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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 *erm*B (80%), *bla*Z (100%) and *van*A (30%) and biofilm formation genes included *hyl* and *ef*3314 (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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Competing interest: The authors have declared that no competing interest exists.

Introduction

Enterococci become one of the most common nosocomial pathogens in patient with high mortality rate. 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 chain, non-spore forming, catalase negative, facultative anaerobic bacteria, resist to adverse condition 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 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 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 *van*A (conferring resistance to Vancomycin), *optr*A (conferring resistance to Linezolid), *tet* (conferring resistance to tetracycline), *erm*B (conferring resistance to Erythromycin), *bla*Z (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 *gel*E , *agg*, *ef3*314, *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 (*van*A, *erm*B, *opt*rA and *bla*Z) and virulence genes (*hyl*, *esp* and *ef*3314) 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 ºC. 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

producer: ODc<ODs≤2×ODc, weak biofilm producer: 2× ODc< OD≤ 4× ODc, medium biofilm producer: 4×ODc< OD, strong biofilm producer.

Antibiotic susceptibility test :

It was performed by standard disc diffusion method using these antibiotic discs: Penicillin (P), Ampicillin (AM), Amoxicillin (AX), Tetracycline (TE), Erythromycin (E), Vancomycin (VA), Nitrofurantoin (F) , linezolid(LZ) and Cefotaxime (CTX) Using Kirby-bauer disk diffusion method.

Briefly antibiotic susceptibility testing a loopful of cultured isolates was inoculated into 2 ml Muller Hinton Broth, and incubated for 4 hours at 37ºC, the concentrates of culture broth was adjusted to a 0.5 Mcfarland standard, then by a sterile cotton swab was dipped into the culture broth. Press the swab against the sides of the suspension tube to remove extra liquid, then apply it to the agar (Muller Hinton Media with 5% sheep RBCS). The swab was swabbed all the plate, moving from side to side all the way from the top to the bottom of the agar before it, the plate was swabbed in three directions by the swab.

The discs of antibiotic were gently pressed to sure their contact with the inoculated plate and incubated at 37ºC. The plates were checked after 18-24h and the zones of inhibition were measured by antibiotic susceptibility scale.

The zone diameter for different antibiotics agents was explained into susceptible, intermediate and resistant categories as in the interpretation table according to (CLSI, 2018) then showed multidrug resistance in our isolates (resistance to ≥3 class of antibiotics).

Detection of Virulence and resistance genes by PCR:

DNA extraction:

Were done according to manfacture 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 cocci 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

Gene	Primer sequences (3'-5')	Length of amplified product	Reference
Hyl	F-ACAGAAGAGCTGCAGGAAATG	276bp	Vankerckhoven *et al*. 2004
	R-GACTGACGTCCAAGTTTCCAA		
Esp	F-AGATTTCATCTTTGATTCTTGG	510bp	Vankerckhoven *et al*. 2004
	R-AATTGATTCTTTAGCATCTGG		
***ef*3314**	F-AGAGGGACGATCAGATGAAAAA	566bp	Creti *et al.*, 2004
	R-ATTCCAATTGACGATTCACTTC		
***Van*A**	F-CATGACGTATCGGTAAAATC	885bp	Patel *et al*., 1997
	R-ACCGGGCAGRGTATTGAC		
blaZ	F-TACAACTGTAATATCGGAGGG	833bp	Bagcigil *et al.*, 2012
	R-CATTACACTCTTGGCGGTTTC		
***optr*A**	F-AGGTGGTCAGCGAACTAA	1395bp	Wang *et al.*, 2015
	R-ATCAACTGTTCCCATTCA		
***erm*B**	F-CATTTAACGACGAAACTGGC	425bp	Schlegelova *et al.*, 2008
	R-GGAACATCTGTGGTATGGCG		

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

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
hyl	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
esp	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
ef3314	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
vanA	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 50 sec.	35	72˚C 10 min.
blaZ	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 50 sec.	35	72˚C 10 min.
optrA	94˚C 5 min.	94˚C 30 sec.	53˚C 1 min.	72˚C 1 min.	35	72˚C 12 min.
ermB	94˚C 5 min.	94˚C 30 sec.	51˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.

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).

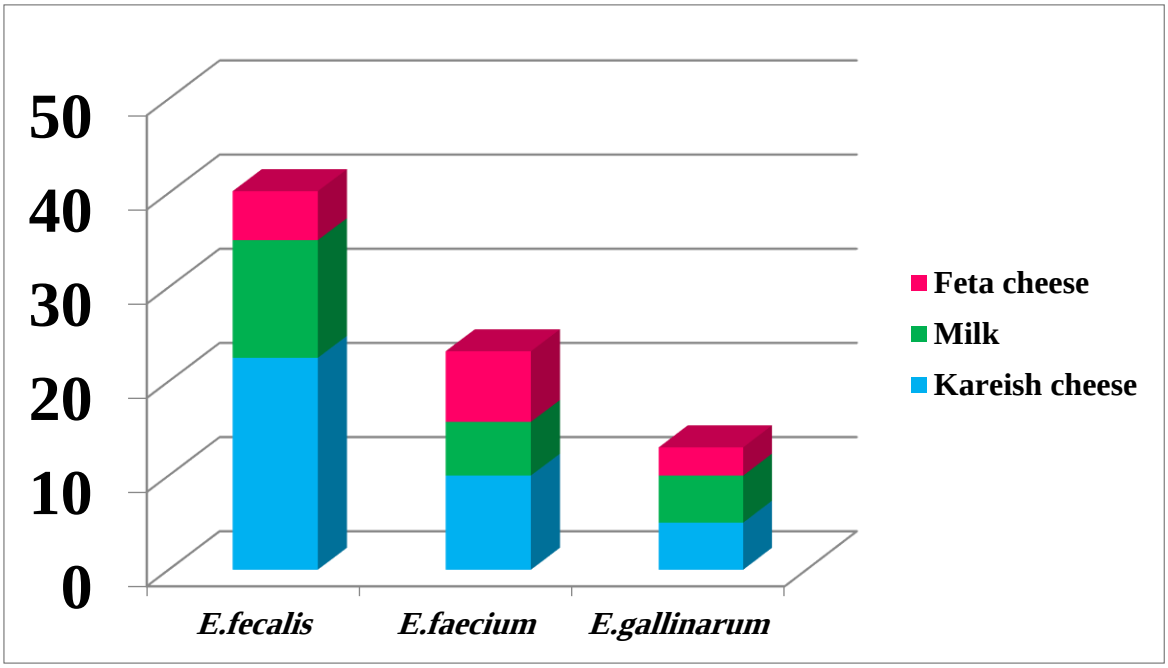

Fig. 1. Incidence of *Enterococcus spp.* in milk samples, kareish cheese and feta cheese samples.

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

Species(n)	Kareish cheese (40)	Milk (40)	Feta cheese (40)	Percentage (%)
E.faecalis	9(22.5%)	5(12.5%)	1(2.5%)	12.5%
E.faecium	4(10%)	3(7.5%)	3(7.5%)	8.33%
E.gallinarum	2(5%)	2(5%)	1(2.5%)	%4.2

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

Isolate No.	Micro titer plate		Genotypic detection		
	Moderate	Weak	*hyl*	*Esp*	*ef*3314
1	+	-	+	+	+
2	+	-	+	+	+
3	+	-	+	+	+
4	+	-	+	+	+
5	+	-	+	+	+
6	+	-	+	+	+
7	+	-	+	+	+
8	+	-	+	+	+
9	+	-	+	+	+
10	-	+	+	-	+

Table 5. Result of sensitivity of different *Enterococcus* isolates to different antibiotic discs

Antibiotic disc	(Sensitive)		(Intermediate)		(Resistant)	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
Penicillin(P)	4	13.33%	6	20.00%	20	66.67%
Ampicillin (AM)	6	20.00%	7	23.33%	17	56.67%
Amoxicillin (AX)	9	30.00%	3	10.00%	18	60.00%
Tetracycline (TE)	12	40.00%	7	23.33%	11	36.67%
Vancomycin (VA)	8	26.66%	11	36.67%	11	36.67%
Linezolid (LZ)	15	50.00%	5	16.67%	10	33.33%
Nitrofurtan (F)	8	26.67%	4	13.33%	18	60.00%
Erythromycin (E)	3	10.00%	7	23.33%	20	66.67%
Cefotaxime (CTX)	8	26.66%	4	13.33%	18	60.00%

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

Isolate No.	*Enterococus* spp.	Multidrug resistance isolates(vitro)	Genotypic detection(vivo)			
			ermB	***optrA***	***blaZ***	***vanA***
1	***E.faecalis***	P, AM, AX, TE,F,CTX	-	-	+	-
2	***E.faecalis***	P, AM, AX,F,E,CTX	+	-	+	-
3	***E.faecium***	P, AM, AX, TE,VA, F,E,CTX	+	-	+	+
4	***E. faecium***	P, AM, AX ,F,E,CTX	+	-	+	-
5	***E.faecium***	P, AM, AX, TE,CTX	-	-	+	-
6	***E.faecium***	P, AM, AX, TE ,F,E,CTX	+	-	+	-
7	***E.gallinarum***	P, AM, AX, E,CTX	+	-	+	-
8	***E.gallinarum***	P, AM, AX ,VA, F,E,CTX	+	-	+	+
9	***E. faecium***	P, AM, AX ,VA, F,E,CTX	+	-	+	+
10	***E.faecalis***	P, AM, AX,F,E,CTX	+	-	+	-

PCR technique could be detect the genes of virulence and antimicrobial resistance in 10 isolates of *Enterococcus*, Virulence gens as *hyl* , *ef*3314 genes distributed among100% and *esp* in 90% of our isolates and antimicrobial resistance genes as *bla*Z, *erm*B, *optr*A and *van*A 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.

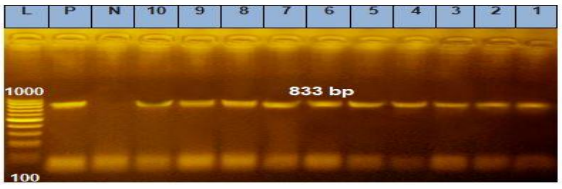

Fig. 2. Agarose gel electrophoresis of *blaZ* gene amplification at 833bp, lane L: ladder (100bp), Lane P: control positive, Lane N: control negative, Lanes 1:10: positive isolates for *bla*Z gene.

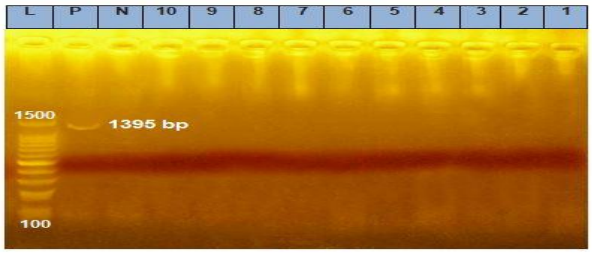

Fig. 3. Agarose gel electrophoresis of *opt*rA gene amplification at 1395bp, laneL: ladder (100bp), Lane P: control positive, Lane N: control negative, Lanes 1:10. negative isolates for *opt*rA gene.

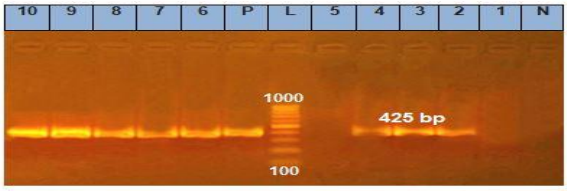

Fig. 4. Agarose gel electrophoresis of *erm*B gene amplification at 425bp, lane L: ladder (100bp), Lane P: control positive, Lanes 2, 3, 4, 6 ,7, 8, 9 and 10 :positive isolates for *erm*B gene while Lanes 1 and 5: negative isolates for *erm*B gene.

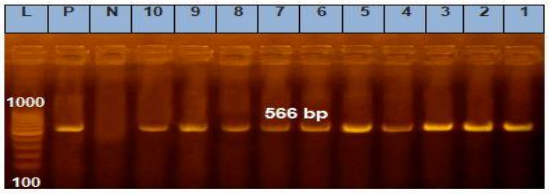

Fig. 5. Agarose gel electrophoresis of *ef*3314 gene amplification at 566bp, lane L: ladder (100bp), Lane P: control positive, Lane N: control negative, Lanes 1:10 positive isolates for *ef*3314 gene.

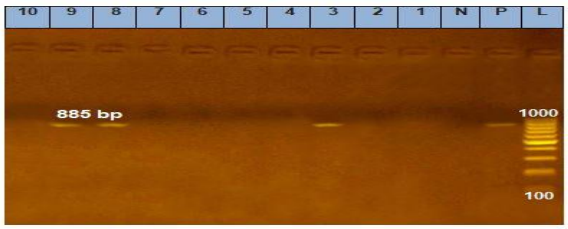

Fig. 6. Agarose gel electrophoresed of *van*A gene amplification at 885bp, laneL: ladder (100bp), Lane P: control positive, Lane N: control negative, Lanes 3, 8 and 9: positive isolates for *van*A gene while Lanes 1, 2, 4, 5, 6, 7 and 10: negative isolates for *van*Agene.

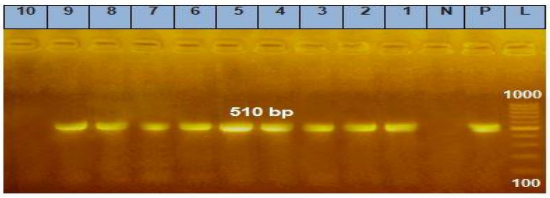

Fig. 7. Agarose gel electrophoresis of *esp* gene amplification at 510bp, lane L: ladder (100bp), Lane P: control positive, Lane N: control negative, Lanes 1: positive isolates for *esp* gene except lane 9.

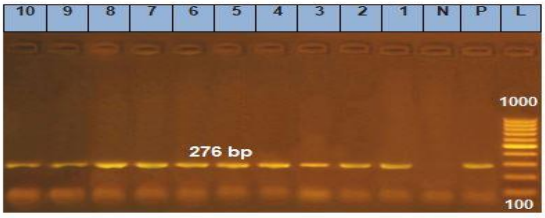

Fig. 8. Agarose gel electrophoresis of *hyl* gene amplification at 276bp, lane L: ladder (100bp), Lane P: control positive, Lane N: control negative, Lanes1:10: positive isolates for *hyl* gene.

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).

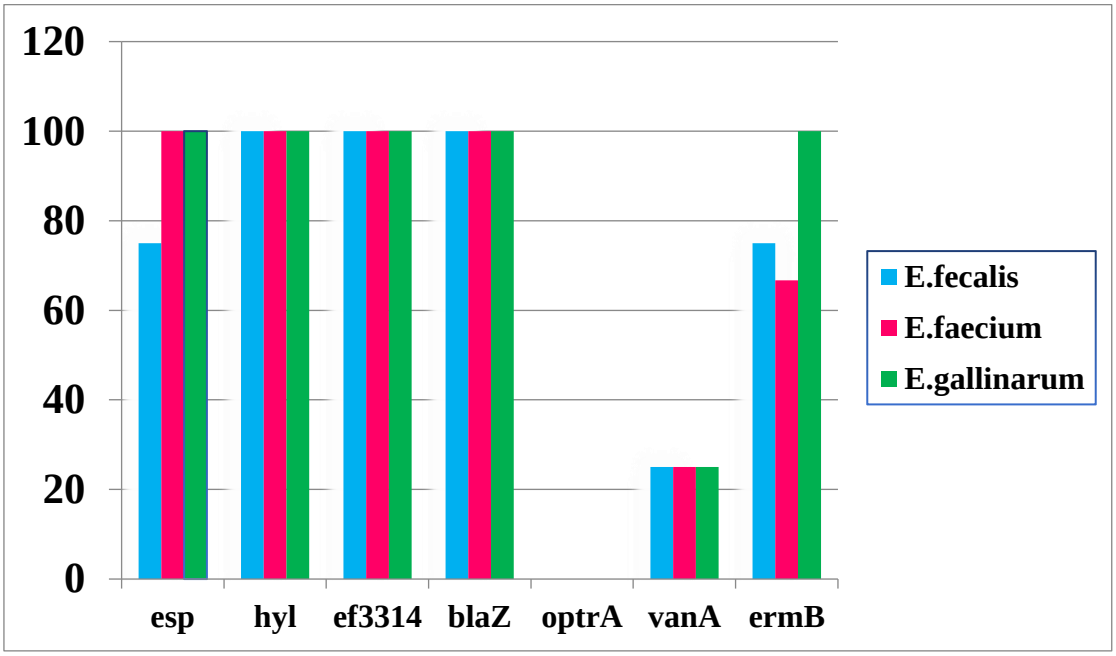

Fig. 9. Percentage of antimicrobial and virulence genes in different isolated strains.

Table 7. Detection of antibiotics genes and biofilm genes among Enterococcus isolates by PCR

Sample	Antibiotics genes				Biofilm genes		
	ermB	*optrA*	*blaZ*	vanA	*hyl*	*esp*	*ef3314*
1	-	-	+	-	+	+	+
2	+	-	+	-	+	+	+
3	+	-	+	+	+	+	+
4	+	-	+	-	+	+	+
5	-	-	+	-	+	+	+
6	+	-	+	-	+	+	+
7	+	-	+	-	+	+	+
8	+	-	+	+	+	+	+
9	+	-	+	+	+	+	+
10	+	-	+	-	+	-	+

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 thoes 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 a*l., 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 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%). Oguntoyinbo 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 *ef*3314 which were studied in this study. *hyl* and *ef*3314 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 *ef*3314 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(*van*A, *bla*Z , *erm*B and *opt*rA) ,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. fecalis , 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 *van*A gene was identified in 100% of *Enterococci* isolates. On the other side, none isolates of *Enterococcus* had *van*A gene that was informed by Franciosi *et al*., 2009).

Other gene of antimicrobial resistance *erm*B 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 *erm*B gene was not identified in any isolates (Gaglio *et al*., 2016).As well as we obtained in this study , existence of *bla*Z 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 *optr*A 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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