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ORIGINAL ARTICLE

Prevalence of Vancomycin Resistant Enterococci in Different Food Samples

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Prevalence, Enterococci, Vancomycin resistant Enterococci, Food Samples

ABSTRACT

Background: *Enterococci are known to be a leading cause of nosocomial infections. The threat of enterococcus infections becomes more serious in light of its increasing antimicrobial resistance especially to vancomycin and quinupristin-dalfopristin that are often considered the last treatment available in serious multi-drug resistant infections. Enterococci also can transfer resistance determinants to other bacterial genera.* ***Objectives:*** *The aim of this study was to evaluate the prevalence of vancomycin resistant enterococci in different food samples of animal and plant origin collected from different supermarkets and groceries.* ***Methodology:*** *Two hundred and fifty different food samples (100 meat and fish, 100 vegetables and 50 pasteurized milk and cheese) were processed and cultured on CATC agar a selective medium for detection of enterococci. API 20 Strep kit was used for species identification. All isolates were tested for antibiotic susceptibility by ATB ENTEROC 5 commercial kit. Vancomycin resistant genes were investigated by PCR among vancomycin non susceptible isolates.* ***Results:*** *Enterococci were isolated from 61.2% of the studied samples (153/250). Unpacked cheese showed the highest frequency (93.3%) followed by meat (80.7%), fish (70.5%) and vegetables (60%). Neither pasteurized milk nor packed cheese was positive for enterococci. E.faecium, was the most prevalent species identified among all isolates 86/153 (56.2 %), followed by E. avium 32/153 (21%), E. faecalis 28 /153 (18.3 %) and E.gallinarum\ E.casseliflavus 7/153 (4.5%). E.faecium was the most prevalent species detected in meat, fish and vegetable {45/67 (67%), 7/12 (58.3%) and 33/60 (55%)} respectively, while E.avium was the most predominant species detected in cheese 7/14 (50%). Isolated Enterococcus species were found to be resistant to erythromycin, tetracycline, rifampicin, chloramphenicol, ciprofloxacin, quinupristin-dalfopristin and vancomycin. Vancomycin resistance was detected in 10/153 (6.5%) of enterococcus isolates. Three vancomycin resistance genes were detected by PCR including vanA, vanC1 and vanC2 genes.* ***Conclusion:*** *our results point out to the fact that raw products of animal origin are possible reservoirs of multi-drug resistant enterococci in the food chain.*

INTRODUCTION

Enterococci are Gram-positive, air tolerant, catalase-negative cocci. They are part of the normal micro-flora in the gastrointestinal tract as well as faeces of vertebrates, being the most abundant Gram-positive cocci in humans'intestine[1]. The genera comprise more than 20 species, but *E.faecium, E.faecalis E.durans, E.avium, E.raffinosus, E.gallinarum, and E.casseliflavus* are the most prevalent species[2-4]. Some *Enterococci* are bacteriocinogenic and capable of inhibiting the growth of certain pathogens and spoilage microorganisms, presenting a great potential in food preservation. Due to the high acid and salt tolerance, enterococci may be used in food fermentations as starter cultures, being responsible for the formation of unique flavors[3,5,6].

However, in immunocompromised individuals *Enterococci* may lead to nosocomial infections causing urinary tract infections, serious wound infections, bacterial endocarditis, meningitis, neonatal sepsis and intra-abdominal and pelvic infections. The majority of these infections are caused by *Enterococcus faecium* and *Enterococcus faecalis*[7]. Antimicrobial agents are widely used as feed additives for growth promotion in animal husbandry[8]. Avoparcin is a glycopeptide antibiotic used for this purpose in poultry, and it appears

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to be associated with the emergence of resistance to glycopeptides in general[9 -11].

Enterococci are intrinsically more resistant than many other bacteria to antimicrobial agents commonly used in hospitals as ampicillin, amoxicillin\ clavulanic acid, tetracycline, erythromycin and vancomycin. However, wide spread use of vancomycin in hospitals likely contributed to the emergence and dramatic increase of vancomycin-resistant enterococci (VRE) over the past 20 years[12]. It was reported that enterococci could be isolated from food of both animal (meat, poultry, turkey, milk, cheeses... etc.) and vegetable origin (potatoes, tomatoes, radish, cucumber, and many others). Enterococci strains isolated from foods were resistant to antibiotics and presented virulence genes[3, 13-14].The differentiation of apparently safe and non-safe enterococcus strains is not simple, especially because virulence genes can be easily exchanged between strains [2, 12, 15-16]. The aim of this study was to evaluate the prevalence of vancomycin resistant enterococci in different food samples of animal and plant origin collected from different supermarkets and groceries.

METHODOLOGY

Samples:

The present study was carried out on 250 different food samples including 83 raw and processed meat (beef, chicken and turkey), 17 fish, 100 vegetables (cucumber, tomato, carrot, onion, radish and green pepper), 35 milk (pasteurized & boiled) and packed cheese (sliced cheddar, Feta and cream cheese) and 15 mozzarella and unpacked cheese (cheddar, molbo, cottage and ras cheese).

Sample processing:

Samples were cut by sterile disposable scalpels into small pieces. Five grams of each sample were placed in a falcon tube containing 45 ml of buffered peptone water, Shook manually and incubated at 37°C for 24 hours.

Isolation and identification of Enterococci

A loopfull was taken from each processed sample, cultivated on CATC agar plates (Merk Germany), and incubated at 37°C for 24 hours. Colonies were identified by colonial morphology and Gram stain. Obtained isolates of enterococci were identified to the species level using API 20 strep (Biomerieux-France) according to the manufacturer instructions.

Antibiotic susceptibility test:

Enterococcus isolates were tested for antimicrobial susceptibility using the ATB ENTROC 5 strip (Biomerieux - France). Four antibiotics were used at single concentration including: penicillin (8µg/ml), ampicillin (8µg/ml), gentamicin (500µg/ml) and Sulphathiazole (1000 µg/ml) while two concentration were used for erythromycin (0.5 & 4 µg/ml), tetracycline (4 & 8 µg/ml), chloramphenicol (8 &16 µg/ml), Rifampicin, (1 & 2 µg/ml), ciprofloxacin (1 & 2 µg/ml), levofloxacin (2-4 µg/ml), vancomycin (4 & 16 µg/ml), teicoplanin (8 &16 µg/ml), nitrofurantoin (32 & 64 µg/ml) and quinupristin-dalfopristine (1 & 2 µg/ml),

ATB test strips were inoculated with microbial suspensions made from fresh cultures with a turbidity equivalent to 0.5 McFarland according to manufacturer's instructions and incubated at 37°C for 24 hours. The growth in the cupules was observed visually. The result obtained classified the isolates as sensitive (no growth), intermediate resistant (growth only at a low concentration when the antibiotic was tested at two concentrations) or resistant (growth was observed at both concentrations).

Detection of vancomycin resistance genes

DNA extraction

Genomic DNA was extracted from each vancomycin resistant enterococcus isolate using prepMan ultra sample preparation reagent (Applied Biosystem, USA) according to the manufacturer instructions. DNA concentration and quality were assessed by measuring optical density at 260 and 280 nm with Nanodrop 2000c spectrophotometer (Thermo scientific, USA).

Polymerase Chain reaction

Each DNA sample was tested by PCR for vancomycin resistant genes; *van*A, *van*B, *van*C1 *and van*C2 using gene-specific primers according to Dutka-Malen *et al* [17]. Primer sequences and size of amplicons are listed in Table 1. PCR was done in a final volume of 50 µl consist of, 45-µL Platinum PCR SuperMix {a ready-made PCR amplification mixture containing: 22 mM Tris\ HCl, pH 8.4; 55 mM KCl; 1.65 mM $MgCl_2$; 220 µM each dNTP; 22 U recombinant Taq DNA polymerase/ml-(Invitrogen)}, 1 µL of each specific forward and reverse primers (200 nM final concentration) and 3 µL of template DNA. Cycling program was as follows; an initial denaturation at 95 °C for 2 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 54° C for 1 min. and extension at 74°C for 1 min. followed by final chain elongation step at 72°C for 10 min. A total of 10µl of PCR products were loaded into 2% agarose gel containing 5 µg/ml ethidium bromide using a 100-bp molecular size marker (Invitrogen) and visualized by transilluminator [17].

Table 1: Primer sequences and size of amplification products

Primer	*Nucleotide sequence of primer (5'–3')*	*Amplicon size (Pb)*
vanA	F- GGGAAAACGACAATTGC	732
	R-GTACAATGCGGCCGTTA	
vanB	F-ATGGGAAGCCGATAGTC	
	R-GATTTCGTTCCTCGACC	635
vanC1	F-GGTATCAAGGAAACCTC	822
	R-CTTCCGCCATCATAGCT	
vanC2	F-CTCCTACGATTCTCTTG	439
	R-CGAGCAAGACCTTTAAG	

F = forward primer; R = reverse primer.

Statistical analysis

The results were tabulated and statistically analyzed in terms of frequencies and percentages using Chi-square test (X 2) or Fisher exact (when the expected frequency was less than 5) at different probability values using computer programs SPSS (Statistical Package for the Social Science; Inc., Chicago, IL, USA) version 15 for Microsoft Windows. P value≤ 0.05 was considered statistically significant and P value≤ 0.001 was considered statistically highly significant.

RESULTS

In our study, Enterococci were isolated from 61.2% of the studied samples (153/250). Unpacked cheese showed the highest frequency (93.3%) followed by meat (80.7%), fish (70.5%) and vegetables (60%). Neither pasteurized milk nor packed cheese was positive for enterococci (Figure 1).

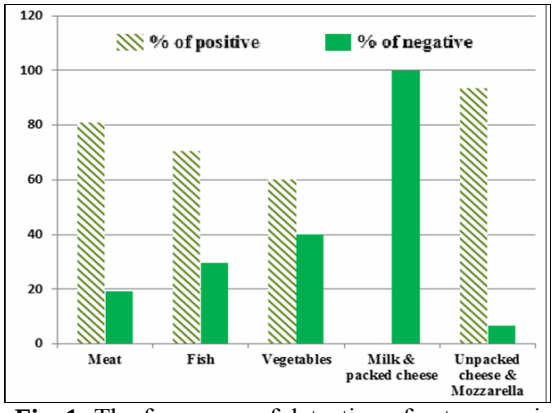

Fig. 1: The frequency of detection of enterococci among the tested food samples

E.faecium was the highest isolated enterococcal species 86/153 (56.2%), followed by *E.avium* 32/153 (21%) with highly significant statistical differences among samples (p<0.001), then *E.faecalis* 28/153 (18.3%) with significant statistical differences among samples (p=0.007), while the percentage of both *E.gallinarum/E.casseliflavus* that cannot be differentiated by the API identification kit were 7/153 (4.5%) with no significant statistical differences among samples (p =0.06) (Figure 2).

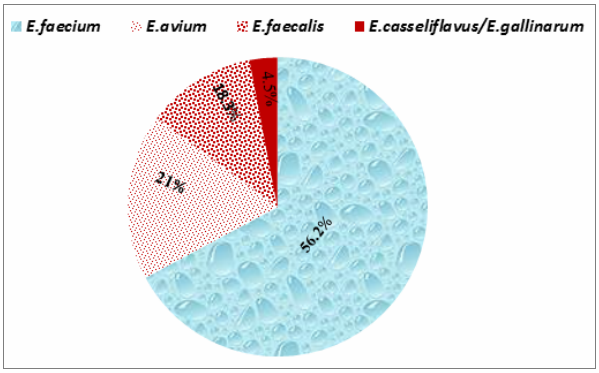

Fig. 2: Frequency of enterococcal species among enterococcal isolates detected in different food samples.

E. faecium was the most prevalent species detected in meat, fish and vegetables (67%, 58.4% and 55% respectively), while *E.avium* was the most predominant species detected in cheese (50%) (Figure 3).

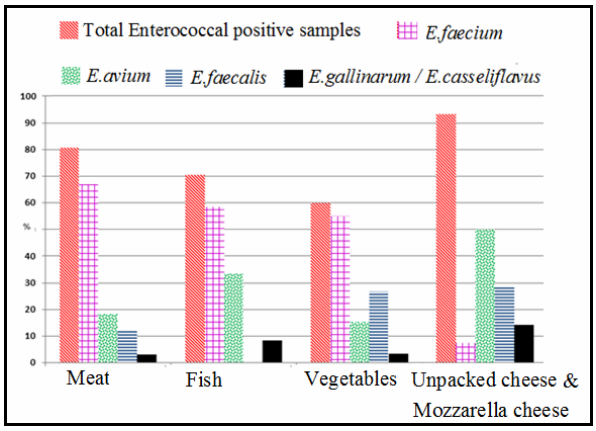

Fig. 3: Frequency of enterococcal species isolated from different food samples.

Antibiotic susceptibility of isolated enterococci revealed that 78%, 76% and 68% of isolates were resistant to quinupristin-dalfopristin, erythromycin and rifampicin respectively, while 96%, 93.5%, 93.5% and 90% of isolates were sensitive to gentamycin/ sulphathiazole, ampicillin/ penicillin, and teicoplanin respectively (Table 3).

Table 3: Antibiotic susceptibility of isolated enterococci

Type of antibiotic	*Sensitive (S)*	*Resistant*		
	No.(%)[1]	**Intermediate resistant (no.)**	**Resistant (no.)**	**Total no (percent)**
Vancomycin	143 (93.5)	4	6	10 (6.5)
Ampecillin/Penicillin	143 (93.5)	0	10	10 (6.5)
Erythromycin	37 (24)	51	65	116 (76)
Tetracycline	72 (47)	4	77	81 (53)
Chloramphenicol	100 (65)	3	50	53 (35)
Rifampicin	49 (32)	21	83	104 (68)
Ciprofloxacin	108 (70)	12	33	45 (30)
Lavoflaxin	123 (80)	2	28	30 (20)
Tecoplanin	138 (90)	6	9	15 (10)
Nitrofurantoin	99 (64.7)	45	9	54 (35.3)
Gentamicin/Sulphathiazole	147 (96)	0	6	06 (4)
Quinupristin-Dalfopristin	34 (22)	17	102	119 (78)

[1] Calculated in relation to the total positive enterococcus samples. (153)

Vancomycin resistance was detected in 10/153 (6.5%) of Enterococcus isolates, 100% of this VRE isolates were resistant to quinupristin- dalfopristin and rifampicin; 90% were resistant to erythromycin and 80% were resistant to tetracycline while ninety percent of VRE were sensitive to lavoflaxin and gentamicin/ sulphathiazole, 80% were sensitive to ampicillin/ penicillin (Table4).

Table 4: Susceptibility of VRE to other antibiotics:

Antibiotic	*Sensitive No. (%)**	*Resistant No. (%)**
Ampecillin/Penicillin	8 (80)	2 (20)
Erythromycin	1 (10)	9 (90)
Tetracycline	2 (20)	8 (80)
Chloramphenicol	7 (70)	3 (30)
Rifampicin	0 (0)	10 (100)
Ciprofloxacin	7 (70)	3 (30)
Lavoflaxin	9 (90)	1 (10)
Tecoplanin	4 (40)	6 (60)
Nitrofurantoin	6 (60)	4 (40)
Gentamicin/Sulphathiazole	9 (90)	1 (10)
Quinupristin-Dalfopristin	0 (0)	10 (100)

* Calculated in relation to the total number of isolated VRE (10).

VRE isolates were investigated for vancomycin resistant genes; *van*A, *van*B, *van*C1 and *van*C2 by PCR. Two isolates of *E.gallinarum* carring were found *van*C1, three isolates of *E.casseliflavus* carring *van*C2, one *E.gallinarum/E.casseliflavus* carring *van*A and the four isolated *E.avium* didn't show any of the four tested vancomycin resistant genes (Figure4).None of the vancomycin resistant enterococci isolated in this study carried *van*B gene.

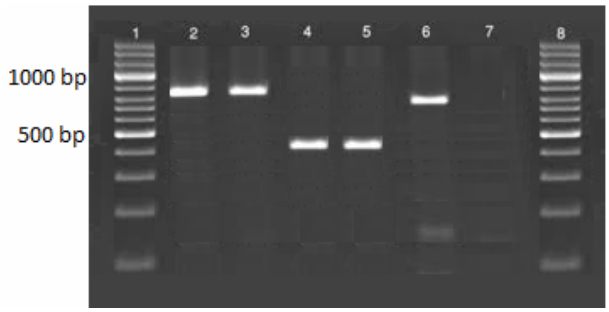

Fig. 4: Ethidium bromide stained 2% agarose gel electrophoresis for PCR products of VRE using different primers for detection of different van genes: lane 1 & lane 8 represent 100 bp DNA molecular size ladder. Lane 2 & 3 show positive amplification of 822 bp fragments specific for *van*C1 gene, lane 4 & 5 show 439 bp fragments specific for *van*C2 gene and lane 6 shows positive amplification of 732 bp fragments specific for *van*A gene. Lane 7 shows negative control.

DISCUSSION

The present study aimed to determine the prevalence of vancomycin resistant enterococci in different food samples of animal and plant origin; raw and processed meat (chicken, beef and turkey), fish, vegetables (cucumber, tomato, carrot, onion, radish and green pepper), pasteurized and boiled milk, packed cheese (sliced cheddar, feta and cream cheese), unpacked cheese (cheddar, molbo, cottage and ras cheese), and mozzarella cheese.

Out of 250 tested food samples, there were 153 (61.2%) positive samples for enterococci with high prevalence in unpacked cheese and mozzarella 93.3% (14/15), meat 80.7% (67/83) and fish 70.5% (12/17), then vegetables 60% (60/100). Neither pasteurized milk

nor packed cheese showed presence of enterococci. Another study was done in Egypt by Salah et al.[18], showed that 80.5% food samples (160/200) were positive for enterococci with 81.5% meat samples (chicken, beef, raw pork and sausage), 80% fish samples, 60% vegetable samples, 87.5% raw milk and milk products were positive for enterococci. Other studies in USA, Brazil, and Poland revealed 47.7% (189/ 396), 2.5% (3/120) and 82.1% (92/112) of different food samples were positive for enterococci respectively[12, 19-20]. However A higher percentage was obtained in USA by Kročko et al [21], who found 100% of 260 food samples collected from raw pork, poultry, un-pasteurized milk and cheese were positive for enterococci however pork and unpasteurized milk samples were not included in our study. On contrast Pesavento et al [22], in Italy, found that 23.6% (311/1315) of meat, ready salads and cheese samples were positive for enterococci. The lower frequencies noticed in their study may be due to differences in water irrigation systems for vegetables and high sanitary condition regarding slicing and packing of cheese.

Our results showed that *E.faecium*, was the most prevelant species of isolated enterococci (56.2%), followed by *E.avium* (21%), *E. faecalis* (18.3%) and *E.gallinarum/E.casseliflavus* (4.5%). In meat samples 67% (45/67) of the isolated enterococcus was *E.faecium* species in agreement with several studies in Belgium, United States, and Brazil [19,23-26] Whereas other investigators reported that *E.faecalis* was the most common species detected in meat samples[12,18, 27-28].This may be related to the age of poultry at the time of slaughter as documented by Aarestrup et al[29], who reported a higher incidence of *E.faecalis* isolates in young poultry, whereas older poultry contained higher numbers of *E.faecium*.

Regarding fish samples *E.faecium* was the most prevalent enterococcus species 58.4% (7/12) with remarkable agreement with other studies done in Argentina, Oman and Spain [30 - 32]

In vegetables, 55 % (33/60) were *E.faecium* in accordance with the results of other studies done by Johnston & Jaykus[25] in USA; Abriouel et al [33], in Netherand and Torre et al[34] in Italy, who found *E.faecium* was the predominant species among vegetables and fruits samples reaching 52%, 50% and 59% respectively. While Salah et al[18], found that *E.faecalis* was the predominant enterococcal species among vegetables samples. Also, McGowan et al.[12], and Gomes et al[19], found that *E.casseliflavus* were the most predominant isolated enterococci in vegetables samples. This disagree in isolated species may be related to the different types of vegetables included or the location from which samples were collected.

In our study, *E.avium* was the predominant species (50%) isolated from cheese followed by *E.faecalis* 28.6%, *E.gallinarum/E.casseliflavus* 14.3%, and *E.faecium* 7.1%. which disagree the results of other investigators who found *E. faecium* was the most common species in cheese samples [19, 22-23, 26, 28, 35-36]. This may be related to the composition of starter bacteria used in cheese manufacturing as it varies for each type of cheese.

Our results showed that packed cheese pasteurized and boiled milk were negative for enterococci. Other study done by Fracalanzza et al[37], in Brazil found that 72% (18/25) of pasteurized milk samples were positive for enterococci.

A specific concern contributing to the pathogenesis of enterococci is their resistance to a variety of antibiotics. The use of antibiotic for a long time in animal feeding as a growth promoter resulted in the spread of transferable antibiotic resistance elements in several ecosystems. Food chains may also have a possible role in the transmission route of resistant enterococci.

Regarding the antimicrobial susceptibility, all isolates of enterococci in our study showed multiple drug resistance. The highest percentage of resistance was observed with quinupristin- dalfopristin (78%). Similar result was also detected by Peters et al[38] in Germany; Novais et al[39], in Portugal and Ristori et al[40], in Brazil with 41%, 53% and 91.4% respectively. Quinupristin-dalfopristin resistance might be caused by the use of virginiamycin, a streptogramin antibiotic like quinupristin-dalfopristin, as a growth promoter in many countries including Egypt[41].It causes cross resistance to quinupristin-dalfopristin and because of this fact, it has been banned in the European Union since 1999.

Our results also revealed that 76 % of isolated enterococci showed erythromycin resistance with remarkable agreement with the results of other investigators including, Jung et al[42], in Korea; Fracalanzza et al[37], in Brazil; Ruzzauskas et al[43], in Lithuania; Ristori et al[40], in Brazil; Liu et al[44] in China and Pesavento et al[22n]in Italy, who found that 64%, 82%, 63.8%, 83.5%, 72.8%, 66% of enterococcal isolates from different food samples were resistant to erythromycin respectively. However lower incidences of erythromycin resistance in enterococci isolated from different food samples were detected in Brazil and Switzerland[19, 45].

Our study revealed that 53% of enterococcus isolates were resistant to tetracycline. Higher tetracycline resistance was detected by Jung et al[42], in Korea; Ristori et al[40] in Brazil and Yurdakul et al[46], in Turkey as they found 85%, 89.3% and 100% of enterococcus isolates from different food samples were resistant to tetracycline respectively. Whereas, lower incidences were found by Fracalanzza et al[37]; Riboldi et al[47], in Brazil; Valenzuela et al[32], in Spain; Ruzzauskas et al[43], in Lithuania and Pesavento et al[22]in Italy, who found that tetracycline resistance in enterococci isolated from different food samples were 31.2%, 6.3%, 8.7%, 45%, and 35.3% respectively.

Our study showed that 6.5 % of enterococcus isolates were resistant to ampicillin. Also, Torre et al.[34] in Italy and Liu et al[44], in China reported that 9.8% and 7.9% of enterococcus isolated from different food samples were resistant to ampicillin respectively. However Peters et al[38]; McGowan et al[12] and Ristori et al[40], found that 1.6%, 0.4% and 0.2% of *Enterococci* isolated from different food samples were resistant to penicillin respectively. In contrast many other studies found no resistance to ampicillin among their enterococcus isolates from different food samples[19,36,47-49]. These results support that ampicillin remain the drug of choice for the treatment of enterococcal infections.

Regarding to vancomycin, our study revealed that 6.5 % (10/ 153) enterococcus isolates were vancomycin resistant. Two isolates were from meat (one undifferentiated *E.gallinarum*/*E.casseliflavus* & one *E.casseliflavus*), 2 from fish (*E.avium* & *E.casseliflavus*), 2 from vegetables (*E.casseliflavus* & *E.avium*) and 4 isolates from unpacked cheese and mozzarella (2 *E.avium* & 2 *E. gallinarum)*. However in another study done in Egypt by Salah et al[18], VRE were detected in 14% (30/212) of enterococci isolated from raw milk, pork, chicken, beef and vegetables, (15 *E.faecalis*, 10 *E.faecium*, 3 *E.avium* and 2 *E.durans*). This difference can be explained by absence of raw milk and pork samples in our study.

A higher percentages of VRE were reported by Citak et al[50], in Turkey who detected VRE in 86.1% (87/101) isolates recovered from Turkish white cheese and Novais et al[39] in Portugal who found VRE in 48% (47/99) isolates recovered from meat samples. Also, Torre et al[34] in Italy reported that 47.6% (50/105) of enterococci isolated from vegetable samples were vancomycin resistant. This high resistance towards vancomycin could be the result of the continuity of using avoparcin in animal feed in some countries or the differences in kinds of samples.

Vancomycin resistant enterococci was also detected by Jung et al[42] in Korea who isolated 7.6% (208/2726) VRE and reported that *E.gallinarum* was the most prevalent species isolated from meat and raw milk samples while Gomes et al[18] in Brazil found 1.14% (3/263) vancomycin resistant *E.faecium* was isolated from different food samples. However Cetinkaya et al[51], in Turkey found that VRE was detected in 4.4% (12/270) of raw milk, dairy products and meat, six isolates were *E.faecium*, 4 were *E. avium,* 1 was *E. gallinarum*, and one *E.durans,* also Pesavento et al[22] in Italy detected VRE in 3.53% (11/312) of beef, cheese and ready to eat salads samples where 5 isolates was *E. faecium*, 2 was *E. faecalis*, and 4 was *E.avium* Whereas other investigators in Iowa, Germany, Athens, Brazil and Canada didn't detect any VRE in their samples[12, 24, 27-38, 49]. This may be due to banning of avoparacin usage in many countries long years ago.

In our study, multiple antibiotic resistance patterns among VRE were detected; 100% were resistant to rifampicin and quinupristin- dalfopristin, 30% resistant to ciprofloxacin and chloramphenicol, 10% resistant to lavoflaxin and gentamycin/sulphathiazole, 90%, 80%, 60%, 40%, and 20%, were resistant to erythromycin, tetracycline, tecoplanin, nitrofurantoin, and ampecillin /penicillin respectively. Our results are in agreement with those of other studies in different countries, indicating the worldwide importance of antibiotic resistant enterococci[15,18,42,51,52, 53.]

VRE were investigated for the presence of resistant genes by PCR using specific primers for *van*A, *van*B, *van*C1 and *van*C2. The results revealed that 2 isolates were *E. gallinarum* as they carry *van*C1 gene, 3 isolates were *E.casseliflavus* as they carry *van*C2, one isolate was *E.gallinarum or E. casseliflavus* carrying *van*A gene, and 4 isolates carried either one of the untested genes or a mutant gene. DNA sequence variation in enterococcal isolates were previously documented by the study of Patel et al[54], in USA (1998), who found sequence variations within *van*A, *van*B, *van*C1 and *van*C2 genes of enterococcal isolates, this explains the undetected genes in our study.

Inspite of *E. gallinarum/E.casseliflavus* are intrinsically resistant to low levels of vancomycin several studies done in Belgium, Switzerland, Australia, Taiwan, Italy, Brazil and India reported that high level resistance to glycopeptides, *van*A and *van*B genes have been acquired by *E.gallinarum* [26, 55-60].This explains the detected *van*A gene in *E.gallinarum/ E.casseliflavus* in our study.

CONCLUSION

Food of animal and plant origin is a possible reservoir of multi-drug resistant enterococci that can be transmitted to the human population through the food chain. The Ministry of Health must control and monitor the implementation of infection prevention and control guidelines and ensure the safety of food, especially dairy products.

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