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Molecular detection of virulence and antibiotic resistance genes in *Enterococcus faecalis* isolated from diseased tilapia fish.

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Abstract

In this study, *Enterococcus faecalis* was isolated from100 samples of apparently healthy and diseased cultured Tilapia fish (*Oreochromis niloticus*) collected from 4 different fish farms at different localities in Kafr El-Sheikh governorate with an incidence of (20%) and it, also isolated from 12 samples (8 water samples and 4 ration samples) at the same infected farms with incidence of 50% from water samples, but the ration samples give a negative result for *Enterococcus faecalis* isolation. The antimicrobial susceptibility test has revealed that isolates were sensitive to vancomycin, amoxicillin, ampicillin, norfloxacin, gentamicin and ciprofloxacin, while resistant to cefotaxime, tetracycline, streptomycin and erythromycin. Molecular detection of some virulence genes from 8 *Enterococcus faecalis* isolates revealed that 3 isolates produced *esp* gene, 7 isolates produced *asa1*gene and all isolates produced *EF3314* gene, while the *cylA* was not detected in any of *Enterococcus faecalis* isolates. Also PCR results of some resistance genes showed that 8 isolates and 7 isolates were found to produce *blaz* and *tetk* genes respectively. Experimental infection test with some isolated *Enterococcus faecalis* revealed that the isolates caused mortality in fish with characteristic disease signs.

Keywords: *Molecular detection, virulence and antibiotic resistance genes, Enterococcus faecalis and diseased tilapia fish.*

Introduction

Aquaculture is one of the last frontiers to increase contributions to food security in the developing world. It now represents the fastest growing agricultural industry in some countries, with freshwater aquaculture dominating total aquaculture production. This global picture is reflected in Africa where aquaculture supply high quality food at low cost to millions of people, generate income for farming and fishing households and play a central role in many local and national economies **(Kitessa *et al.,* 2014).**

Enterococci belongs to the group of lactic acid bacteria (LAB) and are widely distributed in nature. The genera comprise more than 30 species, but *Enterococcus faecium* and *Enterococcus faecalis* are the most prevalent species in foods **(Valenzuela *et al*., 2010).**

Enterococci are widely distributed in nature **(Klare *et al.,* 2003)** also known to be present in the intestine of healthy fish, and are potential probiotic candidates in aquaculture. Enterococci have been known to be resistant to most antibiotics used in clinical practice **(Lukášoá and Šustáˇ cková, 2003)**

In recent years, some opportunistic bacterial fish pathogens have been identified as the causal agents for severe outbreaks in aquaculture facilities. Among them, *Enterococcus* spp. has emerged as one of the important fish pathogens, which severely impacts aquaculture practices worldwide **(Martins *et al.,* 2008)**. *E. faecalis* has been reported as a pathogen causing streptococcal infection in tilapia in

lakes of Egypt, and Thailand **(Abou El-Geit *et al.*, 2013)**. The clinical symptoms recorded in the Tilapia sample infected with *Enterococcus faecalis* were lethargy, abdominal ascites, organ discoloration, necrosis of the spleen and haemorrhages in kidney **(Uma *et al.*, 2017).** The opportunistic fish pathogen, *Enterococcus faecalis* has been reported to cause mass mortality in several fish species in different countries (**Rahman *et al*., 2017).**

Enterococci is considered to be a suitable 'indicator' of fecal contamination in aquatic environment as it survives longer compared to other fecal streptococci and coliforms **(USEPA 2003).** *Enterococcus faecalis*, most prevalent Enterococci, is an enteric bacterium of mammalian intestinal tract. It is notorious as an opportunistic pathogen causing various infections **(Thurlow *et al*., 2010).** Majority of enterococcal infections (80%) are caused by *E. faecalis* due to its ability to acquire virulence traits and resistance to multiple antimicrobials **(Gordon *et al*., 1992).**

Enterococci may carry various genes such as aggregation substances (*asa1*), endocarditis antigen, gelatinase (*gelE*), Extracellular surface protein (*esp*), Cytolysin (*cylA*) and hyaluronidase or adhesion collagen protein have been described in enterococci isolated from foods **(Hammad, *et al.*, 2015).**

Enterococci are intrinsically resistant/tolerant to many antibiotics and are able to acquire drug resistance either by chromosome, transfer of plasmid or transposon acquisition containing genetic sequences that confer resistance in other bacteria (**Ben Belgacem *et al.*, 2010**). As well as transmissible antibiotic resistance plasmids, virulence factors are known to be transmissible by highly efficient gene transfer mechanisms **(Eaton & Gasson, 2001).**

Aim of work: this study describe the isolation, identification, antimicrobial sensitivity patterns and experimental infection of *Enterococcus faecalis* isolated from apparently healthy & diseased *Oreochromis niloticus* fish, farms water and ration samples. In addition to using of polymerase chain reaction (PCR) as sensitive and reliable method for detection of virulence genes and antibiotic resistance genes.

Materials and Methods

Collection of samples

In this study a total number of 100 randomly selected apparently healthy and diseased Tilapia fish (*Oreochromis niloticus*) collected from 4 farms (25 fish from each) the diseased fish showing at least one or more of the external signs as protruding opaque eyes, swollen abdomen, haemorrhage under the pelvic fin region and erosion in the tail, were collected as alive or freshly dead also 8 water samples collected from the same 4 farms (2 ponds in each farm), each water sample taken from water inlet and from inside the pond, and 4 ration samples were collected also from the same 4 fish farms at different localities in Kafr El-Sheikh governorate, Egypt. The fish were transported in sterile polythene bag supplied with aerated chlorine free tap water from fish farms to the laboratory of Animal Health Research Institute Kafr El-Sheikh branch. Also, water samples & ration samples were collected in sterile glass botties to subjected to bacteriological examination.

Bacteriological examinations:

Isolation of *E. Faecalis*

Under aseptic condition samples were taken from skin surface, tail, eye, fins, livers, kidneys, spleen and brain were homogenized in one sample according to the method described by **(Aboyadak *et al*., 2016)** samples from fish primary cultivated on tryptic soy broth (TSB),. Also water samples and ration samples were cultivated separately on tryptic soy broth at 37 ºC for 24h, then each sample (fish, water & ration) were streaked onto enterococci selective differential agar medium (ESD) **(Efthymiou *et al*., 1974)**, tryptic soy agar (TSA), and blood agar plates then the streaked plates was incubated at 37ºC for 24 hours.

Identification of bacterial isolates

All purified isolates were identified by studying colony growth characteristics, stained with Gram's stain and examined microscopically for demonstration of morphology, arrangement and staining reaction of microorganism, also the motility of each isolate was tested. The bacteria isolates were identified according to

schemes of biochemical reactions provided in Bergey's Manual of Systematic Bacteriology **(Holt *et al.*, 1994).**

Antibiotic susceptibility testing

The susceptibility profile of isolated 15 *E. faecalis* isolates to 10 commercial antibiotic disks was determined by disk diffusion method **(Jorgensen and Ferraro, 2009).** The antibiotic disks used in this study were: Streptomycin (S) 10ug, Erythromycin (E)15ug, Vancomycin (VA) 30ug, Amoxicillin (AML) 10ug, Gentamicin (CN) 10ug, Tetracyclin (TE) 30ug, Ampicillin (AMP) 10ug, Cefotaxime (CTX) 30ug, Ciprofloxacin (CIP) 5ug and Norfloxacin (NOR) 10ug. Zones of inhibition formed around the discs were measured and antibiotic sensitivity was assayed from length of the diameter of the zones (in mm). Zone diameters were interpreted as sensitive, intermediate and resistant according to **(CLSI, 2012).**

Detection of some virulence and antibiotic resistance genes of *E. faecalis* isolates by using Polymerase Chain Reaction (PCR).

DNA extraction. DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56ºC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit**.**

Oligonucleotide Primer. Primers used were supplied from **Metabion (Germany)** are listed in table (1).

PCR amplification.

Duplex PCR. Primers were utilized in a 50- µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix **(Takara, Japan),** 1 µl of each primer of 20 pmol concentration, 11 µl of water, and 8 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Uniplex PCR. Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix **(Takara, Japan**), 1 µl of each primer of 20 pmolconcentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the uniplex PCR products and 40 µl of the multiplex PCR products was loaded in each gel slot. A Gelpilot 100 bp Ladder (Qiagen, Germany, GmbH) and generuler 100 bp DNA ladder (Fermentas, Thermo, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1). Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
esp	AGATTTCATCTTT-GATTCTTGG AATTGAT-TCTTTAGCATCTGG	510	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec	72˚C 45 sec	72˚C 10 min.	**Vankerckhoven *et al.*, (2004)**
asa1	GCACGCTATTAC-GAACTATGA TAAGAAA-GAACATCACCACGA	375						
EF3314	AGAGGGAC-GATCAGATGAAAAA ATTCCAATTGAC-GATTCACTTC	566	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec	72˚C 45 sec	72˚C 10 min.	**Creti *et al.*, (2004)**
cylA	ACTCGGG-GATTGATAGGC GCTGCTAAA-GCTGCGCTT	688	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec	72˚C 45 sec	72˚C 10 min.	**Vankerckhoven *et al.*, (2004)**
blaZ	ACTTCAACAC-CTGCTGCTTTC TGACCAC-TTTTATCAGCAACC	173	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec	72˚C 40 sec	72˚C 10 min.	**Duran *et al.*, (2012)**
tetK	GTAGCGACAA-TAGGTAATAGT GTAGTGACAA-TAAACCTCCTA	360						

Experimental infection.

To observe whether *E. faecalis* isolates were pathogenic or not to fish, we conducted an artificial challenge study under laboratory conditions. The Tilapia fish obtained from healthy farm and tested bacteriologically for *E. faecalis* free. Adopted 40 fish free from *E.faecalis* weighing 100 ± 10 gram were distributed in 4 aerated aquaria of 100 cm 40cm 30 cm and acclimated for 7 days before assay **(Keskin *et al.*, 2004)**. During the course of the experiments (21 days) a daily 50% water renovation was provided and feeding the fish with commercial diet. The 40 fish were divided into four groups 1st, 2nd & 3rd (infected group) & the fourth (control or unchallenged group), each group comprised of 10 fish. The colonies of *Enterococcus faecalis* was adjusted by normal saline to a concentration of 1.5×10^8 (McFarland 0.5) CFU/mL. The experiment was done by challenging the 1st, 2nd & 3rd groups fish (infected group) with the live 3 isolates of *Enterococcus faecalis* which carry the same virulence genes with dose 250 ul of 1.5×10^8 CFU/ml into the intraperitoneal cavity according to **(Sharma & Sihag, 2013),** while the fourth control group was injected with physiological saline water. The clinical signs of the disease were recorded continuously during the duration of experiment and fish mortality was recorded at the end of experiment. The liver, brain and kidney from the dead fish were collected and microbiologically examined for reisolation and identification of the inoculated strain by bacterial culture.

Reisolation and identification of *E. faecalis* from artificially infected fish

The samples from skin surface, tail, eye, fins, livers, kidneys, spleen and brain were homogenized in one sample& primary cultivated on tryptic soy broth and incubated at 37ºC for 24h.then streaked onto ESD plates and incubated at 37ºC for 24h. Gram staining were performed to identify Gram-positive cocci in paired and finally, the colonies were further identified using biochemical test (kock's postulates).

Results
Isolation & microscopical examination of *Enterococcus faecalis*

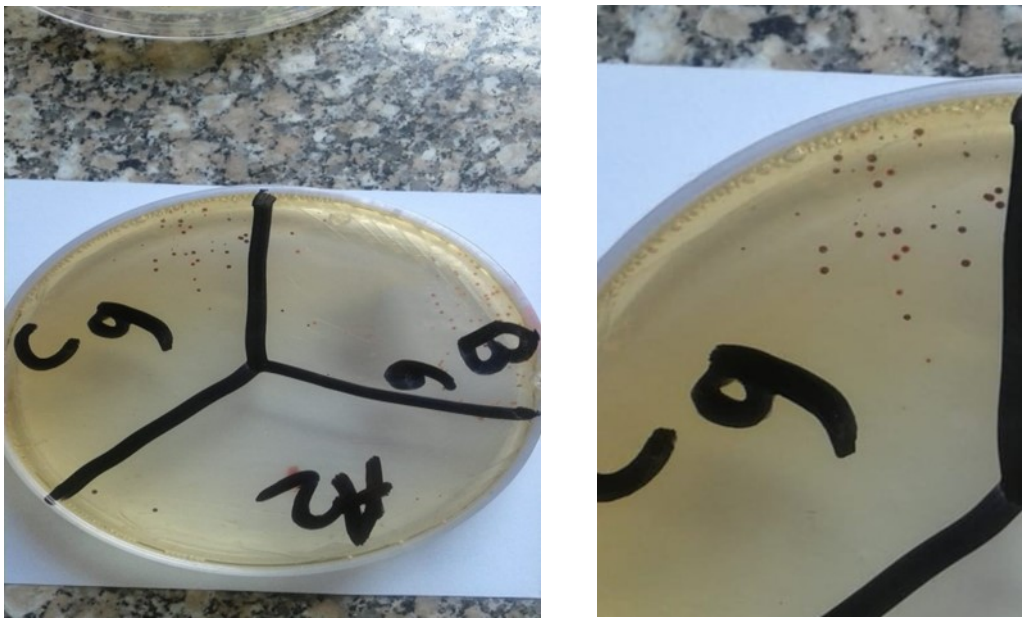


Fig. (1): *Enterococcus faecalis* colonies on (E.S.D) *Enterococcus* selective differential medium showing round, red to magenta colonies

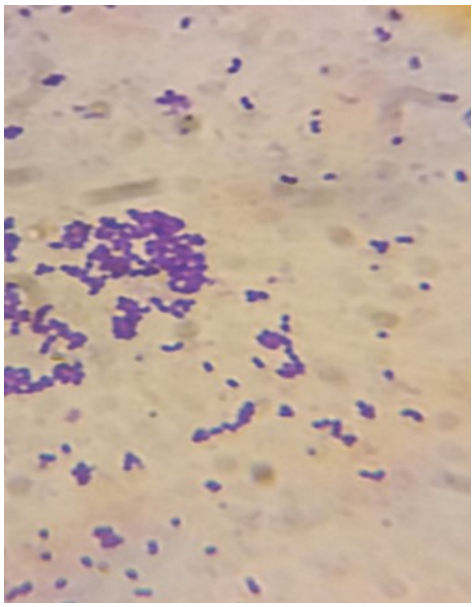


Fig. (2)**:** *Enterococcus faecalis* appear under microscope as Gram positive small cocci in clusters, short chains & diplococci, non sporulated & non capsulated**.**

Table (2). Biochemical characterization of *Enterococcus faecalis* strain

Biochemical tests	*Enterococcus faecalis*
Oxidase test	-
Catalase test	-
Glucose fermentation	+
Vogus proskauer	+
Esculine hydrolysis	+
Starsh hydrolysis	-
Lactose fermentation	+
Sucrose	+
Arabinose	-
Sorbitol	+
Indole test	-
Growth at 6.5% Nacl	+
Growth at 10ºC	+
Growth at 45ºC	+

Table (3). Incidence of *Enterococcus faecalis* isolated from apparently healthy and diseased Tilapia fish, water and ration samples (n= 100, n =8 and n =4) Respectively

Types of samples	No . of Samples	No. of positive samples	Percentage(%)
Tilapia fish samples	100 (25 from each farm)	20 (12 from 1st, farm & 8from2nd farm) but 3rd &4th farms gave negative results	20
Water samples	8 (2 ponds from each farm)	4 ponds (2 farms)	50
Ration samples	4	0	0

The positive 2 fish farms for *Enterococcus faecalis* isolation from fish were positive also to *Enterococcus faecalis* isolation from its water samples &the higher number of *Enterococcus faecalis* isolation was isolated from 1st, farm which showed more symptom & mortality in the farm which collected from it.

Table (4). Antimicrobial susceptibility test for *Enterococcus faecalis* isolates (no=15) random samples

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Cefotaxime (CTX)	-	-	-	-	15	100
Tetracyclin (TE)	2	13.33	1	6.66	12	80
Streptomycin (S)	3	20	3	20	9	60
Erythromycin €	2	13.33	8	53.33	5	33.33
Gentamicin (CN)	8	53.33	4	26.66	3	20
Ampicillin (Amp)	12	80	-	-	3	20
Ciprofloxacin (CIP)	4	26.66	9	60	2	13.33
Amoxicillin (Aml)	13	86.66	-	-	2	13.33
Norfloxacin (NOR)	10	66.66	4	26.66	1	6.66
Vancomycin (VA)	15	100	-	-	-	-

The virulence genes & antibiotic resistance genes in some isolates of *E.faecalis*

The isolates were screened for harboring (*EF3314, asa1,esp* and *cylA*) virulence genes and *blaZ & tetK* antibiotic resistance gene as shown in Table (4)

Table (5). Virulence genes & antibiotic resistance genes of rendamly 8 *Enterococcus faecalis* isolates which isolated from 1st &2nd farms

Isolate	*esp*	*asa1*	*EF3314*	*cylA*	*blaZ*	*tetK*
1	-	+	+	-	+	+
2	+	+	+	-	+	+
3	+	+	+	-	+	+
4	-	-	+	-	+	-
5	-	+	+	-	+	+
6	-	+	+	-	+	+
7	+	+	+	-	+	+
8	-	+	+	-	+	+

Detection of virulence genes in some *Enterococcus faecalis* isolates by PCR:

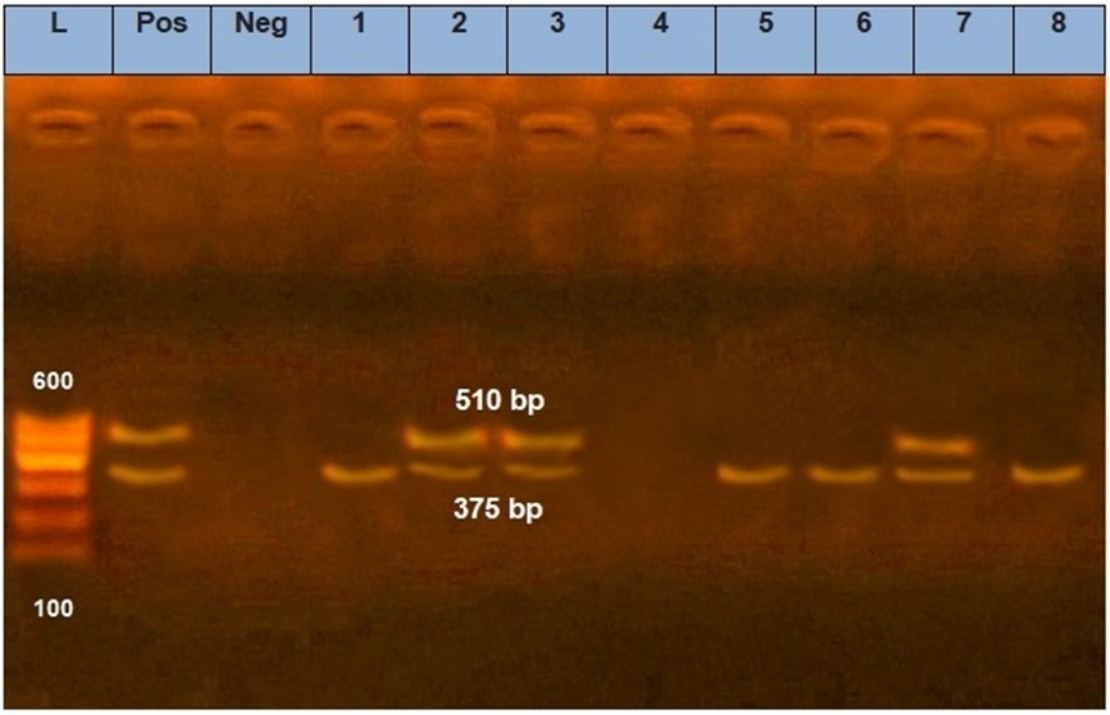


Fig. (3): Agarose gel electrophoresis of multiplex PCR of *esp & asa1* virulence genes of *E.faecalis*
Lane L: 100-600 bp molecular size marker.
Lane Pos: Control positive *E.faecalis asa1* and *Esp* virulence genes at 510 bp & 375bp respectively.
Lane 1, 5, 6, 8 positive to *asa1* gene
Lane 2, 3, 7 positive to *Esp* and *asa1* genes.

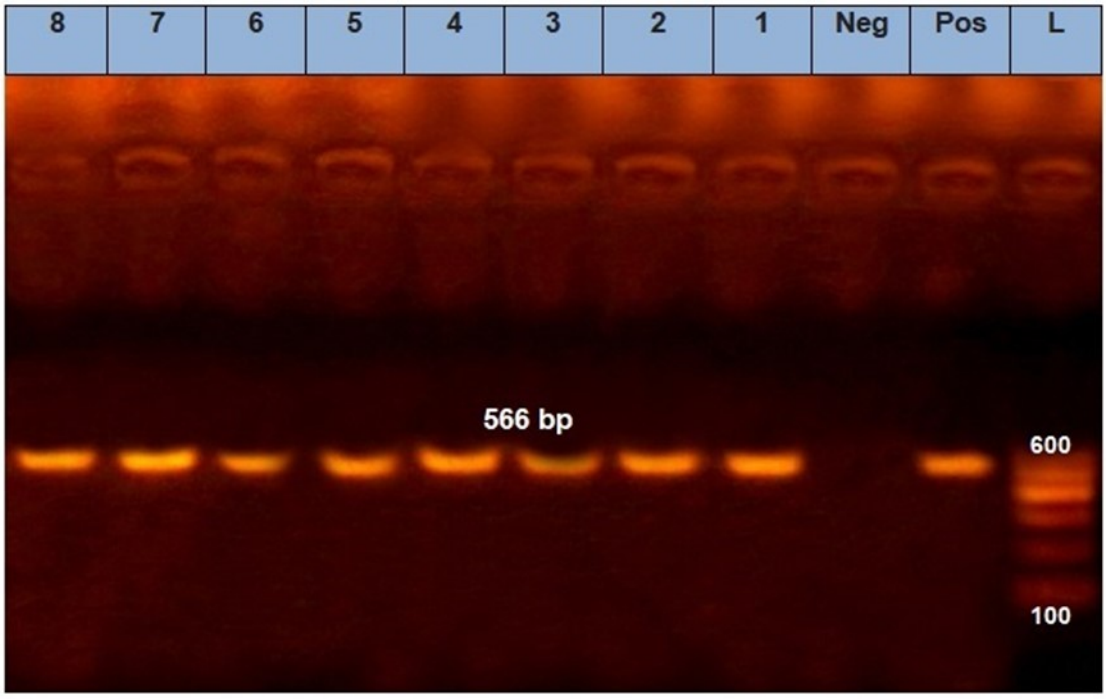
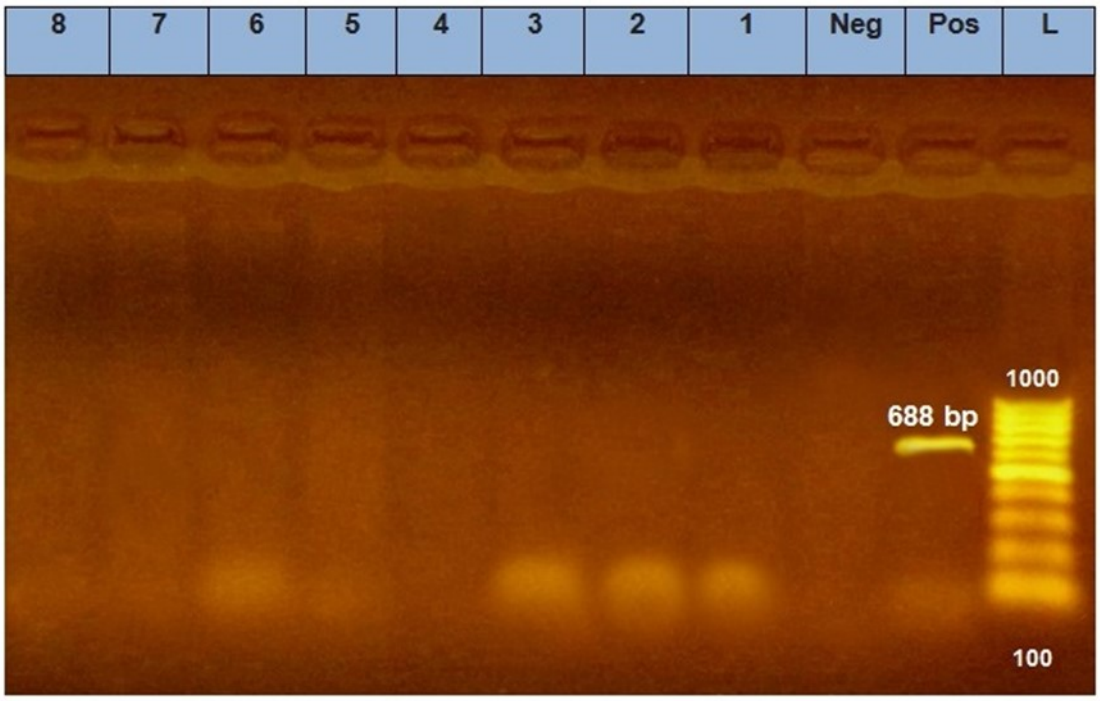


Fig. (4): Agarose gel showing polymerase chain reaction amplification products of *EF3314* virulence gene of *E. faecalis*.
Lane L: 100-600 bp molecular size marker.
Lane Pos.: Control positive *E. faecalis EF3314* virulence gene at 566 bp.
Lane 1, 2, 3, 4, 5, 6, 7 & 8 positive to *EF3314* gene



Fig(5): Agarose gel electrophoresis of PCR of *cylA* virulence gene of *E. faecalis*
Lane L: 100-1000 bp molecular size marker.
Lane Pos: Control positive *E. faecalis cylA* virulence gene at 688 bp.
Lane 1, 2, 3, 4, 5, 6, 7 & 8 negative to *cylA* gene

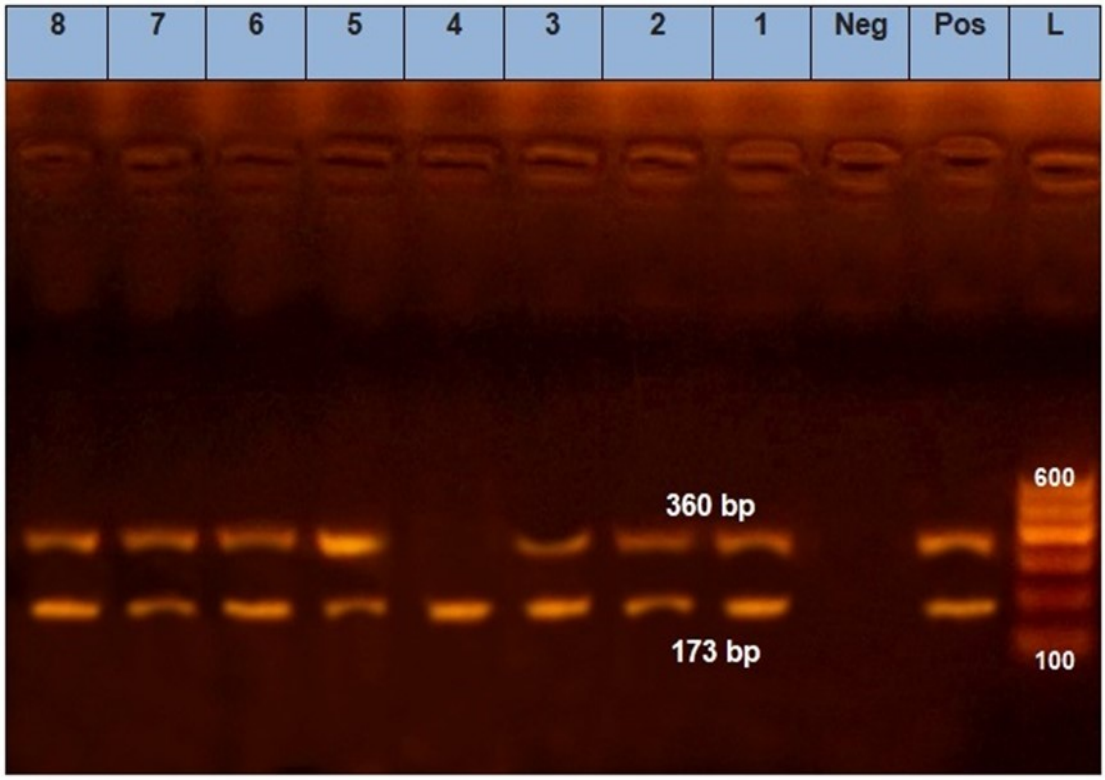


Fig. (6): Agarose gel electrophoresis of m- PCR products of *tetK* and *blaZ* antibiotic resistance genes of *E. faecalis*
Lane L: 100-600 bp molecular size marker.
Lane Pos: Control positive *E.faecalis blaZ & tetK* antibiotic resistance genes at 173 bp & 360 bp respectively.
Lane 1, 2, 3, 5, 6, 7, 8 positive to both *blaZ & tetK* genes.
Lane 4 positive to *blaZ* gene

Table (6). Clinical signs and postmortem lesions in experimentaly infected fish
The first clinical signs appeared within 3 days followed by the death of fish, the total mortality in 3 groups were 13 out of 30 fish.

Groupes of artificially infected fish	No.of fish	No. of dead fish	Mortality %
1st group	10	8	80
2nd group	10	3	30
3rd group	10	2	20

The clinical picture of the experimentally infected Tilapia were seen as: external lesions Figures (7, 8 & 9): eye lesions: in the form of unilateral or bilateral eye redness/opacity; Skin lesions: detached scales, extensive skin congestion, hemorrhage; Fins: congestion at the base of the fins, or even hemorrhagic; Abdomen: slightly distended in some cases; Anal opening: congested with protruded anal opening. The P.M. lesions Figure (10), were seen as: septicemia and hemorrhagic ascites, muscle redness & enlarged liver.

Fig. (7)

Fig. (8)

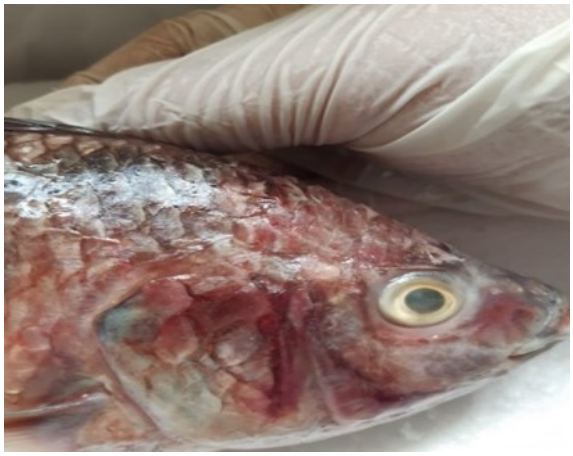


Fig. (9)

Fig. (10)

Fig. (7)**:** Skin congestion, congestion at the base of the fins, Abdomen: slightly distended.
Fig. (8)**:** Erosion in caudal fin, skin congestion, congestion at the base of the fins.
Fig. (9)**:** Diffuse dermal hemorrhages.
Fig. (10)**:** Tilapia fish postmortem internal findings revealed typical signs of septicemia in which severely congested, kidney, spleen, intestine and heart, dark gall bladder, in addition to hemorrhagic ascites, muscle redness.

Reisolation trials from internal organs tissues of artificially infected fish with *E. faecalis*.

The bacteriological examination of homogenized samples from skin, fins & organs has confirmed the presence of the inoculated *E. feacalis.*

Discussion

Enterococci are one of the most common group of bacteria present in foods **(Paganelli *et al.*, 2017).** Also enterococci are widely distributed in nature **(Klare *et al.*, 2003)** and it has been implicated in severe human infections. Enterococci are among the major etiological agents of hospital associated infections **(Hidron *et al*., 2008).** They are characterized by a proneness to acquire resistance determinants (**Arias *et al*., 2010).** and by rapid adaptation to environmental conditions (**Leavis *et al*., 2007).**

In this study, the species *Enterococcus faecalis* was isolated from Tilapia collected from a fresh water. These results agree with those of **Eman, (2007).** The results of bacteriological examination showed that the isolates were gram-positive cocci, non-motile. These findings agreed to that recorded by **Khafagy *et al.* (2009).** Concerning the biochemical tests in this study **Table(2)** *Enterococcus faecalis* was negative for oxidase, catalase, starch hydrolysis and indole these findings agreed to that recorded by **Shoemaker and Klesius, (1997)** who recorded that two of the most important tests for identification of streptococcal pathogens from fish were catalase and starch hydrolysis

test, The isolates grew at 45ºC and grew well at media with 6.5% NaCl this agree with **Khafagy *et al.* (2009).**

In **table (3)** data revealed that *Enterococcus feacalis* was isolated from 100 apparently healthy and diseased Nile Tilapia fish as,20 samples with an incidence of 20% while 8 water samples taken from 4 farms show 2 farms were positive in its water samples for *Enterococcus feacalis* isolation with an incidence of 50% ,but ration samples give negative result in isolation in all farms. These results are agree with those mentioned by **Khafagy *et al.*, (2009).** Who isolated *Enterococcus faecalis* from Nile Tilapia with 23.76% & from Tilapia zilli by 31.90%. Also, **Savaşan *et al.*, (2016).** who isolated *E. faecalis* from 200 fish samples by 13% & **Igbinosa *et al.*, (2016)** who detect *E. faecalis* by 27.66%. From farm water samples, but our results disagree with **El-Sayed & Abou El-Gheit, (2005).** Who detect *E. faecalis* by 5% from farmed and wild Tilapia fish, **El-Kader & Mousa-Balabel, (2017).** Who found that *E. faecalis* was found by 2.7% & **Walaa *et al.*, (2009).** Who detected *E. faecalis* from Nile Tilapia with prevalence of 45,65%.

Enterococci have emerged as very important nosocomial pathogens, and this is attributed, among other factors, to their broad natural and acquired resistance to antimicrobial agents, including glycopeptides, vancomycin and teicoplanin **(Oskoui and Farrokh, 2010).**

The results of antibiotic sensitivity test in **table (4)** showed that, the tested isolates were highly sensitive (100%) to vancomycin and (86.66%) of the tested isolates were sensitive to amoxicillin, (80%) to ampicillin while the isolates were resistant to cefotaxime by (100%), tetracycline by (80%) & streptomycin by (60%).

The results in this study were agree with those of **Kamelia *et al*., (2016)** who detected that the bacteria were sensitive to Ampicillin by 83.3%, Vancomycin by 83.3% but resist to Tetracyclin by 100% but disagree with them in the result of Streptomycin which gave 100% sensitive to the microbe. Also agree with **Arumugam *et al*., (2017)** in some results as Erythromycin & Streptomycin which gave resistant results with the bacteria and agree with those of **Dena, (2004)** who revealed that the bacteria were resistant to Streptomycin. but disagree with (**Rahman *et al*., 2017)** who showed that the bacteria were resistant to Ampicillin & Amoxicillin & **El-Sayed & Abou El -Gheit (2005).** who detected that the bacteria were Ampicillin moderate susceptibility, Cephalocin resistant, Tetracycline moderate susceptibility & Vancomycin moderate susceptibility.

A virulence factor is an effector molecule that enhances the capacity of a microorganism to cause illness. Virulence factors of enterococci play a significant role in the pathogenicity of enterococcal strains. These factors have been intensively investigated in the last few years. The most common and well described virulence determinants in enterococci are aggregation substances (*agg, asa1*), cytolysin (*cyl*), gelatinase (*gelE*), extracellular surface protein (*esp)*, adhesion to collagen (*ace, acm*), and adhesion like endocarditis antigens (*efaAfs* and *efaAfm*) **(Barbosa *et al*., 2010).**

The antimicrobial resistance genes and virulence factors can be transferred to other bacteria, which pose a serious threat to public health through the food chain **(Aslam *et al.*, 2012; Iweriebor *et al.*, 2015).**

The results in **table (5) and Fig (3)** showed that only 3 strains from the examined eight isolates harbor the *esp* gene by (37.5%). This was in agreement with(**Trivedi *et al.*, 2011)** who found *esp* gene by 37% from dairy but disagree with **(Abdeen *et al.*, 2016)** who detected it by 50% in minced meat & **(Chajęcka-Wierzchowska *et al.*, 2016)** who detected it by 100% from retail raw, cooked and ready-to-eat shrimps. Our results showed. Also that *asa1*gene found in 7 from 8 isolates by percent of 87.5 this result was similar to **(Hammad *et al.*, 2014)** who found *asa1*gene of *E. feacalis* by 77.42% from retail ready to eat raw fish.

Our results showed in **table (4) and Fig (4)** showed that all the strains were positive for *EF3314* gene, while PCR couldn't detect *cylA* gene in positive *E. faecalis* isolates **table (4) and Fig (5)**. This is similar to **(Gulhan *et al.*, 2015)** that all isolates were negative for *cylA* gene & **(Savaşan *et al.* 2016)** who showed that none of the strains isolated from fish were observed to show cytolysin activity. This may

attributed to phenotypic expression of cytolysin is encoded by the cytolsin operon (**Medeiros *et al.*, 2014).**

Our molecular detection of virulence genes *esp, asa1*, *EF3314 & cylA*, showed that *EF3314* in table (4) Fig. (3, 4 & 5). & the result of *cylA* may be due to the presence of the *cylA* gene did not correlate completely with its phenotypic expression **(Creti *et al*., 2004).**

As shown in **table (4) and Fig (6)** all the strains were positive for *blaZ* gene but 7 from 8 isolates at percentage of 87.5 were *tetK* gene positive similar results were obtained by **(Feng *et al.*, 2018)** who observed that (96.3%) positive *tetK E. faecalis* gene isolated from subclinical bovine mastitis. lower result to *blaZ* was recorded by **(Di Cesare *et al.*, 2012)** who detect it by 64.44 from seawater & sediment from a Mediterranean aquaculture site.

As screening for the presence of virulence genes to evaluate the potential virulence of *E. faecalis* could be speculative, empirical testing with a disease challenge was conducted. Our experimental pathogenicity test in vivo proved that, the three isolates of the *Enterococcus feacalis* which carry virulence genes were pathogenic **table (6) and Fig (7, 8, 9 & 10).** The infection or pathogenicity process of *Enterococcus* spp. is very complex and is said to involve different virulent and pathogenicity factors which either act together or separately at different stages of infection **(Al-Bahry *et al*., 2014).** *E. faecalis* isolates caused mortality in 1st group with a percent of 80,in the 2nd group it was 30% & in the 3rd group it was 20% . These results are agree with **Woo, (1999)** who mentioned that the pathogenicty of Streptococci may be attributed to the effect of exotoxins produced by the bacterium. Intraperitoneal injection (I/P) was more dangerous and cause 80% mortality, nearly similar result was detected by **(Osman *et al.*, 2016)** who mentioned that *Enterococcus* isolates caused significant morbidity(70%) within 3 days and 100% mortality at 6 days post-injection with general signs of septicemia.

Conclusion

This study revealed that *Enterococcus faecalis* was isolated from fish and farms water samples which consider as indicator of faecal contamination. Vancomycin and Amoxicillin could be used to treat fish infected with *E. faecalis*. Adequate and proper use of manure products and frequent discharge of water from fish pond will reduce both the high level of infection and the potential risk of human health. The presence of antibiotic resistant genes, *blaz* and *tetk* shows that there is a need for judicious use of antibiotics in aquaculture. Molecular detection of virulence genes showed that *EF3314* was the most predominant virulence gene in the examined strains.

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