

## ***Enterococcus faecalis* Infection in the Cultured *Clarias gariepinus* Fish from Ismailia Governorate**

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### **Abstract**

Fish are susceptible to infectious diseases brought on by a variety of phylogenetically distinct bacterial pathogens. This study was aimed at the isolation and identification of *Enterococcus faecalis* in cultured catfish (*Clarias gariepinus*) and the detection of its effect on fish culture. For this purpose, 200 moribund and freshly dead *Clarias gariepinus* were randomly collected from private fish farms in the Ismailia governorate. The clinical signs, postmortem lesions, isolation and identification of *Enterococcus faecalis* as well as molecular identification were investigated. The clinical picture displayed hemorrhages, ulcers, macerated muscles on the flank, redness at the base of fins, enlarged liver, an engorged spleen, a congested kidney, and an inflamed heart. Results of bacterial investigation revealed (53%) of *Enterococcus faecalis*. The highest prevalence was in summer (39.6%), while the lowest was in winter (11.32). The results of molecular identification revealed that all isolates harbored the EF3314 gene of *Enterococcus faecalis* at a fragment size of 566 bp. Finally, it could be concluded that *Enterococcus faecalis* is a threatening bacterial infection in *Clarias gariepinus* culture and it can be easily detected by molecular identification of the causative pathogen

**Keywords:** *Clarias gariepinus*, *Enterococcus faecalis*, PCR, EF3314 gene

### **Introduction**

Aquaculture has become a worldwide economically important industry which requires continuing research with scientific and technical developments and innovations (Bektaş *et al.*, 2017). To cover the increasing need of food in the world, an intensive production in fisheries has been a must which ended with the emergence of diseases (Food and Nations, 2016)

especially bacterial diseases (Pridgeon and Klesius, 2012) at warm temperature. The annual economic loss in the aquaculture industry due to diseases is estimated to be billions of US dollars worldwide (Klesius and Pridgeon, 2011). Bacterial pathogens can survive well in aquatic environments independently without their hosts but when invade fish tissue become responsible for

heavy mortalities (*Oladosu-Ajayi et al., 2011*) Many bacterial species belonging to at least 13 genera have been reported to be pathogenic to aquatic animals, including gram-positive bacteria such as *Enterococci* (*Sudheesh et al., 2012 and Khalil and Abdel-Latif, 2016* . *Enterococcus* spp. is considered commensal microbiota of the oral cavity, genitourinary and gastrointestinal tract of humans and animals, and is widely distributed in the environment, occurring in plants, soil and water. *Enterococcal* species have great genomic plasticity, high versatility to occupy broad ecological roles, able to grow in temperatures ranging from 10 and 45 °C, with optimum growth at 35 °C and salt tolerance (6.5% NaCl) (*Lebreton et al., 2014*). *Enterococcus* spp. may result in streptococcosis. Stress is usually one of the predisposing factors resulting in streptococcosis outbreaks such as rising in the environmental temperature, harvesting, bad handling, transportation, and poor water quality (*Francis-Floyd and Yanong, 2013*).

The present study was planned to investigate *Enterococcus faecalis* infection in the cultured *Clarias gariepinus* from Ismailia governorate through observation of the clinical picture, isolation and identification of the *Enterococcus faecalis* pathogens and molecular identification of *Enterococcus*

*faecalis* gene (*EF3314* gene) using PCR.

### **Material and Methods:**

#### **Fish:**

Two hundred moribund and freshly dead African catfish (*Clarias gariepinus*) of an average body weight of  $310 \pm 10$ g were collected freshly, randomly and seasonally from different private fish farms in Ismailia Governorate, Egypt from March 2019 to February 2020. Moribund fish were transferred to the laboratory of Fish Diseases and Management Department in polyethylene bags containing 1/3 of their volume farm water and the other 2/3 air while dead ones were kept in strong plastic bags and packed in iceboxes. Clinical examinations were done according to *Buller (2004)*.

#### **Bacteriological examination:**

Bacterial isolation was done from skin lesions, external body surface, gills, liver, and kidneys under complete aseptic conditions upon the arrival of the live and/or freshly dead fish and incubated in 9 ml brain heart infusion broth over night at 37 °C. Then a loopful from incubated broth was streaked into the surface of Kanamycin bile esculin azide agar plate (Remel™ KBE Agar) and incubated for 24-48 hrs at 37 °C. The plates were examined for observing the suspected colonies (*Benson et al., 2016*). Identification of suspected colonies was performed by Gram staining and biochemical identification (*2012*).

**Molecular characterization of *Enterococcus faecalis* (Detection of virulence gene (EF3314))**

DNA extraction were done according to manufacture instruction of the QIAamp (Qiagen, Germany). PCR reaction was performed in a total volume of 25 µl

as followed 12.5 µl PCR master mix, 5.5 µl PCR grade water, 1µl for each primer (forward and reverse) and 5µl template DNA. Specific primers used in this study for detection of virulence genes (Table 1) and PCR conditions used were illustrated in table (Table 2).

**Table (1): Oligonucleotide primer sequences.**

Gene	Primer sequence 5'-3')	Length of amplified product (bp)	Reference
EF3314	F-AGAGGGACGATCAGATGAAAAA R-ATTCCAATTGACGATTCACCTC	566	(Creti <i>et al.</i> , 2004)

**Table (2): Temperature and time conditions used for amplification of and *E. faecalis***

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
EF3314	94°C 5 min.	94°C 30 sec.	55°C 30 sec.	72°C 30 sec.	35	72°C 10 min.

**Results**

**Clinical findings**

Most diseased fish displayed hemorrhages on the operculum, mouth, pectoral and anal fins (Fig.1, A). Others showed ulcers and macerated muscles on the flank and abdomen, with mild fin erosions (Fig.1, B). Some cases showed sloughed skin, scattered hemorrhagic patches all over the body surface, and redness at the base of the fins.

Postmortem examination revealed enlarged liver, engorged spleen, congested kidney, inflamed heart and distended intestine (Fig.1, C) and slight inflamed serosa. Many cases showed severe congested liver

and kidney, signs of enteric septicemia, and pale necrotic gills.

**Bacteriological examination**

The bacteria on Kanamycin bile esculin agar gave pinpoint grayish white colonies surrounded by a black zone.

Table (3) summarizes the phenotypic and biochemical characters of the detected *E. faecalis* isolates.

**Molecular characterization of *E. faecalis***

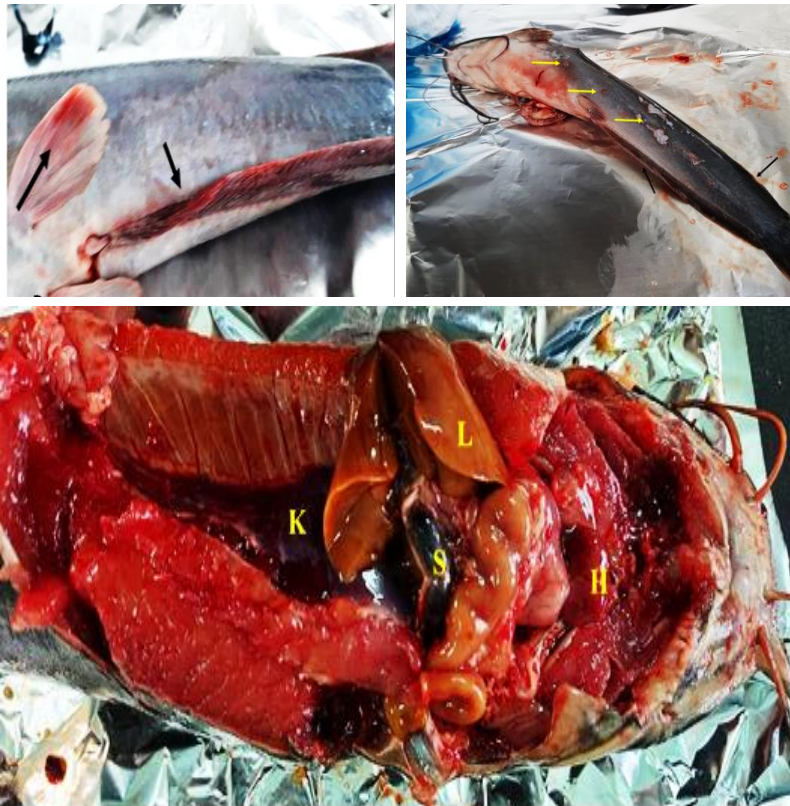
All retrieved isolates harbored EF3314 (a conserved gene of *E. faecalis*) at fragment size of 566 bp (Fig. 2).

**Prevalence of *E. faecalis* among naturally infected *C. gariepinus***

The total prevalence of the detected *E. faecalis* isolates were 53% (106/200). The seasonal prevalence was illustrated in Table (4) as it was the highest in summer (39.6%) and the lowest in winter (11.32%).

#### **Intensity of *E. faecalis* isolates in different organs.**

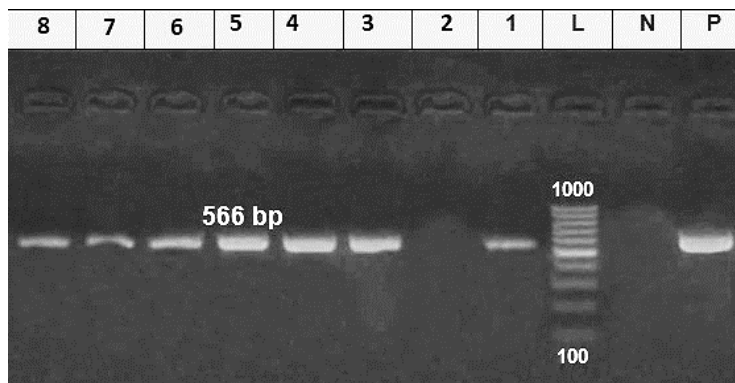
The detected isolates were 207. Liver was the highest organ harbored *E. faecalis* (43.5%) (Table, 5).



**Fig. (1):** *Clarias gariepinus* showing (A) hemorrhages on pectoral and anal fins (black arrows), (B) ulcers and macerated muscle in the flank and abdomen (yellow arrows), and mild fin erosions (black arrows), (C) enlarged liver (L), engorged spleen (S), congested kidney (K), inflamed heart (H) and distended intestine (I).

**Table (3):** Phenotypic and biochemical characteristics of *Enterococcus faecalis* isolates

Basic Characteristics	Properties
Shape	Cocci
Capsule	-ve
Motility	-ve
<b>Spore</b>	<b>-ve</b>
Pigment	-ve
Flagella	-ve
Catalase	-ve
Citrate	-ve
Gelatin Hydrolysis	Variable
Gram Staining	Gram-positive
H <sub>2</sub> S	-ve
Hemolysis	-ve
Indole	-ve
6.5% NaCl	+ve
OF (Oxidative- Fermentative)	Fermentative
Oxidase	-ve
Bile resistance	Bile resistant 40%
Urease	-ve
Voges Proskauer	+ve



**Figure (2):** PCR amplification of *EF3314* gene of *E. faecalis*. Lane marked L:100 bp DNA ladder. P: control positive. N: negative control (DNA free template). Lanes 1–8, the electrophoretic patterns of the representative retrieved isolates with expected fragments of 566 bp.

**Table (4): Seasonal prevalence of *E. faecalis*.**

No. of infected fishes	Winter		Spring		Summer		Autumn	
	No.	%	No.	%	No.	%	No.	%
106	12	11.32	19	17.9	42	39.6	33	31.13

**Table (5): Intensities of *E. faecalis* in different organs of naturally infected *C. gariepinus*.**

Bacterial species	Total No. of isolates	Gills		Kidney		Liver	
		No.	%	No.	%	No.	%
<i>E. faecalis</i>	207	55	26.6	62	29.9	90	43.5

### Discussion

Fish diseases play an important role as a limiting factor for fish production and cause heavy mortalities especially in fish hatcheries affecting fish profiled (*Eissa et al., 2016*).

Pisciculture were increasing nowadays to compensate the shortage of animal protein all over the world among which the African catfish (AFC) culture is increasing and so there is a need to pursue and advance production techniques for its culture (*Onyekwelu et al., 2020*).

In this study, the observed clinical signs in the naturally infected *Clarias gariepinus* with *E. faecalis* infections displayed hemorrhages on operculum, mouth, pectoral and dorsal fins. Other cases showed ulcers and macerated muscle on the flank and abdomen, with mild fin erosions. Some cases displayed petechial hemorrhage and erosions on body surface and hemorrhagic fins. These results are agreed with those obtained by *Cetinkaya et al.*

(2000), *Araújo et al. (2020)*, *Rizkiantino et al. (2020)*, and *Elgohary et al. (2021)* who noted that clinical signs of enterococcus infection in fishes were generalized hemorrhage, scale detachment and skin darkening. These results may attributed to that the bacteria are capable for causing mild lesion changes; however, physiologically, it is suspected that the fish can neutralize the infectious process started by the bacterium (*Abou El-Gheit, 2005*).

Most of the observed postmortem findings in the naturally infected *C. gariepinus* with the bacterial diseases were enlarged liver, engorged spleen, congested kidney, inflamed heart and distended intestine. These results are matched with those obtained by *BRR and Basyoni (2005)* and *Ikpi and Offem (2011)*. These lesions were mainly attributed to the ability of the invading pathogen to multiply inside the host intestine, inducing hemorrhages. Some cases showed slight inflamed serosa. These results

are matched with those obtained by *Oladosu-Ajayi et al. (2011)*, *Udeze et al. (2012)*, and *Das et al. (2018)*. While many other cases showed severe congested liver and kidney, signs of enteric septicemia, and pale necrotic gills. These results are in agreement with *Horsley et al. (2013)* and *Araújo et al. (2020b)*.

All *E. faecalis* isolates were Gram-positive cocci arranged in chains. These results are similar to those recorded by *Araújo et al. (2020a)*, *Osuntokun et al. (2020)*, and *Rizkiantino et al. (2020)*. The bacteria on Kanamycin bile esculin agar gave pinpoint grayish white colonies surrounded by a black zone. This is in agreement with *Elgohary et al. (2021)*.

Regarding the biochemical characterization of *E. faecalis* isolates, they were catalase and oxidase negative, D glucose fermentative, and methyl red, Voges proskaur and indole negative. They grew well in presence of 6.5% NaCl and were positive for glucose fermentation. These results are similar to those recorded by *Arumugam et al. (2017)* and *Sakandar et al. (2018)*.

Regarding the results of PCR, the morphologically and biochemically identified isolates were harbored a virulent gene (EF3314 gene) and gave fragments size of 566 bp., so they confirmed to be *E. faecalis*. The EF3314 gene was always present and specific for *E. faecalis* strains from human, food and animal origin. This result is in agreement

with that reported by *Abou Zeid et al. (2019)* who detected virulent genes from 8 *Enterococcus faecalis* isolates which were (esp gene) from 3 isolates, (asa1 gene) from 7 isolates and (EF3314 gene) from all isolates.

Total prevalence of *Enterococcus faecalis* in this study was 53%. This result is higher than that of *Torky et al. (2006)* and *Udeze et al. (2012)* who recorded a total prevalence of 22.33%, 18.6% respectively. On other hand, these result is nearly similar to that obtained by *Araújo et al. (2020a)* who found the prevalence of *Enterococcus faecalis* as (44.3%) .

Regarding the seasonal prevalence of *E. faecalis*, summer was the highest season of infection (39.6%) followed by autumn (31.13%) then spring (17.9%) and winter (11.23%). These results are in agreement with *Abdel-Naiem (2019)* who recorded the same bacterium in the summer (76%), followed by autumn (68%), then spring (52%) and winter (40%). This may be due to the enterococcus causes septicemia to all fish in the farm primarily during late spring and early summer when water temperature is high.

Regarding the intensity of infection in different organs, liver was the most prominent affected organ 43.5%, followed by kidney 29.9%, then gills (26.6%). In contrast to our findings, *Abdel-Naiem (2019)* recorded the highest prevalence of *Enterococcus faecalis* in gills

(93.2%) followed by liver (64.4%) and kidney (66.1%).

### Concussions

*Enterococcus faecalis* infection flourished in *Clarias garipinus* fish farms during summer season so, adjustment of water criteria especially temperature has become a must in *Clarias gariepinus* aquacultures to avoid stresses which indeed predispose for bacterial diseases. Liver, kidney and gills are affected organs with *E. faecalis*. Liver was the highest organ harboring bacteria followed by kidney then gills. So, we recommend using immunostimulants from the onset of the farming period to avoid *E. faeclais* in *Clarias gariepinis* farms.

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### الملخص العربي

## عدوى المكورات المعوية البرازية في أسماك القرموط المستزرع من محافظة الإسماعيلية

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الأسماك عرضة للإصابة بالأمراض المعدية التي تسببها مجموعة متنوعة من مسببات الأمراض البكتيرية المتميزة نسبياً. هدفت هذه الدراسة إلى عزل وتعريف بكتيريا *Enterococcus faecalis* في القرموط المستزرع (*Clarias gariepinus*) والتعرف على تأثيرها على الاستزراع السمكي. لهذا الغرض، تم جمع 200 نوع من أنواع كلارياس غاريبينوس المحتضرة والميتة حديثاً بشكل عشوائي من المزارع السمكية الخاصة في محافظة الإسماعيلية. تم فحص العلامات السريرية وأفات ما بعد الوفاة وعزل والتعرف على *Enterococcus faecalis* وكذلك جين التعريف الجزيئي. أظهرت الصورة السريرية نزيفاً وتقرحات وعضلات متعسنة على الخاصرة واحمرار في قاعدة الزعانف وتضخم الكبد واحتقان الطحال واحتقان الكلى والتهاب القلب والأمعاء المنتفخة. أظهرت نتائج التحاليل الجرثومية (53%) من بكتيريا *Enterococcus faecalis*. كان أعلى انتشار في الصيف (39.6%)، بينما كان أدنى انتشار في الشتاء (11.32%). أظهرت نتائج التعريف الجزيئي أن جميع العزلات تحتوي على جين EF3314 من *Enterococcus faecalis* بحجم شظوية 566 زوج قاعدي. أخيراً، يمكن الاستنتاج أن *Enterococcus faecalis* هي عدوى بكتيرية مهددة في ثقافة *Clarias gariepinus* ويمكن اكتشافها بسهولة عن طريق التحديد الجزيئي لمسببات الأمراض