Molecular detection of virulence and resistance genes of Enterococcus species isolated from milk and milk products

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Abstract

Enterococcus species are one of the most serious pathogens that could pose risk to animal and human also a reservoir of genes encoding antibiotic resistance which can be transferred to other microorganisms. In the last decade, Enterococci become the most frequently reported cause of an abdominal or pelvic infection, septicemia, and enterococcal meningitis in humans. So, this study aimed to focus alight on virulence of these microbes and to show the linkage between antimicrobial resistance and biofilm formation in Enterococcus spp. A total of 120 samples of raw milk and cheese were collected from different markets in Qena province, Egypt. These samples were suspected for microbial analysis as culture, biochemical method, VITEK2 system and PCR technique for detection of resistance and virulence genes. The conventional identification showed that 30 isolates were suspected to be Enterococcus spp., VITEK 2 system confirmed the isolates as Enterococcus and classified them into 15 isolates as Enterococcus faecalis (E. faecalis), 10 isolates as Enterococcus faecium (E. faecium) whereas the least species were 5 isolates were Enterococcus gallinarum (E. gallinarum). also, our isolates showed their ability to form moderate and weak of biofilm with percentage%66.67% and 16.67% respectively. Also, the results of antimicrobial susceptibility showed high resistance against penicillin, Erythromycin, Amoxicillin, Nitrofurantoin and Cefotaxime. Our study is fueling the concern on a significant relationship between biofilm formation and specific types of antimicrobial resistance in Enterococcus spp. as Cefotaxime, Penicillin, Ampicillin and Erythromycin. Ten isolates were selected and tested by PCR for detection antimicrobial resistance and biofilm genes. The isolates obtained in this study harbored different antimicrobial resistance genes included ermB (80%), blaZ (100%) and vanA (30%) and biofilm formation genes included hyl and ef3314 (100%), esp (90%). Finally, we included, the deterrence of the contamination by Enterococcus spp. in milk and its products becomes necessary by applying strict hygienic measure.

Keywords: Antimicrobial resistance, Dairy products, Enterococcus, VITEK2 system.

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Introduction

Enterococci become one of the most common nosocomial pathogens in patients with high mortality rates. In the last decade, Enterococci become the second most frequently reported cause of surgical wound infections, urinary tract infections and the third most frequently reported cause of bacteraemia in humans and endocarditis (Said et al., 2021).

Enterococci are gram-positive cocci that commonly in pairs or short chains, non-spore forming, catalase negative, facultative anaerobic bacteria, resist to adverse conditions as low PH (4.5-10.0), high salinity and high temperature (5-65ºC) (Bondi et al., 2020). Genus Enterococcus classified into 67 species such as; E. faecalis, E. faecium, Enterococcus durans, Enterococcus casseliflavus and E. gallinarum (Parte et al., 2014).

Several virulence factors contribute to the ability of Enterococci to cause several diseases (Ashraf et al., 2019) as Enterococcal surface protein encoded by esp gene that promote the colonization of various surfaces and adherence to abiotic surface, so it plays a great role in biofilm formation also Hyaluronidase (hyl) gene act as spreading factors, that are able to breakdown the hyaluronic acid its targets the mucopolysaccharides in the connective tissue and cartilage, thus promoting dissemination of Enterococci within the host’s tissues so increases bacterial invasion.

Other surface protein that shares to the virulence determinants as ef3314 gene species specific for E. faecalis strains isolated of food and animal sources (Creti et al., 2009). The significant importance of ef3314 is related to biofilm-associated proteins (Cucarella et al., 2001). Also, gelatinase (Cosentino et al., 2010) and aggregation substance are a virulence factor, cytolysin (cyl), bacteriocins, hemolysin/cytolysin, a serine protease and collagen-binding protein. Enterococci can cause severe infections with the ability to resist antimicrobial agents and biofilm formation (Kristich et al., 2014).

Enterococcus spp. are resistant to various antimicrobial agents usually used in hospitals like Vancomycin, Tetracycline, B-lactams, Erythromycin glycopeptides, linezolid (Abbo et al., 2019), numerous genes are responsible for antimicrobial resistance, these genes as vanA (conferring resistance to Vancomycin), optrA (conferring resistance to Linezolid), tet (conferring resistance to tetracycline), ermB (conferring resistance to Erythromycin), blaZ (conferring resistance to B-lactamase) (Mannu et al., 2003).

Enterococci can form biofilm which contribute to their virulence and antibiotic resistance and facilitate adaptation to tough environmental conditions (Moghadam et al., 2015), several genes involved in biofilm production these genes were detected by PCR as geIE, agg, ef3314, ace, esp and hyl genes (Vankerckhoven et al., 2004).

The antimicrobial resistance shared with the ability of a biofilm production this made genus Enterococcus as one of the most serious pathogens that could pose risk to health and life. That is why it is important to study and control of these microbes in different groups, both in human and animals to prevent dangerous development of epidemics in hospital environments or massive damages among farm animals.

Molecular technique is the most accurate and effectively methods that can rapidly identify Enterococci spp. s in a short period through species-specific protein segments and can detect genes responsible for antimicrobial resistance and biofilm formation of Enterococci spp. (Fang et al., 2012).
The aim of this study was identification of Enterococcus spp. from raw milk, kareish and feta cheese by Vitek identification system, detection of biofilm formation, evaluation the susceptibility of Enterococcus isolates to several types of antibiotics, and molecular detection of resistance genes (vanA, ermB, optrA and blaZ) and virulence genes (hyl, esp and ef3314) by PCR.

Materials and methods

Ethical approval:

All procedures in the present study were performed and approved in accordance with the Ethics Committee of the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt.

Sample collection:

A total of 120 samples (40 samples for each of milk, kareish and feta cheese) were collected under aseptic condition from different markets in Qena province, during the period between January to February 2021, the samples were transported in ice box immediately to microbiological laboratory, faculty of veterinary medicine, south valley university.

Preparation of samples:

One grams of each sample was incubated in 9 ml of brain heart infusion broth over night at 37 °C (in milk samples), but in cheese samples one grams of each sample was taken and homogenized by sterile food blender for 1 min. then was incubated in 9 ml of brain heart infusion

Isolation and identification of Enterococcus spp.:

A loopful from incubated broth was streaked into the surface of bile aesculin azide agar plate then incubated for 24-48 hrs at 37 °C. The plates were examined for observing the suspected colonies (Benson et al., 2016). Identification of suspected colonies was performed by Gram staining and biochemical identification as catalase, oxidase and hydrolysis of aesculin (Igbinosa & Beshiru, 2019).

Identification of Enterococcus spp. by the VITEK 2 system:

In this study, Vitek GP (Gram positive) identification according to the cards with reference number 21342 was used to Enterococcus spp. identification (Philipp et al., 2015).

Detection of biofilm production by microtiter plate:

Biofilm formation

Isolates were tested for biofilm production by microtiter plate method as described earlier (stepanovi´ et al., 2007) with slight modifications. In details, our isolates were grown in TSB overnight at 37 °C. The optical density of suspension adjusted to almost 0.5 McFarland standard (at absorbance = 620 nm in a plate reader (ELx800, Biotek, USA). culture was diluted by 1:200 with TSB containing 1% glucose. Then 200 µl of each diluted culture was dispensed into three wells of a 96-well microtiter plate and incubated for 24 hr at 37 °C. Then the wells were washed with sterile phosphate-buffered saline (PBS) for three times for removal of non-adherent cells and inverted the plate and left it for air dryness. Then put 200µl of methanol in each well and removed it after 30 min then left plate for dryness 15 min. Adherent cells were dyed with 200 µl of crystal violet 1% for 15 minutes. Extra stain was washed with water gently. The dye attached to the adherent cells was resolubilized with 200 µl of glacial acetic acid 33%. In control wells, broth with no bacteria was added. In this study the ODc value was 0.8. Classification as followings: OD≤ ODnc, non-biofilm
Detection of Virulence and resistance genes by PCR:

DNA extraction:

Were done according to manufacture instruction of the QIAamp (Qiagen, Germany).

PCR Protocol:

PCR reaction was performed in a total volume of 25 µl as followed 12.5 µl PCR master mix, 5.5 µl PCR grade water, 1µl for each primer (forward and reverse) and 5µl template DNA. Specific primers used in this study for detection of virulence and antimicrobial resistance genes (Table 1). and PCR conditions used were illustrated in table (Table 2).

Statistical analysis:

By using SPSS software version 22 could be known relationship between different antibiotics resistance and biofilm formation among Enterococcus isolates. SPSS software was used for categorical variables and P value <0.05 was considered as significant

Results

Bacterial examination for the collected 120 samples (40 samples for each of milk, kareish and feta cheese) were carried on bile aesculin azide agar revealed that 30 samples produced dew drop like colonies surrounded by dark brown to black precipitate. Staining and biochemical tests showed gram positive cocii arranged in pair, negative catalase and oxidase test. The results of phenotypic identification showed that 30 suspected isolates (10 suspected isolates from milk samples, 15 suspected isolates from kareish cheese samples and 5 suspected isolates from feta cheese samples).
table 1. oligonucleotide primers sequences used to detect specific genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (3’-5’)</th>
<th>Length of amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyl</td>
<td>F-ACAGAAGAGCTGCAAGAAATG R-GACTGACGTTCAGTTTCCAA</td>
<td>276bp</td>
<td>Vankerckhoven et al. 2004</td>
</tr>
<tr>
<td>Esp</td>
<td>F-AGATTTTCATCTTTGATCTTGG R-AATTGATTTTACATCTGG</td>
<td>510bp</td>
<td>Vankerckhoven et al. 2004</td>
</tr>
<tr>
<td>ef3314</td>
<td>F-AGAGGGACGATCAGATGAAA R-ATCCCAATTGACAGATTCATTTC</td>
<td>566bp</td>
<td>Creti et al., 2004</td>
</tr>
<tr>
<td>VanA</td>
<td>F-CATGACGTATCGTAAATC R-ACCGGGCAGRGTATTGAC</td>
<td>885bp</td>
<td>Patel et al., 1997</td>
</tr>
<tr>
<td>blaZ</td>
<td>F-TACAACCTGTAATATCGAGGG R-CATTACACTCTTGGCGGTTTC</td>
<td>833bp</td>
<td>Bagcigil et al., 2012</td>
</tr>
<tr>
<td>optrA</td>
<td>F-AGGTTGTCAGCGAACTAA R-ATCAACTGTTCCCATTCA</td>
<td>1395bp</td>
<td>Wang et al., 2015</td>
</tr>
<tr>
<td>ermB</td>
<td>F-CATTACACGAGAATACTG</td>
<td>425bp</td>
<td>Schlegelova et al., 2008</td>
</tr>
</tbody>
</table>

Table 2. Condition of virulence and resistance genes in PCR protocol

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primary denaturation</th>
<th>Secondary denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>No. of cycles</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>hyl</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>55°C 30 sec.</td>
<td>72°C 30 sec.</td>
<td>35</td>
<td>72°C 7 min.</td>
</tr>
<tr>
<td>esp</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>50°C 40 sec.</td>
<td>72°C 45 sec.</td>
<td>35</td>
<td>72°C 10 min.</td>
</tr>
<tr>
<td>ef3314</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>55°C 40 sec.</td>
<td>72°C 45 sec.</td>
<td>35</td>
<td>72°C 10 min.</td>
</tr>
<tr>
<td>vanA</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>50°C 40 sec.</td>
<td>72°C 50 sec.</td>
<td>35</td>
<td>72°C 10 min.</td>
</tr>
<tr>
<td>blaZ</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>50°C 40 sec.</td>
<td>72°C 50 sec.</td>
<td>35</td>
<td>72°C 10 min.</td>
</tr>
<tr>
<td>optrA</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>53°C 1 min.</td>
<td>72°C 1 min.</td>
<td>35</td>
<td>72°C 12 min.</td>
</tr>
<tr>
<td>ermB</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>51°C 40 sec.</td>
<td>72°C 45 sec.</td>
<td>35</td>
<td>72°C 10 min.</td>
</tr>
</tbody>
</table>

Using of VITK 2 system for identification of Enterococcus spp. between the suspected isolates, the isolates was differentiated into 15 isolates as E. fecalis (50%) followed by 10 isolates as E. faecium (33.33%) whereas the least 5 isolates were identified as E. gallinarum (16. 67%) (Table 3). These 30 isolates were classified into moderate, weak and negative biofilm production (%66.67%, 16.67%, 16.67%) respectively as illustrated in (Table 4).

Incidence of Enterococcus spp. in milk samples, kareish cheese and feta cheese samples is shown in Fig. 1.

By using disc diffusion technique, all 30 isolates were resistant against different antibiotics by different average as Penicillin and Erythromycin resistant (66.67%), Ampicillin resistant (56.67%), Amoxicillin, Nitrofurantoin and Cefotaxime resistance (60%), Tetracycline and Vancomycin
resistance (36.67%), these result are shown in Table (5) and some *Enterococcus* isolates show multiple drug resistance as shown in Table (6).

![Bar graph showing incidence of Enterococcus spp. in milk samples, kareish cheese and feta cheese samples.](image)

**Fig. 1.** Incidence of *Enterococcus spp.* in milk samples, kareish cheese and feta cheese samples.

**Table 3.** Results of *Enterococcus* identification by using VITEK2 system.

<table>
<thead>
<tr>
<th>Species</th>
<th>Kareish cheese (40)</th>
<th>Milk (40)</th>
<th>Feta cheese (40)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>9 (22.5%)</td>
<td>5 (12.5%)</td>
<td>1 (2.5%)</td>
<td>12.5%</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>4 (10%)</td>
<td>3 (7.5%)</td>
<td>3 (7.5%)</td>
<td>8.33%</td>
</tr>
<tr>
<td><em>E. gallinarum</em></td>
<td>2 (5%)</td>
<td>2 (5%)</td>
<td>1 (2.5%)</td>
<td>4.2%</td>
</tr>
</tbody>
</table>

**Table 4.** The correlation between phenotypic ad genotypic biofilm production among *Enterococcus* isolates

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Micro titer plate</th>
<th>Genotypic detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moderate</td>
<td>Weak</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 5. Result of sensitivity of different *Enterococcus* isolates to different antibiotic discs

<table>
<thead>
<tr>
<th>Antibiotic disc</th>
<th>(Sensitive)</th>
<th>(Intermediate)</th>
<th>(Resistant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of isolates</td>
<td>%</td>
<td>No. of isolates</td>
</tr>
<tr>
<td>Penicillin (P)</td>
<td>4</td>
<td>13.33%</td>
<td>6</td>
</tr>
<tr>
<td>Ampicillin (AM)</td>
<td>6</td>
<td>20.00%</td>
<td>7</td>
</tr>
<tr>
<td>Amoxicillin (AX)</td>
<td>9</td>
<td>30.00%</td>
<td>3</td>
</tr>
<tr>
<td>Tetracycline (TE)</td>
<td>12</td>
<td>40.00%</td>
<td>7</td>
</tr>
<tr>
<td>Vancomycin (VA)</td>
<td>8</td>
<td>26.66%</td>
<td>11</td>
</tr>
<tr>
<td>Linezolid (LZ)</td>
<td>15</td>
<td>50.00%</td>
<td>5</td>
</tr>
<tr>
<td>Nitrofurant (F)</td>
<td>8</td>
<td>26.67%</td>
<td>4</td>
</tr>
<tr>
<td>Erythromycin (E)</td>
<td>3</td>
<td>10.00%</td>
<td>7</td>
</tr>
<tr>
<td>Cefotaxime (CTX)</td>
<td>8</td>
<td>26.66%</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 6. The correlation between phenotypic and genotypic antibiotics resistance among *Enterococcus* isolates

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th><em>Enterococcus</em> spp.</th>
<th>Multidrug resistance isolates (vito)</th>
<th>Genotypic detection (vivo)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>ermB</em></td>
<td><em>optrA</em></td>
</tr>
<tr>
<td>1</td>
<td><em>E. faecalis</em></td>
<td>P, AM, AX, TE, F, CTX</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>E. faecalis</em></td>
<td>P, AM, AX, F, E, CTX</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td><em>E. faecium</em></td>
<td>P, AM, AX, TE, VA, E, F, CTX</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td><em>E. faecium</em></td>
<td>P, AM, AX, F, E, CTX</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td><em>E. faecium</em></td>
<td>P, AM, AX, TE, CTX</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td><em>E. faecium</em></td>
<td>P, AM, AX, TE, E, CTX</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td><em>E. gallinarum</em></td>
<td>P, AM, AX, E, CTX</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td><em>E. gallinarum</em></td>
<td>P, AM, AX, VA, E, F, CTX</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td><em>E. faecium</em></td>
<td>P, AM, AX, VA, F, E, CTX</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td><em>E. faecalis</em></td>
<td>P, AM, AX, F, E, CTX</td>
<td>+</td>
</tr>
</tbody>
</table>

PCR technique could detect the genes of virulence and antimicrobial resistance in 10 isolates of *Enterococcus*, Virulence genes as *hyl*, *ef*314 genes distributed among 100% and *esp* in 90% of our isolates and antimicrobial resistance genes as *blaZ*, *ermB*, *optrA* and *vanA* distributed in 100%, 80%, 0%, and 30% respectively. The most isolates that harbored virulence factors was *E. faecium* isolates. Finally, by using SPSS software showed significant linkage among biofilm formation and different types of antibiotics such as (Cefotaxime, Penicillin, Ampicillin and Erythromycin) These results are illustrated in (Figs 2-8) and (Table 7).

Percentage of antimicrobial and virulence genes in different isolated strains are shown in Fig. 9.

Fig. 2. Agarose gel electrophoresis of *blaZ* gene amplification at 833bp. lane L: ladder (100bp), Lane P: control positive, Lane N: control negative, Lanes 1:10: positive isolates for *blaZ* gene.
Discussion

Virulent Enterococcus spp. caused a major public health risk which could be transmitted to human by eating contaminated food that was considered as a major pathway for the food borne microorganism transmission to human. The existence of Enterococci in milk and its products was represented as an indication of lacking sanitary conditions during the production, collection of milk and processing of cheese (Larsen et al. 2010). Enterococci were considered not only potential microorganism, but they carry antibiotic resistance genes, so the infection by these microbes consider a serious health hazard this because they can’t be treated with known antibiotics (Pesavento et al., 2014).
There were various traditional diagnostic ways for identification of Enterococcus spp. such as isolation on selective media like bile aesculin azide agar and biochemical tests as oxidase and catalase test (Shukla et al., 2013). In this study Enterococcus spp. recovered from 25%, 37.5% and 12.5% in milk, kareish and feta cheese respectively on bile aesculin azide agar. The incidence of Enterococcus isolates from total examined samples was (25%), its nearly similar to this reported by Tyson et al. (2017) (28.6%). Higher incidence of Enterococcus (100%) was reported by Trivedi et al. (2011) while lower incidence of Enterococcus (20%) was recorded by Ozmen et al. (2010).

Vitek2 compact system is a dependable, fast technique and more accurate in detection and identification of Enterococcus spp.
Kareman et al. (2016), several studies supported identification of Enterococci by VITEK 2 system (Othman et al., 2019). Our 30 isolates were identified by VITEK2 system into 15 isolates as E. faecalis (%50), 10 isolates as E. faecium (%33.33) and 5 isolates as E. gallinarum (%17), this result was reinforced by those reported by Ligozzi et al. (2002) and Ashraf et al. (2019) who used VITEK2 system and found that E. faecalis with the highest percentage. In our study, it was found that the most prevalent Enterococcus spp. isolated from milk and dairy products was E. faecalis and E. faecium this result was supported by this reported by Aslam et al. (2012).

In the present study, the percentage of Enterococcus spp. in raw milk was (25%), it agreed with Kim et al. (2012) which reported similar percentage (25.8%) while lower incidence (96%) was found in Australia by McAuely et al. (2015). Besides, incidence of Enterococcus spp. in Kareish cheese samples was (37.5%), higher incidence (34.5%) was reported by Pesavento et al. (2014) in retail cheese. The percentage of Enterococci in feta cheese in this study was (12.5%) while Furlaneto-Mia et al., (2014) reported that all the examined feta samples were contaminated with Enterococcus spp.

Biofilm had been concerned in the pathogenesis of enterococcal infection (Sandoe JAT et al., 2003). The percentage of biofilm formation in Enterococcus isolates (47%) comparable to that was described in last studies (38–93 %) (Comerlato et al., 2013; Gozalan et al., 2015), also associated with E. faecalis (86.67 %) E. faecium (80 %), this result higher than that reported by Comerlato et al. (2013). In this study, it was found that Enterococcus spp. were biofilm producer with various degree of intensity, more frequently by E. faecalis isolates than other species, this result was supported by Necidova et al. (2009). Also, E. faecalis had virulence genes more than the other species as reported by GolobM et al. (2019). Our result was reinforced by Gawryszewska et al. (2017) who recorded that the most frequently species associated with diseases was E. faecalis.

In the present study, we noted a significant relationship between biofilm production and antimicrobial resistance. The structure of biofilm provides an ideal microenvironment for development and facilitates transmission of mobile genetic elements among bacteria (Sieńko et al., 2015).

Enterococci were resistant to a wide variety of antibiotics used therapeutically (Mannu et al., 2003), our results showed multidrug resistance, high percentage of resistance were observed to B-Lactamase, Erythromycin, Cefotaxime and Nitrofurantoin. These antimicrobial groups are often used in human and veterinary medicine, especially in enteric infections. The resistance to penicillin between our isolates was 87% of E. faecalis, 50% of E. faecium and 40 % E. gallinarum, the frequency of Ampicillin resistance in Enterococcus isolates was (56.67%) and it was higher than which told by Wu et al. (2016),but Tuncer et al. (2013) noted that all Enterococcus isolates spp. were sensitive to Ampicillin. Generally, the resistance of B-lactamase spp. were sensitive to Ampicillin. Generally, the resistance of B-lactamase group in the current study (61%) this percentage was higher than (42.8%) that reported by Mohamed and El-sayed et al. (2016).
The resistant to Erythromycin in this study with different percentage in different species of Enterococcus (60% of E. faecalis isolates, 70% of E. faecium isolates and 80% of E. gallinarum isolates), mostly the resistance level of Erythromycin detected in Enterococci isolates (66.67%) was higher than those informed via Ashraf et al. (2019), in other side this result was lower than that reported by Ahmadpoor et al. (2021) (77.7%), also all isolates highly resist to cefotaxime (60%), which was lower than that noted by El Malt et al. (2015) (67.6%). In current study, 36.67% of Enterococcus isolates were resist to vancomycin, while Pesavento et al., (2014) reported low percentage of resistance to vancomycin among Enterococcus isolates (3.53%). Oguntiyinbo and Okueso. (2012) reported similar vancomycin resistance (33.7%).

In the previous few years, PCR was used due to molecular technique more accurate and decrease misidentification of conventional identification methods (Fang et al.,2012). Of the most important virulence genes of Enterococci were hyl, esp and ef3314 which were studied in this study. hyl and ef3314 were the most predominant genes with percentage 100% (10/10), this result higher than 26.3% that recorded by Xin et al., 2020). In this study, esp was detected with 90 % (9/10), that was higher than that noted by (Neda et al.,2022) (51.7%). Among the 10 biofilm-producing strains, 9 isolate had esp gene, so a strong link between the biofilm formation and presence of the esp gene as recorded(Tsikrik onis et al., 2012) however other investigators unsuccess to find as an relationship (Comerlato et al., 2013).Also the percentage of esp gene in E.fecalis was (75%) , E. faecium(100%),else hyl and ef3314 gene had major role in biofilm production .

In other side, a several of genes had been detected especially for antimicrobial resistance in different Enterococcus spp. as(vanA, blaZ , ermB and optrA ) ,the result of vancomycin resistance phenotypically (36.67%) , In the other side genotypically had nearly percentage of van A gen(30%) (3/10), in E. faecalis , E. faecium and E. gallinarum , This percentage was higher than that found by Erbas et al., (2016) while Furlaneto-Maia et al., (2014) detected that the vanA gene was identified in 100% of Enterococci isolates. On the other side, none isolates of Enterococcus had vanA gene that was informed by Franciosi et al., 2009).

Other gene of antimicrobial resistance ermB gene was detected in (6/10) (60%), which was lower than % 92.6 this percentage was noted in Turkey ( Erbas et al., 2016), While the ermB gene was not identified in any isolates (Gaglio et al., 2016).As well as we obtained in this study , existence of blaZ gene in (10/10) (100%) that responsible for resistance of B-lactmase group, existing of this gene was altered between studies for examples Carla et al.,2011(%15) in meat samples. Finally, none of all isolates screened by PCR not carried optrA gene. In the other side, Wedad et al.,2021 noted high percentage of the optrA gene in her study.

Conclusion

VITEK2 system has a great role in identification different species of Enterococcus that contaminated our samples as E. fecalis, E. faecium and E. gallinarum , PCR was more accurate method for detection of virulence and
resistance factors that confirm pathogenicity of Enterococcus, so must be strict hygiene in milk and its product especially in markets.

**Conflict of interest statement**

The authors declare that they have no conflict of interest.

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**References**


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CLSI. (clinical and laboratory standards institute) (2018). Performance Standards for Antimicrobial Susceptibility Testing, Clinical and Laboratory Standards Institute 950 West Valley Road, Suite 2500. Wayne, PA 19087 USA.


El-Malt RMSA (2015). Molecular studies on esp gene in multiple antibiotic resistant Enterococci. A thesis (M. V. Sc), Bacteriology, Immunology and Mycology department, Faculty of Vet. Medicine, Zagazig University.


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Kareman AE, and Wageih SE, Radwa ME (2016). Comparative study between molecular and non-molecular methods used for detection of Vancomycin Resistant Enterococci in Tanta University Hospitals, Egypt. Life Science Journal; 13(1s).


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Emerging Infect. Dis, 16: 682–684.


Ozmen ST, Celebi L and Temiz A (2020). virulence genes, antibiotic resistance and plasmid profiles of Enterococcus faecalis and Enterococcus faecium from naturally fermented Turkish food. J.Appl.Microbiol, 1365-2672.04763


Pesavento GC, Calonico B, Ducci A, Magnanini and A Lo Nostro (2014). Prevalence and antibiotic resistance of Enterococcus spp., isolated from


