

ISOLATION, IDENTIFICATION, MOLECULAR, AND HISTOPATHOLOGICAL INVESTIGATIONS OF TWO PATHOGENIC *Enterococcus* SPECIES FROM TILAPIA IN EGYPTIAN FARMS

Gamal A. El-Nobi, Mohamed Hassanin, Mohamed El-Hady, Shimaa Aboshabana*

Department of fish disease and management, Faculty of Veterinary Medicine, Zagazig University, Egypt

*Corresponding author, E-mail: drshimaa07@yahoo.com

Abstract: The rapid increase in global aquaculture have exposed many diseases in aquaculture field, enterococci are one of them. Tilapia is one of the most important and economic fish species in the world. Hundred and thirty diseased farmed Nile tilapia (*Oreochromis niloticus*) had been investigated for Enterococci collected from different farms in Abbassa, Sahl Elhusseineya, Altal alkabir, Bahr Albaqar, Al Manzalah, Ismailia, Port said during both spring and summer seasons. Two species were identified by automated identification and antibiotic sensitivity testing system (ID&AST) as *E. faecalis* and *E. faecium*. In vitro antibiotic susceptibility testing for these two species showed high susceptibility to ampicillin, penicillin, streptomycin, and gentamycin while, *E. faecalis* was sensitive to vancomycin but *E. faecium* was resistant to it. These species were confirmed by 16s rRNA as Enterococcus. The two species showed sensitivity and resistance to many antibiotics. Ninety fish with an average weight 50-250 g were exposed to pathogenicity test. Fish were randomly divided into three groups, each contain thirty fish then divided into triplicate (10 fish per replicate). Fish of the first and second groups were intraperitoneally injected with 0.2 ml of bacterial suspension of the 2 species, with comparison of a control group they showed high pathogenicity level (77% for *E. faecium* and 73% for *E. faecalis*), also the histopathology revealed vascular congestion and infiltration with inflammatory cells in examined organs tissues (brain, liver, kidney, spleen and heart). In conclusion, *E. faecalis* and *E. faecium* were the most common subspecies of Enterococci showing high pathogenicity for Nile tilapia fish in Egypt.

Key words: identification; Tilapia; *Enterococcus*; pathogenicity; histopathology; PCR

Introduction

Aquaculture is one of the fastest growing food production sectors globally (1, 2). The development of intensive aquaculture has led to the emergence of various bacterial diseases (2). Bacteria are the leading causative agents of diseases in freshwater fishes all over the world (3). *Aeromonas*, *Edwardsiella*, *Pseudomonas*, *Flavobacterium*, *Vibrio*, and *Streptococcus* are major genera of fish pathogens causing diseases in different tropical freshwater fishes (4, 5).

The genus *Streptococcus* is large and complex, accommodating a wide range of Gram-positive bacteria (6). The most relevant *Streptococcus* spe-

cies that cause disease in the tilapia farming globally are *S. iniae*, *S. agalactiae*, *S. dysgalactiae* and *Lactococcus garviae* (7, 8, 9). Several biotypes have been isolated from fish and the most pathogenic are those belonging to D serogroup, otherwise known as the Enterococci (6).

According to Lancefield Group, *Enterococcus faecalis* is a streptococcal bacterium classified into Group D Streptococcus based on bacterial serological groups and other species, such as *E. faecium* and *Streptococcus bovis*. This bacterium is Gram-positive, catalase negative, and γ hemolytic if cultured on a 5% sheep blood agar medium (10).

Enterococcus species has emerged as one of the important fish pathogens, which severely impacts aq-

uaculture practices worldwide (11), causing economically important losses in both freshwater and marine cultured fish (6). Enterococci are part of the enteric microflora and are indicators of fecal contamination of the aquatic environment (6). Several *Enterococcus species* have been implicated in disease outbreaks in aquaculture facilities worldwide, including Egypt (11, 12, 13). *E. faecium* and *E. faecalis* are the most frequently encountered enterococcal species (14, 15).

Among the freshwater species infected, Nile tilapia (*Oreochromis niloticus*) is one of the highly susceptible species (6, 13). Clinical signs recorded on infected fish include septicemic signs, lethargy, anorexia, exophthalmia, abdominal distension and hemorrhage on the skin and base of fins (11, 13). Furthermore, degenerative, and necrotic pathologic changes were observed in the internal organs of infected fish (13, 16).

Since *Enterococcus species* has been recognized as a causative agent of hemorrhagic septicemia in fish with high mortalities (13), this study was conducted to isolate and identify pathogenic *Enterococcus species* associated with mass and/or chronic mortalities of cultured Nile tilapia (*Oreochromis niloticus*). Furthermore, histopathological alterations following experimental infection were recorded in the organs of infected fish.

Material and methods

Fish sampling

One hundred and thirty Nile tilapia (*Oreochromis niloticus*), weighing 50-250 g, were collected from some fish farms with mass and/or chronic daily mortalities. The samples were collected from different farms in Abbassa, Sahl Elhusseineya, Altal alkabir, Bahr Albaqar, Al Manzalah, Ismailia, Port Said during both spring and summer seasons. Fish were transported alive to the Fish Diseases and Management laboratory at faculty of Veterinary Medicine, Zagazig University, Egypt. Fish were exposed to clinical and bacteriological examination according to Osman et al. (9). The whole study was approved by ZU-IACUC committee (no: ZU-IACUC/2/F /137/2020).

Bacteriological examination

Isolation and biochemical identification

Samples, aseptically taken from brain, and different organs of dissected fish, were inoculated in

tryptic soya broth (Oxoid, USA) and incubated at 30°C for 24 hours followed by streaking on nutrient agar (Himedia, India) supplemented with 5% defibrinated sheep blood then suspected colony were streaked Edward media for 24-48 hours at 30° c (Himedia, India) for isolation of pure streptococci and enterococci colonies. Conventional bacteriological identification including gram staining, catalase and oxidase production were applied to the isolated bacterial colonies for primary characterization. The suspected bacterial isolates were inoculated into semisolid nutrient agar tubes and incubated at 30°C for 24 hours for motility testing and to be preserved for further identification (17).

Fifteen suspected isolates were selected to be biochemically identified using Automated identification and antibiotic sensitivity testing ID&AST System, MA120 (Render, China). Streptococcus/Enterococcus ID&AST (CAT No: MA120-SE) identification kits were used for the identification of the isolated bacteria according to the manufacturer's instructions.

Molecular characterization of the bacterial isolates

Three randomly selected representative isolates were used for molecular identification using polymerase chain reaction (PCR). Genomic DNA was extracted from the bacterial colonies using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. PCR identification of the selected isolates was conducted in T3 thermocycler (Biometra, Germany) using *Enterococcus* genus-specific primers for the 16S rRNA gene (forward primer 5'ATCAGAGGGGGATAACAC-TT3', and reverse primer 5'ACTCTCATCCTTGTTCCTC-TTC3' as described by (18)). The cyclic conditions included primary denaturation at 94°C for 5 min and 35 cycles comprising denaturation at 94°C for 30 sec, annealing at 50°C for 40 sec, extension at 72°C for 40 sec, and a final extension at 72°C for 10 min. Amplified PCR products were visualized in a gel documentation system to determine the size of the PCR amplicons (337 bp). A standard 100 bp DNA ladder (Fermentas, USA) was used as a molecular marker.

Antibiotic sensitivity assay

After microbial identification using automated identification and antibiotic sensitivity testing system ID&AST System, MA120 (Render, China).

Streptococcus/Enterococcus ID&AST (CAT No: MA120-SE) we performed antibiotic sensitivity testing for the isolated species.

Experimental pathogenicity testing of the isolated bacteria

Ninety apparently healthy Nile tilapia (with an average body weight of 45 ± 5 g), obtained from the Fish Research Unit at Faculty of Veterinary Medicine, Zagazig University, were used to study the pathogenicity of two isolated and identified *Enterococcus* species (*E. faecium* and *E. faecalis*). Fish were kept in glass aquaria and acclimatized to the laboratory condition for two weeks. Fish after being acclimatized were randomly divided into three groups, each contain thirty fish then divided into triplicate (10 fish per replicate). Fish of the first and second groups were intraperitoneally injected with 0.2 ml of bacterial suspension containing 3×10^8 CFU/ml of *E. faecium* and *E. faecalis*, respectively according to Zahran et al. and Fawzy et al. (13, 16). The third group was kept as a control and intraperitoneally injected with 0.3 ml of physiological saline. All groups fed three times daily using basic diet formula (Table 1) at a rate of 5% of their biomass and were observed for 4 weeks post-inoculation. The mortalities, clinical signs and postmortem lesions were recorded according to Zahran et al. (13).

Histopathological examination

Brain, liver, kidney, spleen, and heart specimens, collected from freshly dead fish, were immediately fixed in 10% neutral buffered formalin, and then processed for staining of tissue sections

with hematoxylin and eosin (H&E) according to Suvarna et al. (19). Stained sections were inspected using light microscope for any pathological changes in the examined tissues.

Results

Clinical observation of naturally infected fishes

Clinical examination of diseased *O. niloticus* revealed signs of septicemic eye lesion in the form of unilateral or bilateral eye opacity and exophthalmia (Figure 1A), skin lesions as detached scales, ulcers, hemorrhage, or dark discoloration, hemorrhages at the base of fins, abdomen in some cases distended with ascites, congested protruded anal opening (Figure 1B). Postmortem examination of diseased fish showed enlarged spleen, hemorrhagic or pale liver, hemorrhagic kidney (Figure 1C), and brain also showed hemorrhagic condition (Figure 1D).

Isolation and identification of bacterial isolates

Seventy-seven suspected isolations were isolated from the examined diseased *O. niloticus*. The suspected colonies on nutrient sheep blood agar 5% showed greenish hemolysis (alpha hemolysis) (Figure 2A). The colonies on Edward's media at 27-30 °C for 48 hours showed very small, opaque, rounded with entire edges or creamy rounded, large colonies (1-2mm). The isolated colonies were non motile gram-positive cocci arranged in pairs and some-times short chains (Figure 2C), oxidase negative and catalase negative. Regarding to these results the number of isolates suspected to be Enterococci or streptococci were 77 isolates (Table 2).

Table 1: Basal diet formula for fish nutrition

Ingredients	Percentage of diet
Ground yellow corn	26.5
Soy bean meal	22
Fish oil	5
Meat meal	20
Fish meal	25
Mineral and vitamin mixture (premix)*	1.5
Calculated chemical analysis	
Crude protein	39.9
Crude lipid	10.89
Crude fiber	3.68
Ash	9.11
Moisture	10.58

*Minerals mix: Each kg contains manganese 60g, iron 80g, copper 5g, zinc 40g, selenium 0.15 and iodine 0.35 g. Vitamins mix. Provide (g, mg or LU kg diet) Vit. A 5000 LU, D, 2,000 LU, E 100mg, K, 10.0 mg. C 1,000 mg B1 10mg, B2 15.0 mg, B6 7.5mg, B12 0.1mg, Biotin 0.2mg, Folic acid 0.4 mg, choline 10g inositol 3,000.0 mg, pantoic acid 500mg, Nicotinic acid 100mg, P-Aminobenzoic acid 50,0mg

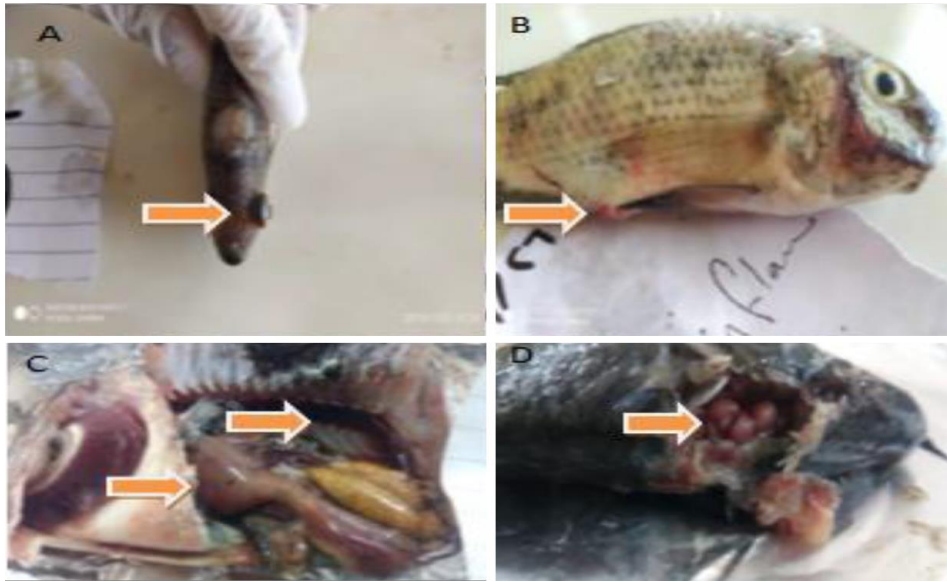


Figure 1: Naturally infected Nile Tilapia with Enterococci infection showing unilateral exophthalmia (A), protruded inflamed anus (B), enlarged congested liver and kidney (C), and brain hemorrhages (encephalitis) (D)

Table 2: phenotypic and biochemical characterization of enterococci and streptococci

Characterization tests	Enterococci	Streptococci
Hemolysis on blood agar	Alpha hemolysis (incomplete)	Beta hemolysis (complete)
Shape of colonies on Edwards media	very small, opaque, rounded or creamy rounded, large colonies	very small, dew like or creamy rounded, rounded
Catalase test	negative	negative
Oxidase test	negative	negative
Gram stain	Gram positive arranged in pairs or short chains	Gram positive arranged in short or long chains
Motility	Non motile	Non motile

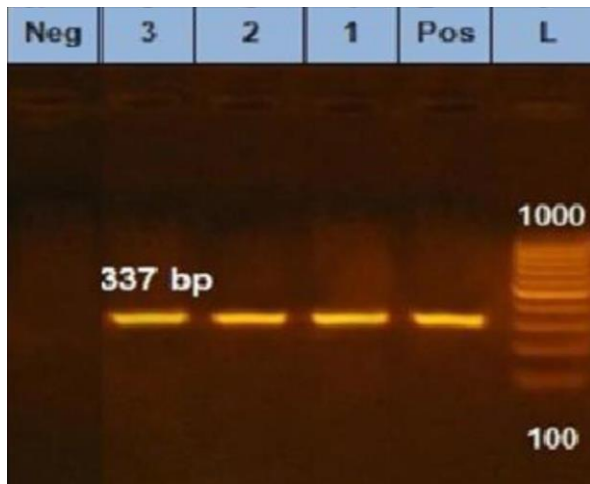


Figure 2: Agarose gel electrophoresis of *Enterococci spp* showing: L: 100 bp DNA ladder (marker) (size 1000bp). Pos: positive control. Neg: negative control. Lanes: 1 to 3 amplifications of 337 bp of 16 sRNA gene of *Enterococci spp.* isolates

Out of the 15 selected from 77 suspected bacterial isolates, 10 isolates were identified as Enterococci species (four *E. faecium* and six *E. faecalis*) and five of them were other species of streptococci (*Streptococcus agalactia*).

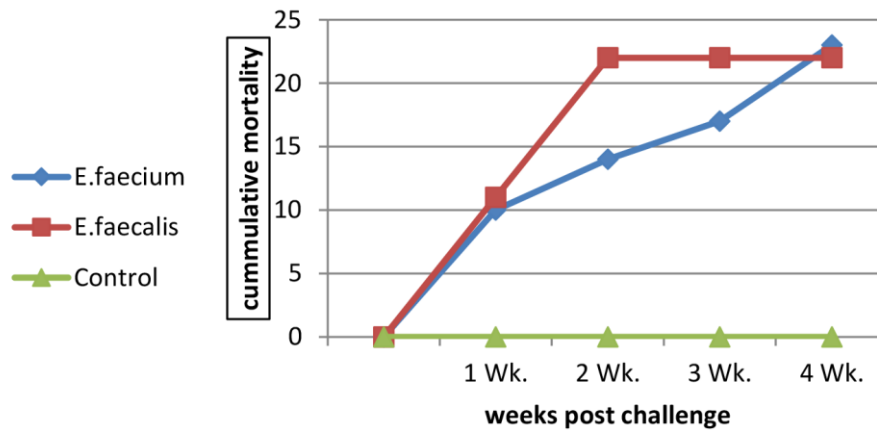
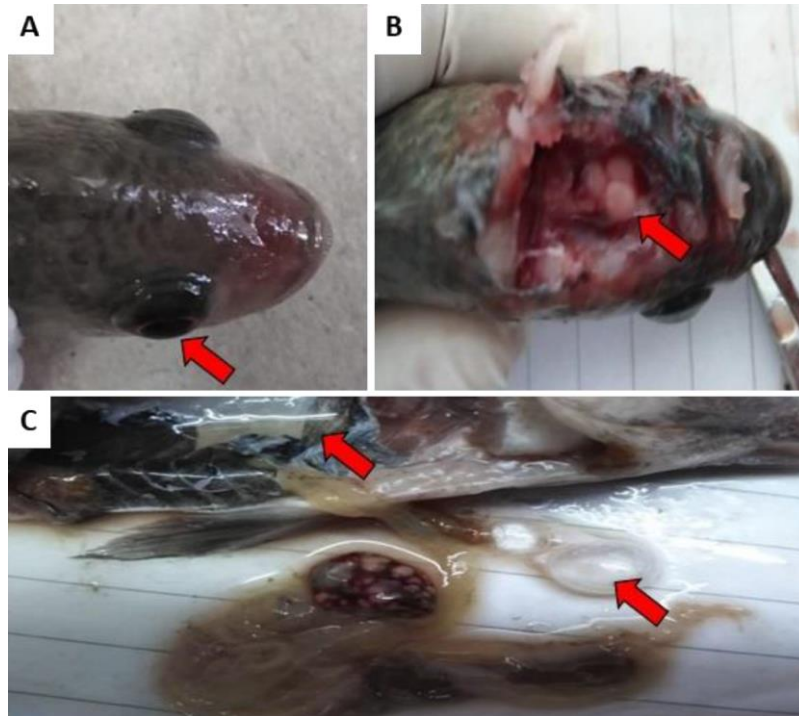
Molecular characterization

Regarding the samples identified as Enterococci by Automated ID&AST System, MA120 (Render, China) some samples confirmed by PCR to 16 sRNA gene for Enterococci at 337 bp (Figure 2).

Table 3: Results of antibiotic sensitivity test of *E. faecium* and *E. faecalis*

Antibiotic	<i>E. faecium</i>	MIC	<i>E. faecalis</i>	MIC
Ampicillin	S	≤0.12	S	≤1
Penicillin	S	≤0.06	S	≤0.5
Streptomycin	S	≤1000	S	≤1000
Gentamycin	S	≤500	S	≤500
Erythromycin	S	≤0.12	I	=2
Linezolid	S	≤1	S	≤1
Doxycycline	S	≤0.25	I	=8
Vancomycin	R	R	S	=1

MIC: minimum inhibitory concentration. S: sensitive. I: intermediate resistance. R: resistant

**Figure 3:** The cumulative mortality of Nile tilapia experimentally infected with pathogenic *Enterococcus faecium* and *E. faecalis* strains during the 4 weeks post infection**Figure 4:** Experimentally infected Nile Tilapia fish expressing disease symptom, A- Unilateral exophthalmia b- Encephalitis in brain, and C-Inflamed spleen with focal caseation

Antibiotic sensitivity assay

Susceptibility of these isolates was assessed against 8 antibiotics shown in Table (3). Both species showed good sensitivity to ampicillin, penicillin, streptomycin, gentamycin, linezolid. *E. faecium* showed sensitivity to erythromycin, doxycycline while was resistant to vancomycin. Regarding *E. faecalis* was sensitive to vancomycin while intermediate sensitivity to erythromycin and doxycycline.

Clinical signs and postmortem findings of experimentally infected O. niloticus

Clinical signs and postmortem findings of experimentally infected fish (Figure 4) were similar to that of naturally infected fishes. The experimentally infected *O. niloticus* showed restlessness; loss of appetite, loss of equilibrium, nervous manifestation, respiratory distress, and reduced activities as there were loss of any reflex action. Also, fish showed fin rot, loss of scales, emaciation, and dark body coloration. Signs of septicemia appeared as erythema and hemorrhages on mouth, bases of fins, operculum, around anal opening, through 3 weeks post inoculation fish showed ulceration on body and slight unilateral exophthalmia (Figure 4A). After dissection, the infected fish showed generalized hyperemic appearance with congestion of all internal organs as brain appeared inflamed (encephalitis) (Figure 4B). Liver appeared friable with distended and enlarged gall bladder with bile, congested spleen with focal caseation, in addition to that serosanguinous fluid appeared in abdomen (ascites) (Figure 4C).

Histopathological findings of experimentally infected O. niloticus

Enterococcus faecium

Examined sections of cerebrum revealed congestion of the cerebral blood vessels, neuronal and axonal degeneration, demyelination, vacuolations, and multifocal malaciatic areas. Cerebellar tissue showed Purkinje cells degeneration and necrosis, molecular and medullary axonal degeneration, demyelination, and gliosis (Figure 5A* and A**). Liver showed hepatic degeneration, periportal vacuolations and macrosteatosis, focal periportal proliferation and aggregation of melano-macrophages (Figure 5B). Kidney showed congestion of renal blood vessels, perivascular edema, hemorrhages, tubular degeneration, glomerular lobulation and shrinkage,

mild interstitial lymphocytic infiltration and marked proliferation, and aggregation of melano-macrophages (Figure 5C). Multi-focal necro-granulomatous lesions were seen replacing the splenic parenchyma, represented by central necrosis entangling dead neutrophils and lymphocytes and surrounded by large number of macrophages, lymphocytes, and neutrophils. The surrounding sinusoids appeared congested, and the lymphoid tissue of the white pulp was atrophied (Figure 5D). Toxo-pathologic changes were seen in cardiomyocytes appeared degenerated (vacuolated and hyalinized) or atrophied due to presence of interstitial edema and some were necrotic (myomalacia). The blood vessels were mildly congested, and a few round cells were detected between the affected muscle fibers (Figure 5E).

Enterococcus faecalis

As regarding *enterococcus faecalis*, the brain revealed peculiar histo-morphologic changes with an outstanding necrosis of the neurons, purkinje cells and granular cells of both cerebral and cerebellar counterparts. The cerebral blood vessels were moderately dilated and surrounded by inflammatory fluid rich in lymphocytes and polymorph cells. The nerve tracts, axons and neuropils were vacuolated, demyelinated or degenerated and fragmented. Multifocal malaciatic areas were encountered elsewhere in the cerebral and cerebellar tissue. Focal astrogliosis and microgliosis were also seen (Figure 6A). Regarding liver, there were marked peri-portal and par-central hepatocellular degeneration vacuolation and macrosteatosis. The hepato-portal areas showed characteristic degeneration and dissociation of the pancreatic tissue and proliferative aggregation of the melano-macrophages (Figure 6B). Nephrotoxic lesions were encountered in this group as, tubular degeneration, tubulitis, glomerular atrophy, interstitial hemorrhages, lymphocytic infiltrations and aggregations and melano-macrophages proliferation. A few renal tubules were cystic (Figure 6C). Spleen showed characteristic proliferative aggregations of melano-macrophages, both perivascular and interstitial were detected. The blood vessels were markedly congested and dilated, and the lymphoid tissue was clearly depleted (Figure 6D). In heart, there were interstitial oedema, hemorrhages, myocardial degeneration and atrophy beside focal myomalacia were encountered (Figure 6E).

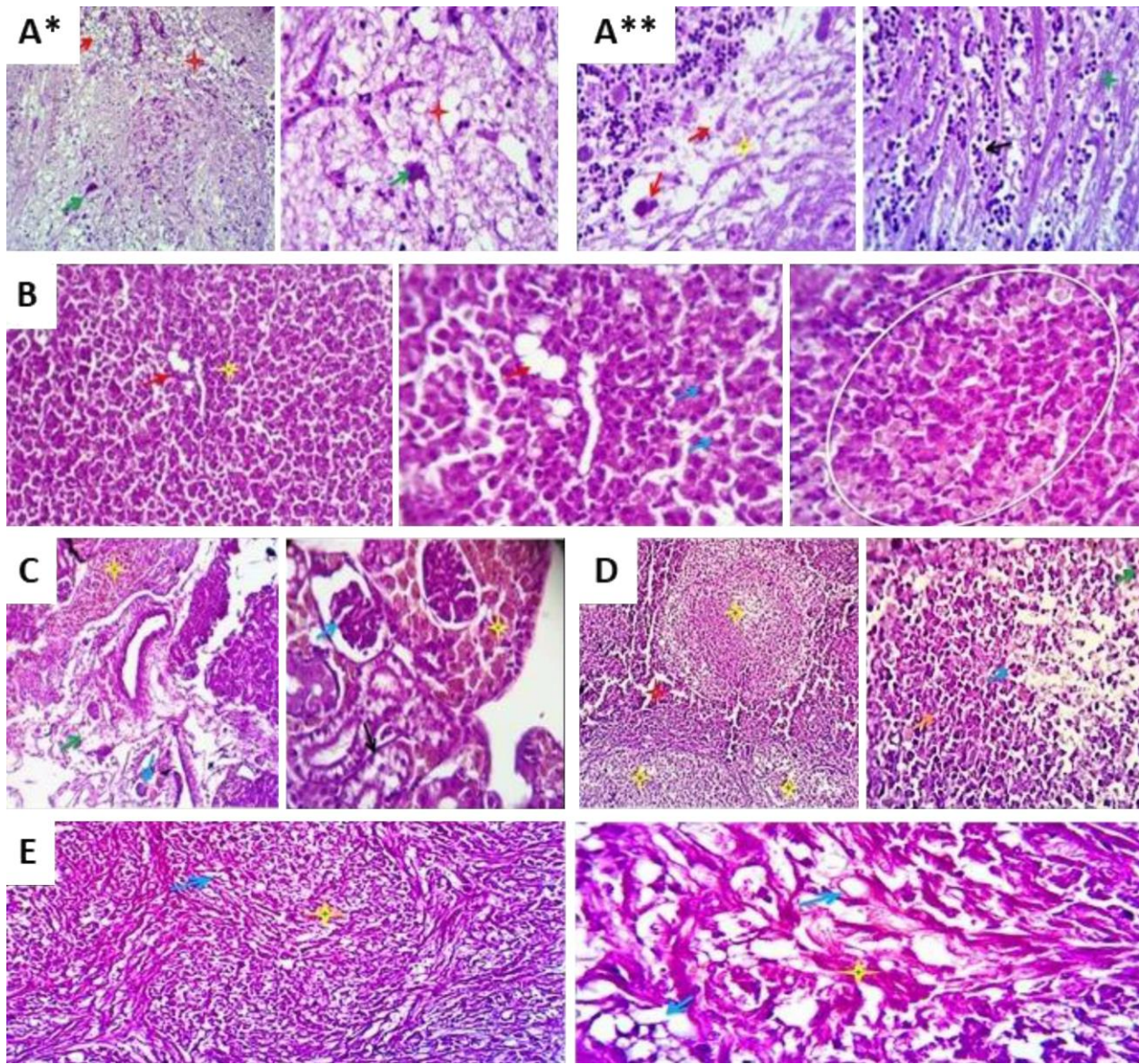


Figure 5: Photomicrograph from *Tilapia nilotica* fish infected with *Enterococcus faecium* showing

A*and **) Brain: congestion of the cerebral blood vessels (orang arrow), neuronal and axonal degeneration, demyelination, vacuolation and multifocal malaciatic areas (red and green arrows and red stars) (H&E X100, 200 and 400). B) Liver: focal hepatic degeneration, periportal vacuolations (blue arrows), macro-steatosis (red arrows), characteristic focal periportal proliferation and aggregation of melano-macrophages (Circle) (H&E X200, 400). C) Kidney: congestion of renal blood vessels, perivascular edema, hemorrhages (green arrow), tubular degeneration (hydropic) and glomerular lobulation and shrinkage (black and blue arrows) beside mild interstitial lymphocytic infiltration and marked proliferation and aggregation of melano-macrophages (yellow stars) (H&E X200, 400). D) Spleen: multi-focal necro-granulomatous lesions (yellow stars) replacing the splenic parenchyma, each represented by central necrotic tissue entangling dead neutrophils and lymphocytes (blue arrow) and surrounded by large number of macrophages, lymphocytes and neutrophils and melano-macrophages (orange and green arrows). The surrounding sinusoids appears congested, and the lymphoid tissue of the white pulp is atrophied (red star) (H&E X100, 400). E) Heart: degenerated cardiomyocytes (vacuolated and hyalinized) (blue arrows) or atrophied (yellow stars) due to presence of interstitial edema and some are necrotic (myomalacia) (orange arrow) (H&E X200, 400).

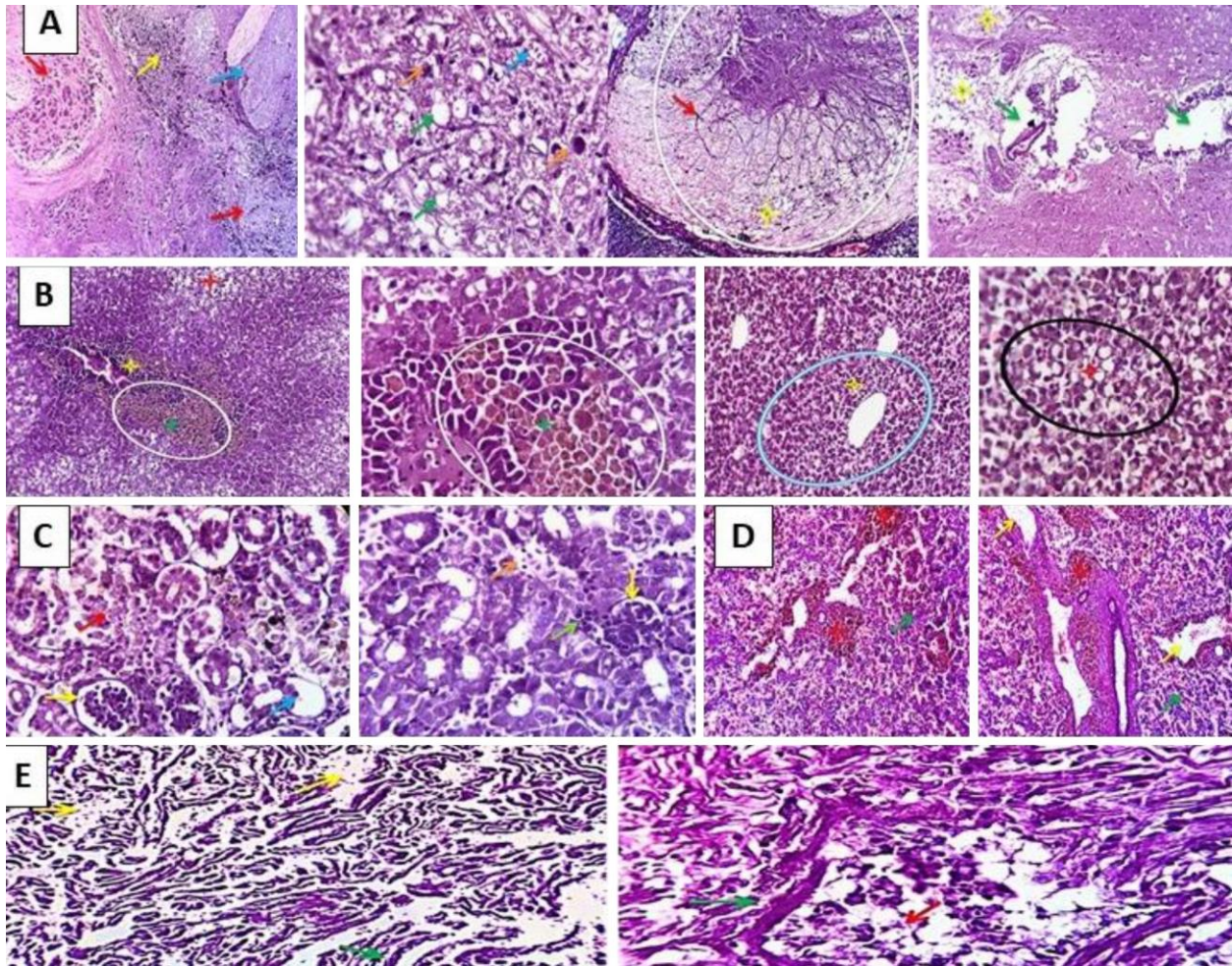


Figure 6: Photomicrograph from of *Tilapia nilotica* fish infected with *Enterococcus faecalis* showing

A) Brain: outstanding necrosis of the neurons, Purkinje cells and granular cells of both cerebral and cerebellar counterparts (red and orange arrows and yellow star). The cerebral blood vessels were moderately dilated and surrounded by inflammatory fluid rich in lymphocytes and polymorph cells (green arrows). Focal astrogliosis and microgliosis are also seen (yellow arrow) (H&E, X100, 200, 400). B) Liver: marked peri-portal and paracentral hepatocellular degeneration vacuolation and macrosteatosis (blue and black circles and yellow and red stars). The hepato-portal areas showed characteristic degeneration and dissociation of the pancreatic tissue and proliferative aggregation of the melano-macrophages (white circles) (H&E, X100, 200, 400). C) Kidney: tubular degeneration, tubulitis (red arrows), glomerular atrophy (yellow arrows), interstitial hemorrhages (orange arrow), lymphocytic infiltrations and aggregations (green arrow) and melano-macrophages proliferation (white arrow). A few renal tubules are cystic (blue arrow) (H&E, X400). D) Spleen: proliferative aggregations of melano-macrophages (red stars). The blood vessels are markedly congested and dilated (yellow arrows) and the lymphoid tissue are clearly depleted (green arrows) (H&E, X100). E) Heart: interstitial edema, hemorrhages (yellow arrows), myocardial degeneration and atrophy (green arrows), beside focal myomalacia (red arrow) (H&E, X100, 400)

Discussion

The result of clinical examination and post-mortem finding of diseased fish *O. niloticus* showed signs of septicemia and inflammation. This result is totally agreed with Arumugam et al. and Osman et al. (1, 9) as they isolated *E. faecalis* from diseased tilapia and recorded the same signs on examined fish.

The result of microbial identification showed characteristics of *Enterococcus* strain in agreement with Khafagy et al. (20) as their results showed gram-positive cocci arranged in pairs and sometimes short chains, negative for oxidase and catalase and nonmotile. In agreement with Rahman et al., (5) who used Kenner-Fecal (KF) streptococcal agar as a specific media for isolation; they founded Gram positive, cocci, non-motile, cata-

lase and oxidase negative isolates. Our results revealed that the two identified species showed greenish (alpha) hemolysis on blood agar these results supported by El-Bouhy (21) who mentioned that *E. faecalis* showed alpha or no hemolysis on blood agar while Zeid (22) documented that they gave alpha hemolysis only. Other synonymous terms are incomplete hemolysis or partial hemolysis. Alpha hemolysis is caused by hydrogen peroxide produced by the bacterium, oxidizing hemoglobin producing the green oxidized derivative methemoglobin.

The result of biochemical identification applied by Automated identification and antibiotic sensitivity testing of suspected isolates showed two subspecies of Enterococci: *E. faecium* and *E. faecalis*. Sergelidis et al. (15) were identified their isolates depending on biochemical characterization by semi-automated system WIDER (Francisco Soria Melguizo, Madrid, Spain) using the Gram positive minimal inhibitory concentration/identification panels, which was nearby method to that used in our experiment. Also, Arumugam et al. (1) documented biochemical characterization of *E. faecalis* included Gram strain, catalase test, and growth at 6.5% NaCl/ 45°C, using Rapid HiStrep™ biochemical test kit specific for *Streptococcus* species. The identified subspecies were *E. faecium* and *E. faecalis*, in agreement with the result of Osman et al. (9) who isolated *E. faecalis* from Nile tilapia using Vitek 2 (Biomérieux, France). Also, Rizkiantino et al. (23) concluded that *E. faecalis* were found as the cause of streptococcosis, infecting and causing mild lesions in red tilapia. Owing to the current state of sewage pollution in Egyptian fish farms loaded with many pathogens, *E. faecalis* has been concern with significant health risks to animals and humans (5, 24).

Globally, the use of antimicrobial agents is regulated differently from country to country, being either very strict or under-regulated. Possible hazards associated with drug abuse in fish farming are the presence of residues in food and the development of antibiotic resistance in the bacterial population (9). Antibiotic sensitivity test resulted in that all isolated strains were sensitive to ampicillin, penicillin, streptomycin, and gentamycin and this disagree with Economou et al. and

Jahan and Holley (25, 26) who mentioned that acquired resistances observed toward chloramphenicol, erythromycin, fluoroquinolones, tetracycline, penicillin, ampicillin, aminoglycosides (gentamicin, kanamycin, and streptomycin) and glycopeptides (vancomycin) in enterococci from other microorganisms, via plasmids or transposons, could be observed. While this partially differ from Rahman et al. (5) who mentioned that all of the *E. faecalis* isolates from Nile tilapia exhibited varying levels of susceptibility to nitrofurantoin, azithromycin, gentamycin, levofloxacin, and vancomycin and showed resistance to amoxicillin, ampicillin, cefradine cefuroxime, erythromycin and penicillin. Arumugam et al. (1) found that the *E. faecalis* showed resistance to amoxiclav, ampicillin, erythromycin, gentamicin, kanamycin, nitrofurantoin.

The result of amplified patterns obtained by PCR with tested Enterococcus strains; isolates were confirmed by 16s rRNA primer. All strains gave almost a common band with the molecular weight observed also by Arumugam et al. (1) who used 16s rRNA as specific gene as method of identification of enterococcus species.

Regarding the pathogenicity study of isolated *E. faecium* and *E. faecalis* it revealed that percent of mortalities was 77% and 73% respectively while Rahman et al. (5) concluded that percent of mortalities for *E. faecalis* different nine isolates was ranged from 40% to 98%. The secreted virulence factors of *E. faecalis* such as hemolysin, cytolysin, and surface proteins such as the M proteins are most likely to be responsible for these clinico-pathological characteristics and high mortality rates (5).

The result of histopathological findings illustrated that infection with *enterococcus spp.* resulted in different types of degenerative damage in all examined organs tissues (brain, liver, kidney, spleen, and heart). Also, Fawzy et al. (16) observed degenerative and necrotic pathologic changes in the internal organs. Zahran et al. (13) mentioned that histopathological and transmission electron microscope examination revealed degenerative and necrotic changes in the heart and liver at all time-points post-challenge. Lipoteichoic acid is found in *E. faecalis*, and it is one of the most important virulence factors found in

Gram-positive bacteria's cell walls. It is responsible for pathogenesis and plays an important role in inflammatory reactions (27). Inflammation causes a series of chemical and morphological changes in affected tissues including leucocyte migration and the formation or increase in the number of granulocytes. Chronic inflammatory reactions are easy to observe by histological approaches in fish (28).

From these findings we conclude that *E. faecalis* and *E. faecium* were the most common subspecies of Enterococci showing high pathogenicity for Nile tilapia fish in Egypt causing histopathological degenerative damage. In vitro antibiotic susceptibility testing for the isolated bacterial species showed that high susceptibility to ampicillin, penicillin, streptomycin, linezolid, and gentamycin while *E. faecium* showed resistance to vancomycin.

References

1. Arumugam U, Stalin N and Rebecca G. Isolation, Molecular Identification and Antibiotic Resistance of *Enterococcus faecalis* from Diseased Tilapia. *Int J Curr Microbiol App Sci* 2017; 6: 136-146.
2. Pridgeon JW and Klesius K. Major bacterial diseases in aquaculture and their vaccine development. *Anim Sci Rev* 2012; 7: 1-16.
3. Zorrilla I, Chabrillón M, Arijó S, et al. Bacteria recovered from diseased cultured gilthead sea bream (*Sparus aurata* L.) in southwestern Spain. *Aquaculture* 2003; 218: 11-20.
4. Plumb J A. Infectious diseases of tilapia. In *Tilapia aquaculture in the Americas*. (Edited by Costapierce, B. A. & Rakocy, J.E.) 1997; 212-228.
5. Rahman M, Rahman MM, Deb SC, et al. Molecular identification of multiple antibiotic resistant fish pathogenic *enterococcus faecalis* and their control by medicinal herbs. *Sci Rep* 2017; 7:1-11.
6. Athanassopoulou F and Roberts RJ. Streptococcal infections of farmed fish (In Greek and English). *J. Hell. Vet. Medical Soc* 2004; 55: 136-144 .
7. Evans JJ, Klesius PH, Shoemaker CA. First isolation and characterization of *Lactococcus garvieae* from Brazilian Nile tilapia, *Oreochromis niloticus* (L.) and pintado, *Pseudoplatystoma corruscans* (Spix & Agassiz). *J Fish Dis* 2009; 32: 943-51.
8. Netto LN, Leal CA, Figueiredo HC. *Streptococcus dysgalactiae* as an agent of septicemia in Nile tilapia, *Oreochromis niloticus* (L.). *J Fish Dis* 2011; 4:251-254.
9. Osman KM, Al-Maary K S, Mubarak AS et al. Characterization and susceptibility of streptococci and enterococci isolated from Nile tilapia (*Oreochromis niloticus*) showing septicemia in aquaculture and wild sites in Egypt. *BMC Vet Res* 2017; 13: 357.
10. Haslam DB and Geme JWS. Classification of streptococci. In *Principles and practice of pediatric infectious diseases*, 5th ed. Eds., Long, S., Prober, C. and Ficher M. Amsterdam, NL: Elsevier 2018.
11. Martins ML, Mouriño JL, Amaral GV, et al. Hematological changes in Nile tilapia experimentally infected with *Enterococcus* sp. *Braz J Biol* 2008; 68:657-661.
12. El-Refaey AME. Studies on major bacterial diseases affecting fish; Tilapia *Oreochromis niloticus*, catfish, *Clarias gariepinus* and mullets in Port Said, Egypt with special references to its pathological alterations. *Researcher* 2013; 5: 5-14.
13. Zahran E, Mahgoub H A, Abdelhamid F, Sadeyen J, Risha E. Experimental pathogenesis and host immune responses of *Enterococcus faecalis* infection in Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 2019; 512:734319.
14. Ben Braek O and Smaoui S. Enterococci: Between Emerging Pathogens and Potential Probiotics. *Biomed Res Int* 2019; Article ID 5938210, 13.
15. Sergelidis D, Abraham A, Papadopoulos T et al. Antimicrobial susceptibility of *Enterococcus* spp. isolated from freshwater fish and personnel and equipment of fish markets in northern Greece. *J Hell Vet Medical Soc* 2013; 64: 239-248.
16. Fawzy N, Osman K, Ibrahim M, et al. Streptococcosis in tilapia: Clinico-pathological picture of experimentally infected tilapia. *Life Sci J* 2014; 11:1005-1012.
17. Manero A and Blanch AR. Identification of *Enterococcus* spp. with a biochemical key. *Appl Environ Microbiol* 1999; 65: 4425-430.
18. Matsuda K, Tsuji H, Asahara T, et al. Establishment of an analytical system for the human fecal microbiota, based on reverse transcription quantitative PCR targeting of multicopy rRNA molecules. *Appl Environ Microbiol* 2009; 75: 1961-1969.
19. Suvarna Kim S, Christopher Layton and Bancroft John D. *Bancroft's Theory and practice of histological techniques*, 7th Edition 2013.
20. Khafagy AAR, Eid HMI, Abou El-Atta MEI, et al. Isolation of *Enterococcus faecalis* from tilapia in lake Tamsah in Ismailia Governorate. *SCVMJ* 2009; IVX: 45-57.
21. El-Bouhy AM. Studies on streptococcosis in some freshwater fishes in relation to aquatic birds. Ph.D. Thesis, Faculty of Veterinary Medicine Zagazig University 2002.
22. Zeid M. Studies on streptococcosis among cultured and wild *Oreochromis niloticus*. MV Sci (Doctoral dissertation) Faculty of Veterinary Medicine Suez Canal University 2004.

23. Rizkiantino R, Wibawan IWT, Pasaribu FH et al. Isolation and characterization of the *Enterococcus faecalis* strain isolated from red tilapia (*Oreochromis hybrid*) in Indonesia: A preliminary report. *J Surv Fish* 2020; 7: 27-42.

24. Wang S, Hibberd ML, Pettersson S, et al. *Enterococcus faecalis* from healthy infants modulates inflammation through MAPK signaling pathways. *PLOS ONE* 2014; 9: e97523 .

25. Economou V, Sakkas H, Delis G, et al. Antibiotic resistance in *Enterococcus* spp. Friend or foe? In: Singh O. V., editor. *Foodborne Pathogens and Antibiotic Resistance*, First Edition. John Wiley & Sons, Inc. 2017: 365–395.

26. Jahan M and Holley RA. Transfer of antibiotic resistance from *Enterococcus faecium* of fermented meat origin to *Listeria monocytogenes* and *Listeria innocua*. *Lett Appl Microbiol* 2016; 62: 304–310.

27. Park OJ, Han JY, Baik JE, et al. Lipoteichoic acid of *Enterococcus faecalis* induces the expression of chemokines via TLR2 and PAFR signaling pathways. *J Leukoc Biol.* 2013; 94: 1275–1284.

28. Koppang EO, Fischer U, Satoh M, et al. Inflammation in fish as seen from a morphological point of view with special reference to the vascular compartment. *Curr Pharm Des* 2007;13: 3649-3655.