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Genotyping and resistance genes of *Enterococcus Faecalis* isolated from different food sources in Egypt

Ashraf A. Abd El-Tawab[1], Fatma I. El-Hofy[1], Mohamed A. Abdelmonem[2], Eman K. Amin[1]

[1] *Bacteriology, Immunology and Mycology Department, Faculty of Veterinary Medicine, Benha University, Egypt.*

[2] *Department of Microbiology, Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Foods (QCAP), Dokki, Giza, Egypt.*

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ABSTRACT

Enterococcus species are considered as a major etiological agent of nosocomial infections, they are commonly isolated from different sources of food. So, this study was conducted for detection of *Enterococcus* spp. from fish (n=10), herbs (n=30), drinking water (n=20), dairy products (n=30), and meat products (n=20) samples from Cairo Governorate, Egypt. PCR was done for detection of some antibiotic resistant genes *(mphC, norA, tetK, floR, vanA).* The results revealed that from 110 food samples analyzed, 11.8% were positive for *Enterococci*. Moreover, *E. faecalis* was detected by percentage 53.8%. *E. faecalis* isolates were resistant to vancomycin (100%), erythromycin (57.1%), ciprofloxacin (14.3%), chloramphenicol (42.9%), tetracycline (57.1%), while none of the *E. faecalis* isolates were resistant to ampicillin, penicillin or norfloxacin. Genotypic characterization revealed that *tet*K and *flo*R genes were present in the all 7 *E. faecalis* isolates. While, *van*A gene was detected in 3 isolates, and *mph*C gene was detected only in 2 isolates. The results of our investigation indicated high levels of contamination with multi-resistant *E. faecalis* strains of serious concern with the isolation of strains resistant to vancomycin, considering that vancomycin is the alternative agent for patients who are intolerant to penicillin or who have *Enterococci* infections with high level resistance to penicillin.

1. INTRODUCTION

Enterococcus (from Greek έντερο, éntero, "intestine" and κοκκος, coccos, "granule") is a large genus of lactic acid bacteria of the phylum Firmicutes (http://www.en.wiki-pedia.org/Enterococcus_faecalis, 2019). It is normal inhabitant in the gastrointestinal tracts of humans and other mammals. So, it is used as an indicator for faecal contamination and poor hygienic measures during manufacture process of dairy and meat products (Buyukyoruk *et al.*, 2014; Nashy, 2017). *Enterococcus* can be readily isolated from foods, Once rejected from the environment by means of human faeces or animal ejecta, it is able to colonies diverse niches because of its exceptional aptitude to resist or grow in hostile environments. Therefore, *E. faecalis* is not only associated with warm-blooded animals, but also occur in soil, surface waters and on plant and vegetables. Also, it can contaminate finished products during food processing (Pesavento *et al.*, 2014; Abdeen *et al.*, 2016).

Enterococci are Gram-positive non-spore forming, catalase and oxidase-negative, facultative anaerobic cocci that often occur in pairs diplococci or short chains (Van Tyne and Gilmore, 2014; Nashy, 2017). *Enterococci* can tolerate different environmental conditions, such as high temperature, it can grow at temperature ranging from 10-45 °C up to 60 °C for 30 min, and NaCl 6.5% (Sanlibaba and Senturk, 2018). It can cause life-threatening infections in humans, especially in the nosocomial environment, (Iweribor *et al.*, 2015). *Enterococcus* spp., particularly *E. faecium* and *E. faecalis*, are important in public health. They cause urinary tract infections, bacteremia, peritonitis, and endocarditis in humans (Fisher and Philips, 2009).

Recent studies indicated that the proportion of *E. faecalis* infections has increased mainly owing to an increased number of antibiotic resistant *E. faecalis* isolates (Golob *et al.*, 2019). *E. faecalis* is resistant to many commonly used antimicrobial agents (aminoglycosides, aztreonam, cephalosporins, clindamycin, the semisynthetic penicillins nafcillin and oxacillin, and trimethoprim–sulfamethoxazole). Resistance to vancomycin in *E. faecalis* is becoming more common (http://www.en.wiki-pedia.org/ Enterococcus faecalis, 2019).

In this article, the aim was to provide information about the *E. faecalis* recovered from selected Egyptian foods, focusing on genotypic characteristics and antibiotic resistance.

2. MATERIAL AND METHODS

2.1. Sampling:

A total of 110 samples (10 Cray fish , 30 herbs (15 Basil, 10 Camomile, 5 Calendula), 20 water, 30 dairy products (15 milk and 15 cheese), 20 meat products (10 beef burger, and 10 minced meat) were collected randomly from different places and sales markets in Cairo Governorate, Egypt. All The collected samples were labeled, aseptically put into

* Corresponding author: **Ashraf A. Abd El-Tawab**. Bacteriology, Immunology and Mycology Department, Faculty of Veterinary Medicine, Benha University, Egypt.

clean, dry, and sterile containers, kept in ice box and transferred to the laboratory of microbiology to be analyzed for detection and isolation of *Enterococcus faecalis*.

2.2. Isolation of Enterococcus: (NMKL 125, 2005; ISO 7218, 2013; ISO 6887–1, 2017)
For water samples, 100 ml of water sample was filtrated using membrane filter with vacuum pump; the membrane filter was placed on *Enterococcus* agar (Slantz and Bartley agar). Then, it was incubated at 44 ± 1 °C for 48 h then the membrane was examined for all characteristic colonies.
For solid samples, 10 g of each sample were mixed with 90 ml maximum recovery diluents to prepare the initial suspension. By mean of a sterile pipette 1 ml of the initial suspension was transferred to 9 ml of maximum recovery diluents to make serial dilution.
For milk samples, 10 ml of the samples were centrifugated at 5000 rpm for 10 min and the supernatant was discarded. A loopful of homogenates was plated on the surface of medium.
By using sterile pipette 0.1 ml was added to a sterile Petri dish containing *Enterococcus* agar. The plate was inverted and incubated at 44 ± 1 °C for 48 h. *Enterococcus* are indicated by all raised colonies with a dark red color, confirmed by inoculation on bile-aesculin agar, incubated at 44 ± 1 °C for 2 hours, and read immediately. All typical colonies showed a tan to black color in the surrounding medium.

2.3. Molecular detection of E. faecalis isolates and resistance genes:
Genomic DNA of the isolates were extracted using DNA Purification Kit QIAamp DNA Mini Kit (Cat. No. 51304–Qiagen) according to Sambrook *et al.* (1989) with modification. Determination of *Enterococci* at genus level was performed using specific gene primers as shown in table 1. The presence of antibiotic resistance genes was identified by PCR in isolated strains.

Table 1 Oligonucleotide primers sequences for detection of *E. faecalis* and resistance genes

Gene	***Primer Sequence 5'-3'***		***Amplified Product***	***Reference***
16SrRNA	F	GTT TAT GCC GCA TGG CAT AAGAG	310 bp	Zoletti *et al.* (2006)
	R	CCG TCA GGG GAC GTT CAG		
*mph*C	F	GAGACTACCAAGAAGACCTGACG	722 bp	Schlegelova *et al.* (2008)
	R	CATACGCCGATTCTCCTGAT		
*nor*A	F	TTCACCAAGCCATCAAAAAG	620 bp	Pourmand *et al.* (2014)
	R	CTTGCCTTTCTCCAGCAATA		
*tet*K	F	GTAGCGACAATAGGTAATAGT	360 bp	Duran *et al.* (2012)
	R	GTAGTGACAATAAACCTCCTA		
*flo*R	F	TTTGGWCCGCTMTCRGAC	494 bp	Doublet *et al.* (2003)
	R	SGAGAARAAGACGAAGAAG		
*van*A	F	CATGACGTATCGGTAAAATC	885 bp	Patel *et al.* (1997)
	R	ACCGGGCAGRGTATTGAC		

2.4. Antibiotic susceptibility:
Only *E. faecalis* isolates were tested for their susceptibility to 8 antimicrobials by a disk diffusion technique (CLSI–M100, 2018). The 8 antibiotics tested comprised fluoroquinolones (ciprofloxacin 5 mg, and norfloxacin 10 mg), glycopeptides (vancomycin 30 mg), macrolides (erythromycin 15 mg), penicillin (ampicillin 10 mg, and penicillin 10 mg), tetracyclines {tetracycline (30 mg), and phenicols (chloramphenicol (30 mg).

3. RESULTS

3.1. Isolation and detection of E. faecalis isolates:
Out of examination of 110 food samples, only 13 samples were positive for enterococcus species. *E. faecalis* was detected by using specific gene primers as shown in figure (1). Only 7 out of the 13 samples were considered as *E. faecalis* with percent 53.8% (Table 2).

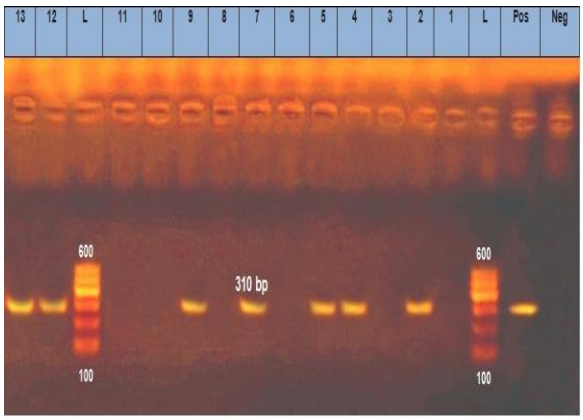

Fig. 1 Gel electrophoresis for molecular identification of *E. faecalis*. Lane L: molecular weight marker (100–600 bp), Lane pos: positive control, Lane neg: negative control, Lane (2, 4, 5, 7, 9, 12, 13): are positive isolates.

Table 2 Prevalence of *Enterococcal* spp. in different food sources in Egypt. Percentage in this table as 1 from 10 (100%) isn't correct

Food Source	Samples	No. of Enterococcus Species		E. faecalis*	
		No.	***%***	***No.***	***%***
Cray fish	10	1	10	1	100
Camomile	10	3	30	1	33.3
Calendula	5	1	20	1	100
Basil	15	1	6.7	0	0
Water	20	5	25	2	40
Beef burger	10	2	20	2	100
Milk	15	0	0	0	0
Cheese	15	0	0	0	0
Total	110	13	11.8	7	53.8

* The percentage of *E. faecalis* was estimates according to total number of positive isolates.

3.2. Antimicrobial sensitivity test:
The sensitivity test of seven *E. faecalis* isolates from cray fish, cammomile, calendula, water, and beef burger samples were done against eight different antimicrobial agents. According to the readings of inhibitory zones of different antibiotic discs on seven *E. faecalis* isolates, the results clearly shows that the all tested isolates showed multi-resistance to different antibiotics. All the 7 isolates were resistant to vancomycin, but they were sensitive to ampicillin and penicillin as shown in table (3).

3.3. Detection of resistance genes of E. faecalis:
A PCR was designed to detect *tet*K for tetracyclines, *flor*R for chloramphenicol, *mph*C for macrolides, *nor*A for quinolones, and *van*A for vancomycin genes in seven antibiotic resistant *E. faecalis* as shown in figures (2-5).
The results showed that all the isolates (100%) had a band compatible with *tet*K and *flor*R, while only two isolates

(28.57%) had a band compatible with *mph*C and only three isolates (42.86%) had a band compatible with *van*A

Table 3 The antibiogram of *E. faecalis* isolates according to CLSI–M100 (2018)

	Sample ID						
Antibiotic Discs	2	4	5	7	9	12	13
Vancomycin, 30 µg	R	R	R	R	R	R	R
Erythromycin, 15 µg	R	S	R	R	R	I	I
Ciprofloxacin, 5 µg	S	I	S	I	R	S	S
Chloramphenicol, 30 µg	R	R	S	I	R	S	I
Tetracyclin, 30 µg	R	S	S	S	R	R	R
Norfloxacin, 10 µg	I	I	S	I	I	S	S
Ampicillin, 10 µg	S	S	S	S	S	S	S
Penicillin, 10 µg	S	S	S	S	S	S	S

Where: R: Resistant. S: Susceptible. I: Intermediate.

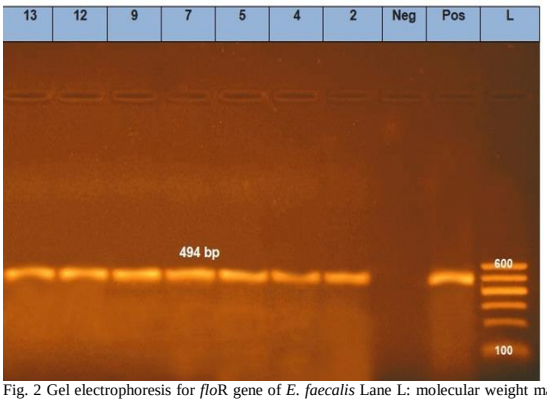

Fig. 2 Gel electrophoresis for *flo*R gene of *E. faecalis* Lane L: molecular weight marker (100–600 bp), Lane pos: positive control, Lane neg: negative control, Lane (2, 4, 5, 7, 9, 12, 13): are positive.

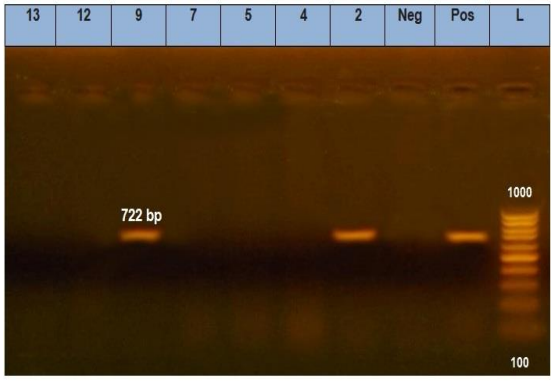

Fig. 3 Gel electrophoresis for *mph*C gene of *E. faecalis.* Lane L: molecular weight marker (100–1000 bp), Lane pos: positive control, Lane neg: negative control, Lane (2, 9): are positive.

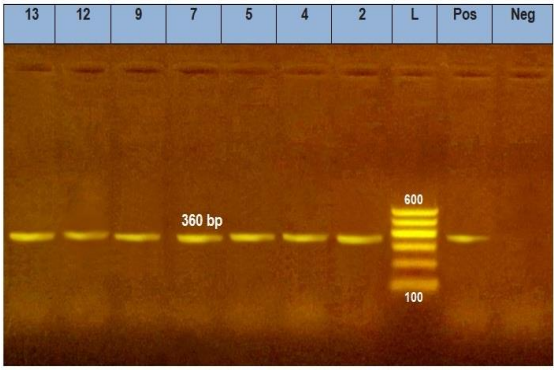

Fig. 4 Gel electrophoresis for *tet*K gene of *E. faecalis.* Lane L: molecular weight marker (100–600 bp), Lane pos: positive control, Lane neg: negative control, Lane (2, 4, 5, 7, 9, 12, 13): are positive.

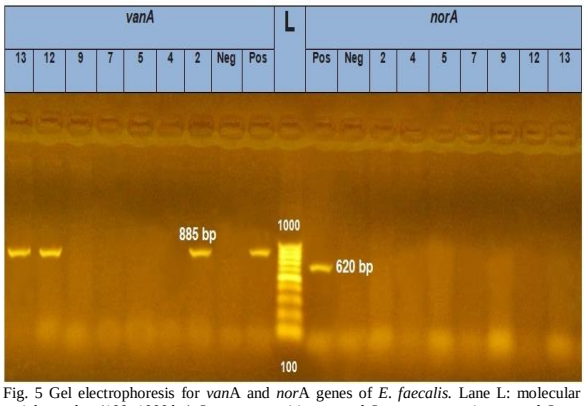

Fig. 5 Gel electrophoresis for *van*A and *nor*A genes of *E. faecalis.* Lane L: molecular weight marker (100–1000 bp), Lane pos: positive control, Lane neg: negative control, Lane (2, 12, 13): are positive for *van*A gene

4. DISCUSSION

Enterococci are ubiquitous in nature, exist at high levels in food and can cause severe diseases in humans. They represent one of the leading agents of nosocomial infections especially urinary tract infections in hospitalized patients (Kafil and Asgharzadch, 2014). *Enterococci* resist to adverse environmental conditions such as low pH, high salinity and high temperatures. So, this takes account for their ability to colonize different habitats and for their potential for easy spreading through the food chain (Fracalanzza *et al.*, 2007). In recent years, *Enterococci* developed resistance to multiple anti-microbial drugs. Antibiotic resistance may be considered to be both the cause and the effect of the adaptation of certain isolates to hospital environment (Cosentino *et al.*, 2010). Therefore, this study aimed to characterize the spread of some antibiotic resistant genes among *Enterococcus faecalis* isolates from different food samples (*tet*K, *flo*R, *mph*C, *nor*A, *van*A.

The classical microbiological techniques currently in use for *Enterococcal* detection and identification are satisfactory in most situations, and the ability to grow on bile esculin agar. Interestingly, *Enterococci* naturally determine their frequent finding in food as contaminants. *Enterococci* can also contaminate finished products, such as fermented food (yogurt and sausages) (Kučerová *et al.*, 2009).

The incidence of *Enterococcus* among the examined samples was 13 out of 110 with percentage 11.8%, (Cray fish, Basil, Camomile, Calendula, Water, and beef burger were 10%, 6.7%, 30%, 20%, 25%, and 20% respectively), which was close to that reported in Assuit, Egypt (8%) (Moustafa *et al.*, 1975). While it was lower than that obtained in Menofia, Egypt (75%) (Abdeen *et al.*, 2016), and in Sharkia and Dakahlia, Egypt (59%) (Nashy, 2017). in the current work, Genus-specific gene of *E. faecalis* was detected in only 7 samples out of the 13 *Enterococcal* isolates by PCR with percentage 53.8%.

The total percentage of *E. faecalis* isolates agreed with the results isolated from dairy products in Pakistan (57%) (Javed *et al.*, 2010). While it was higher than that isolated in Brazil (26.8%) from fresh herbs, vegetables, meat and dairy products (Gomes *et al.*, 2008), Italy (13.9%) from retailed products (as cheese, ham, and ready to eat salads) (Pesavento *et al.*, 2014), Japan (32.2%) from raw fish (Hammad *et al.*, 2014), Tunisia (27.3%) from fermented food and vegetable products (Rehaiem *et al.*, 2016), and China (15.3%) from

different water samples (Wei *et al.*, 2017). Also, Mcgowan-spicer *et al.* (2008) in Athens, isolated *E. faecalis* from 23 samples of fresh vegetables and fruits, as well as it was isolated from 55 (vegetables, raw meat and dairy products) samples in Porto Alegre, South Brazil by Medeiros *et al.* (2014).

As mentioned in table (2), *E. faecalis* isolates were isolated from different food sources with a percentage of 100%, which was highly predominated in both raw fish and meat, followed by 40% in both fresh herbs and water. The higher prevalence rate of *E. faecalis* was obtained from raw fish and meat samples (100%), which was higher than those of previous studies reported the occurrence of *E. faecalis* isolated from fresh meat in both Italy (44.3 %) (Pesavento *et al.*, 2014), and Brazil (15 %) (Gomes *et al.*, 2008). As well as *E. faecalis* isolated from fresh fish in Japan (32.2 %) (Hammad *et al.*, 2014). The incidence of *E. faecalis* in fresh herbs samples (40 %), which was higher than that obtained in Brazil (2.5 %) (Gomes *et al.*, 2008). On the other hand, the incidence of *E. faecalis* in water samples (40 %), which is lower than that obtained in China (57.1 %) (Wei *et al.*, 2017).

One of the most important concerns regarding the presence of *E. faecalis* in the food chain is the possible transmission of antibiotic resistance (Franz *et al.*, 2001). Indeed, this bacterium has a remarkable ability to acquire new mechanisms of resistance and can also transfer resistance determinants to other bacteria by conjugation (Sanlibaba and Senturk, 2018; Sanlibaba *et al.*, 2018; Golob *et al.*, 2019). Antibiotic-resistant *E. faecalis* are widespread in meat products, dairy products, and ready-to-eat foods (Pesavento *et al.*, 2014; Rehaiem *et al.*, 2016).

In our study the *in vitro* sensitivity tests of 7 *E. faecalis* isolates revealed that the tested isolates were resistant to vancomycin (100%), erythromycin (57.1%), ciprofloxacin (14.3%), chloramphenicol (42.9%), tetracycline (57.1%), while, none of the *E. faecalis* isolates were resistant to ampicillin, penicillin or norfloxacin. in El-Menofia Governorate, Egypt. Hammad *et al.* (2015) stated that most of samples were resistant to vancomycin, erythromycin, ciprofloxacin, chloramphenicol and tetracycline with percentage 62.5%, 12.5%, 37.5%, 12.5% and 62.5%, respectively. While, the results of studies of resistance to antibacterial agents of *E. faecalis*, which were isolated from raw milk and cottage cheese in Ukraine by Horiuk *et al.* (2018) were compatible with current results in the resistant to vancomycin (79.5%) and tetracycline (77.1%), but different in their resistance to both norfloxacin (52.1%) and ampicillin (27.1%). Significant differences in rates of multiple-drug resistant *E. faecalis* detection were observed for different countries which could be due to the different regulations and policies pertaining to antibiotic use in animals, the sensitivity of detection methods, number and kinds of examined samples (Gulhan *et al.*, 2015; Rehaiem *et al.*, 2016).

5. CONCULOSION

From this study we can conclude that, the high degree of contamination of most foods analyzed is an indicator of how high the probability of colonization by these microorganisms of the human intestine. *Enterococci*, however, do not represent a serious risk to the immunocompetent population and should be considered not only as potential pathogens, but also as a reservoir of genes encoding for antibiotic resistance that can be transferred to other pathogenic and nonpathogenic microorganisms. Moreover, considering that *Enterococcus* spp. isolated from foods and clinical samples, are becoming resistant to an increasing number of antibiotics.

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