Genotyping and resistance genes of *Enterococcus Faecalis* isolated from different food sources in 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 tetK and floR genes were present in the all 7 *E. faecalis* isolates. While, vanA gene was detected in 3 isolates, and mphC 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 ἐντερόκοκκος, énterokókkos, “granule”) is a large genus of lactic acid bacteria of the phylum Firmicutes (http://www.en.wikipedia.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 (Buyukyorum 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.wikipedia.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
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 means 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Amplification Product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>F GGT TAT GTC GCA AAG GAT CAT AAGG</td>
<td>710 bp</td>
<td>Ziekin et al. (2008)</td>
</tr>
<tr>
<td>aprC</td>
<td>F GAG ACT ACG CAG ACC GAC CTG GAC</td>
<td>722 bp</td>
<td>Schlegel a et al. (2008)</td>
</tr>
<tr>
<td>norA</td>
<td>F TGC AGG GCT CAA GAA GCT GAA</td>
<td>620 bp</td>
<td>Pournamd et al. (2014)</td>
</tr>
<tr>
<td>sepK</td>
<td>F GAT GAA GAA TAT CTA TAT GAT</td>
<td>300 bp</td>
<td>Duran et al. (2012)</td>
</tr>
<tr>
<td>floR</td>
<td>F TTG GCC GCT TCT TGC CAT AGT</td>
<td>404 bp</td>
<td>Dittke et al. (2008)</td>
</tr>
<tr>
<td>vanA</td>
<td>F CAT GCG ATG GTC TAA GTC</td>
<td>835 bp</td>
<td>Patel et al. (1997)</td>
</tr>
</tbody>
</table>

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).

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

<table>
<thead>
<tr>
<th>Food Source</th>
<th>Samples</th>
<th>No. of Enterococcus Species %</th>
<th>E. faecalis %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cray fish</td>
<td>10</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Camomile</td>
<td>10</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>Calendula</td>
<td>10</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Basil</td>
<td>10</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Water</td>
<td>10</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>Beef burger</td>
<td>10</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Milk</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cheese</td>
<td>10</td>
<td>15</td>
<td>0</td>
</tr>
</tbody>
</table>

* 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, camomile, 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 the the tested isolates were positive to ampicillin and penicillin as shown in table (3).

3.3. Detection of resistance genes of E. faecalis: A PCR was designed to detect tetK for tetracyclines, florR for chloramphenicol, mphC for macrolides, norA for quinolones, and vanA 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 tetK and florR, while only two isolates
(28.57%) had a band compatible with mphC and only three isolates (42.86%) had a band compatible with vanA

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

<table>
<thead>
<tr>
<th>Antibiotic Discs</th>
<th>2</th>
<th>4</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin, 30 µg</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Erythromycin, 15 µg</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>Ciprofloxacin, 5 µg</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Chloramphenicol, 30 µg</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>I</td>
</tr>
<tr>
<td>Tetracycline, 30 µg</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Norfloxacin, 10 µg</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Ampicillin, 10 µg</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Penicillin, 10 µg</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>


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 (tetK, floR, mphC, norA, vanA).

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 (20.8%) 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 (Rehaim 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 iso-
lated 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 pre-
vious 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 Horutik 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. CONCLUSIONS

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 microorga-
nsisms of the human intestine. Enterococci, however, do not
represent a serious risk to the immunocompetent population
and should be considered not

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