectively), as shown in Table (1). These consequences are nearly similar to those previously recorded by (Khafagy et al. 2009). This percentage contrasts with the results obtained by (Elgohary et al. 2021) and Fawzy et al. (2014), who

isolated *Enterococcus* bacteria from the brain of *O. niloticus*. This difference in the rate of infections could be attributed to the stage of infection, age, size, and individual variation among fish (*Elgohary et al., 2021*).

16SrRNA plays an important role in genetics as its region is highly conserved. It is considered a standard marker in phylogenetic analysis to distinguish species (*Nagpal et al., 1998*).

Recent studies show that different *enterococcal* strains isolated from different oceans elucidate the unique nucleotide location and evolution of *Enterococci* and other species (*Prichula et al., 2016*).

In addition, many publications have recently been published on the *16S rRNA* sequences of *Enterococcus* species and the phylogenetic relationships obtained from the analysis of these sequences (*Galloway-Pena et al., 2011*).

The isolates were sensitive to linezolid, as (*Arias and Murray, 2012*) recorded that linezolid was used to treat serious invasive infections caused by vancomycin-resistant *Enterococci*. The isolates were sensitive to vancomycin and levofloxacin; these results deal with (*Rahman et al., 2017*) on the contrary (*Ali, 2022*) discussed that most of tested isolates were resistant to vancomycin and levofloxacin. Isolates were resistant to ampicillin, erythromycin and tetracyclin this results were obtained (*Osman et al., 2017*). The isolates were sensitive to

Imipenem; these results were the same as (*Ali, 2022*) and were sensitive to streptomycin and Clindamycin results disagree with (*Mezalira et al., 2019*) who reported natural resistance of *E.faecalis* includes aminoglycosides and clindamycin. This change is accepted because there are various factors that can influence the antibacterial activity of the same isolate, particularly in lake water. These factors include the type of disease involved, where the bacteria came from, how it is isolated, and the type of culture medium it grows in (*Jones et al., 1986*) as well as whether it has been exposed to antibiotics before (*Kuster et al., 2014*)

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الدراسات الجزيئية علي انواع المكورات المعوية التي تصيب عادة الاسماك المستزرعة

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تهدف هذه الدراسة إلى دراسة مدى انتشار ونشاط مضادات الميكروبياتية لبكتيريا المكورات المعوية البرازية المعزولة من 100 سمكة بلطي نيلي وأظهرت عتامة العين، ونزيف، وتصبغ داكن في الجسم كله، وانتفاخ البطن، ونزيف، وتقرح الجلد. تم جمع الخياشيم والجلد والكلى والطحال من كل سمكة للحصول على النتائج البكتريولوجية. وتم جمع العينات بشكل عشوائي في الفترة من مايو إلى أكتوبر 2022 من المزارع السمكية الخاصة الواقعة في محافظة الإسماعيلية. تم تأكيد المستعمرات البيضاء الدقيقة التي تم الحصول عليها كيميائياً واختبارها ضد عوامل مضادة للميكروبات المختلفة عن طريق فحص انتشار القرص. تم فحص جميع العزلات باستخدام تفاعل البوليميراز المتسلسل. كان معدل انتشار بكتريا المعوية البرازية في جميع الأعضاء التي تم فحصها 100/69 (69%). وكانت أعلى نسبة انتشار للعدوى في الخياشيم بين الأعضاء المجمعة الأخرى، يليها الكبد والكلى والطحال بنسبة (28.9%، 26.7%، 24.1%، 20.3%) على التوالي. أظهرت العزلات المستردة مقاومة للأمبيسلين والتتراسيكلين والإريثروميسين. كانت العزلات حساسة للينزوليد والإيمبيينيم والليفوفلوكساسين.