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Original Research Article

Study of *gyrA* S83R Mutation Rate in Vancomycin Resistant *Entrococcus Faecium*

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HIGHLIGHTS

- There is a strong association between the presence of point mutation at the position of Ser83 and vancomycin resistant phenotype.
- Resistance in *Enterococcus* spp. is highly mediated by mutations in *gyrA* gene which plays a major role in interaction with vancomycin.
- Vancomycin is the only drug that can be consistently relied on for the treatment of infections caused by multidrug resistant enterococci.
- VRE has presented a serious challenge for the Iranian medical community.

ABSTRACT

Enterococci are among Gram-positive cocci and are common inhabitants of the human gastrointestinal tract and enough potent to cause serious infections such as bacteremia, urinary tract infections (UTIs) and endocarditis. Enterococci are not generally considered as highly infectious bacterium. However, the main reason for treatment failure in enterococcal infections is acquired resistance to glycopeptide antibiotics, specifically vancomycin. Most enterococcal infections in human such as gastroenteritis, intestinal infections, and endocarditis have been caused by *E. faecalis* and *E. facieum*. They are holding the second place of most frequent cause of hospital infections since 1990. In present study g*yrA* S83R polymorphism rate in vancomycin resistant *Enterococcus faecium* isolated from environment, food industries, and hospitals of Tehran were examined by ARMS-PCR technique. DNA was extracted from the bacterial colonies using standard column method, after separating the samples into two groups of antibiotic resistant and antibiotic susceptible by antibiogram test. A fragment of *gyrA* gene was amplified using PCR method to investigate point mutation of S83R position. ARMS-PCR technique was applied to detect the presence or absence of mutation using a set of specified primers which can be annealed when the mutation is present. The results were statistically analyzed by chi-square test ($p < 0.05$) using SPSS 19th version. The results showed that there was a significant correlation between the presences of S83R polymorphism with vancomycin resistance trait in *Entrococcus faecium.* Therefore, this technique could be used as a diagnostic tool to detect vancomycin resistance cases of *E. faecium* in patients and environment.

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Introduction

Enterococci have been recognized as a common cause of serious infections such as bacteremia diverticulitis, meningitis and endocarditis. They are most commonly isolated from infections of urinary tract, and wounds (French, 1998; Morandi et al., 2006; Brinster et al., 2007; Sood et al., 2008; Nallapareddy et al., 2011).

The enterococci readily colonize the bowel, spread rapidly in nature, between healthcare employees or other industries and mostly can be transmitted from patient to patient in the hospitals (French, 1998; Woodford, 1998; Rathnayake et al., 2012). Antibiotic resistance would be considered as an important risk factor for the elderlies. The enteroccocal infections may be treated with vancomycin, which is common medication used to reduce the symptoms of patients. However, vancomycin resistance enterococci (VRE) infections are more difficult to deal with, because the effective antibiotics against resistant bacteria are rare and the antibiotic resistant species can widely transfer among patients. As a result, the frequency of VRE infections is increasing (Cloete, 2003; Hollenbeck and Rice, 2012). The great majority of enterococcal infections are caused by *Enterococcus faecalis* and *Enterococcus faecium*.

Enterococcus faecalis is the most common isolate among enterococci. However, *Enterococcus faecium* has become increasingly important after the emergence of vancomycin resistance (Huycke et al., 1998; Weinstein and Hayden, 2000; Cloete, 2003; Fisher and Phillips, 2009).

The major cause of enterococcal infections is related to *E. faecalis* (Sood et al., 2008). *E. faecium* causes a substantial proportion of infections acquired in the hospital setting. In the late 1980s, clinical isolates of the vancomycin resistance enterococci species began to appear, which induced significant changes in microbial identification of enterococci in the clinical laboratories and infection control units (Huycke et al., 1998; Murray, 2000; Giraffa, 2002; Sood et al., 2008; Fisher and Phillips, 2009). The phenotypic and genotypic bases of several VRE have been characterized. *E. faecium* is equipped with a variety of inherent antibiotic-resistance genes as well as the acquired ones. Since many clinical isolates of *E. faecium* are resistant to vancomycin and fluoroquinolones, there are very limited treatment choice for *E. faecium* infections (Huycke et al., 1998; Weinstein and Hayden, 2000; Rice, 2006; Fisher and Phillips, 2009).

Pathogen resistance to antibiotics can be the result of variations in drug target, molecular bypass, active efflux (or decreased entry), and chemical modification of the compound. Several antibiotics exert their growth inhibitory activity against bacteria through the interaction with critical bacterial pathways such as DNA synthesis. Therefore, alteration of the molecular targets are the basic mechanism of bacterial resistance (Wright, 2011). DNA gyrase (topoisomerase II) and topoisomerase IV (*parC* and *parE*) are the enzymes involved in DNA synthesis that may be modified in the resistant bacteria (Giedraitienė et al., 2011). The genes of *gyrA* and *gyrB* are the relevant genes of DNA gyrase that their single mutations may emerge high level of flouroquinolone resistance in pathogenic gram positive bacteria; as the minor changes in the amino acid sequence of enzymes can alter the protein structure required for antibiotic binding and action. For example, mutation of Ser to an amino acid with a bulkier side chain (Leu, Trp, Ile) at position 83, or Asp87 to Asn, Tyr, or Gly are the common modifications resulted in fluoroquinolone resistant species (Wright, 2011). Mutations of *gyrA* or *parC* are not directly relevant to the vancomycin mechanism of action but these mutations have been reported in VER species (López et al., 2011; Miller et al., 2014).

Recent studies have shown that in *E. faecium*, vancomycin resistance can be related to mutations in the gyrA gene. All common species comes with amino acid changes in *gyrA* protein (S83I, S83Y, S83R or S83I-E87G) with/without changes in *parC* protein (S80I or S80R or S80l) (Korten et al., 1994; Morandi et al., 2006; Tenover, 2006; Rathnayake et al., 2011; Teymournejad et al., 2011; Ardebili et al., 2015).

The origins of *Enterococcus* species have variation from environmental to animal and human sources. The distribution of *Enterococcus* species varies throughout Asia. In Iran *E. faecalis* and *E. faecium* are the most commonly isolated species from clinical, industrial, and environmental sources (Fatholahzadeh et al., 2006).

In this study, we have analyzed vancomycin resistance E. faecium isolates from various origins (Giraffa, 2002; Moreno et al., 2006; Ogier and Serror, 2008), including environment, food industries and hospitals, for point mutation in *gyrA* at position 83 (serine-to-arginine substitutions).

Materials and Methods

Bacterial isolates and growth conditions

This research is a cross sectional and purely descriptive study which is carried out over a 13 month period (January to February 2016) in three origins;

1. Hospitals (Besat Nahaja General Hospital, Laleh Hospital), 2. Environment (Tajrish -Shemiranat river water) and 3. Food industries (milk and the dairy product-Industrial Estate) of Tehran city.

Proper clinical specimens (urine, blood, wound and hospital area swabs), Milk and dairy samples (Swabs from all the equipment in the production line, before and after pasteurization) and river water samples (sampling depth profile is 12-25 cm from the surface) were collected and appropriately transported to microbial laboratory

for isolation on Sheep Blood agar aerobic cultures, and biochemical identification of enterococci. Membrane filtration technique were used on various sample dilutions to achieve isolated colonies (0.45 µm membrane filter, Millipore Corporation, Bedford, MA, USA).

Isolation/identification of enterococci

162 of *E. faecium* isolates were identified from 737 different samples of all three origins. The non-hemolytic streptococci-like colonies (0.5–1 mm size) of enterococci were isolated on Blood agar plate and small dark-red magenta colonies were seen on MacConkey agar. The enterococci colonies were then confirmed by positive gram stain, growth in 6.5% NaCl broth, negative catalase test, positive bile-aesculin (bile insolubility) test, and Enterococcus species were identified by special fermentation reactions of sugar (glucose, lactose, mannitol, sorbitol, and arabinose) (Lee, 1972; Gardini et al., 2001; Michaux et al., 2011).

Antibiotic susceptibility test

The vancomycin susceptibility of enterococci isolates were confirmed using Laboratory Standards Institute (CLSI) disk diffusion method on Mueller-Hinton 2% NaCl with 30µg vancomycin disks (Mast, Merseyside, and U.K) and with 35°C for 24 hours incubation condition. Zone diameter (ZD) of inhibition ≥17mm, 15-16mm, ≤14 mm or less defined as susceptible, intermediate-resistant and full-resistant to vancomycin (Jorgensen and Turnidge, 2015). The vancomycin MIC was determined by the broth macro dilution method based on Clinical and Laboratory Standards Institute (CLSI) criteria (Dudley et al., 2013).

Briefly, vancomycin serial dilution was performed in Mueller Hinton broth tubes with the starting bacterial density of 5×10^6 CFU/mL. The tubes were then incubated at 37 °C for 18 h and the MIC was defined by lowest concentration of antibiotic with no visible bacterial growth. Finally, *E. faecium* isolates were considered as susceptible, intermediate-resistant, and full-resistant to vancomycin when the MIC was equal to 4 µg/mL, 8-16µg/mL and ≥32 µg/mL, respectively. The controls for vancomycin sensitive enterococci (VSE) and vancomycin resistance species were *E. faecalis* ATCC 51299 and E faecalis ATCC 29212 (BD-BBL™ Sensi-Disc™ Antimicrobial Susceptibility).

DNA Extraction

All the resistance and sensitive isolates were investigated for vancomycin resistance genes. DNA was extracted by Genomic DNA Purification kit protocol (#k0512, Fermentas, Thermo Fisher Scientific). Briefly, enterococci cells were grown in LB medium for 24 h. The cultures were harvested at 5000 g for 10 min. Then, 10-20 mg of bacterial cells were re-suspended to a 1.5-mL screw-capped micro tube containing 200 µL of TE buffer (10 mM Tris-HCI, 1 mM EDTA). Then a mixture of 200 µL of previous sample with 400 µL lysis buffer incubated at 65° C for 5 min. DNA was directly extracted from the sample by adding 600 µL of chloroform and mixing for 3-5 min. The samples for 2 min with 10,000 g (4°C) were centrifuged (Eppendorf 5415R; Hamburg, Germany), and the upper aqueous layer was separated and freshly transferred to micro-centrifuge tube, and also 800 µL prepared precipitation solution was added to the tube. Gently, the solution was mixed by several inversions at room temperature for 1-2 min, then the sample was centrifuged (Eppendorf 5415R) at 10,000 g (4°C) for 2 min. After decanting the supernatant, DNA pellet was dissolved in 100µL NaCl solution by gentle vortexing. For the DNA precipitation, 300 µl of cold ethanol was added (10 min at -20°C- 10,000 rpm, 3-4 min). Finally, the pellet was washed with cold 70% ethanol and in a laminar air flow, the pellet was then air dried, and a re-suspension of dried pellet was made in 100 µL nuclease-free water and kept at −20°C or −80°C.

Concentration, Purity Determination and DNA Integrity

DNA concentration was measured spectrophotometrically and visually after electrophoresis in agarose gel. For spectrophotometric assay of DNA, a UV-visible spectrophotometer (Agilent Technologies, Santa Clara, USA) was used and wavelengths of 260 and 280 nm (A_{260} and A_{280}, respectively) were set for Absorbance measurement. The estimation of DNA purity was made by absorbance quotient (OD_{260}/OD_{280}). A ratio of <1.7 indicated protein contamination, however a ratio of >1.9 indicated RNA contamination. An absorbance value of 1.7 < ratio (R) < 1.9 was considered to be purified DNA. Usually 100 ng/µL of DNA were obtained.By resolving DNA extracts on a 0.8% agarose gel, the integrity of genomic DNA was tested (Gel electrophoresis, Bio-Rad, USA), and then the test was completed with ethidium bromide staining. Each DNA sample's molecular weight was identified, based on the electrophoretic migration of sample DNA compared with a known size marker (Fermentas, Thermo Scientific).

Polymerase Chain Reaction for amplification

Extracted DNAs were further amplified by PCR (PCR Master Mix (2X), #K0171). Specific Primers *gyrA* F[5′-GAAATCAGAGAAAACATCCATGATG 3′], *gyrA* R[5′-ATATTCGTGGCCATACCTACG -3′] were chosen in this study for PCR amplification of the 539 bp product containing Ser83 region of gyrA gene of vancomycin

Table 1. Antibiotic resistance behavior of enterococci isolates with disk diffusion.

Antimicrobial agent: vancomycin	ZD of *E. faecium* isolates (n=162)		
	≥ 17 mm	15-16 mm	≤ 14 mm
Sources	**S***	**I***	**R***
Environment	47	0	14
Food industrials	10	0	10
Hospitals	56	0	25

* S: susceptible; I: intermediate-resistant; R: full-resistant

resistance and susceptible isolates of *E. faecium* .

Primer design and Detection of virulence of point mutation in *gyrA* genes at Ser83 by ARMS-PCR (21). All the isolates were investigated for vancomycin resistance genes. Identification of point mutation in *gyrA* at position 83 (serine-to-arginine substitutions) for each isolates of VRE and VSE was performed by a separate PCR with specific primers as follows; Two sets of primers, wild type, and mutant type were designed. Wild type primers for the detection of normal DNA of *gyrA* Ser83 Region Forward [5′-TAAATACCATCCTCATGGAGACA3′], *gyrA* Ser83 region Reverse [5′-GTGCTTCGGTATAACGCATA3′], and the mutant ones were previously designed by Zima gene Co (Iran, Tehran) for gyrA (*gyrA*-1F [5′-TAAATACCATCCTCATGGAGACC 3′] and *gyrA*-1R (5′- GTGCTTCGGTATAACGCATA 3′).

The PCR assay was carried out in a 25 µL solution containing 10 mM Tris-HCl (pH = 8.3), 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$, 0.5 U TaqDNA polymerase (HT Biotechnology, Cambridge, UK), and each primer (40 pmol). Initial denaturation of PCR cycle was at 95°C for five minutes, then 35 cycles of denaturation at 94°C for 50 seconds, and annealing was done at 60°C for 40 seconds and then followed by extension at 72°C for 50 seconds, with a final extension at 72°C for 5 minutes. An Eppendorf Mastercycler (Eppendorf, Hauppauge, NY) was used for PCR. PCR products were visually identified on horizontal 1.0% agarose gels with 0.5 x TBE buffer, loaded with 5 µL of reaction mix and stained with ethidium bromide after electrophoresis. The images were captured under U.V. illumination by a video system (Gel DOC 1000 system, BioRad).

Statistical analysis

For Statistical analysis tables of frequently were prepared and Chi-Square test was used for the relationship between variables and difference was considered remarkable at $p < 0.05$ by SPSS software version 19 (Chicago, IL, USA).

Results and Discussion

Resistant species identification and isolation

According to the biochemical tests, of 737 samples, a total of 162 isolates belonged to *E. faecium*. 59, 18, and 85 of which were from environment, food industry, and hospital respectively. The disk diffusion patterns of *E. faecium* to vancomycin antibiotics are presented in Table 1. Of 162 isolates of *E. faecium,* 49 strains were resistant to vancomycin by disk diffusion method, but resistance of 41 strains to vancomycin was confirmed by micro-dilution broth.

Antimicrobial susceptibility

Of 162 isolates of *E. faecium*, resistance of 41 and sensitivity of 121 isolates to vancomycin were confirmed by micro-dilution broth method. So, antibiotic susceptibility test revealed that 11, 7, and 23 isolates obtained from environment, food industry, and hospital respectively, were vancomycin-resistant (Table 2).

Point Mutation at Ser83 determined by ARMS-PCR

PCR technique was developed for the identification of the Ser-to-Arg mutation at amino acid position 86 in the *gryA* of *E. faecium* isolates. The amplification of Isolates with the wild-type amino acid Ser83R were not performed with the reverse mutation primer whereas the mutated amino acid isolates generated a 152-bp PCR product. Common mutations in VRE strains occurred in Ser83 region of gyrA gene. The occurrence of nucleic acid alterations in these codons changed the amino acid profile from Serine to Arginine. Forty-one isolates of *E. faecium* strains were examined for the presence of point mutations related to vancomycin resistance. The DNA extraction of the152 bp fragment was confirmed on a 1% agarose gel (Fig. 1). The PCR used for gyrA genes amplification by designed primers and the efficacy of the method was determined by gel electrophoresis. To identify point mutations, the

Table 2. Results of MIC for vancomycin in *E. faecium* strains.

Antimicrobial agent (vancomycin)	*E. faecium* isolates (n= 59) Environment			*E. faecium* isolates (n= 18) Food industries			*E. faecium* isolates (n = 85) Hospital		
Sensitivity Status	S*	I*	R*	S*	I*	R*	S*	I*	R*
MIC (µg/mL)	≥ 4	8 - 16	≤ 32	≥ 4	8 - 16	≤ 32	≥ 4	8 - 16	≤ 32
Number	48	-	11	11	-	7	62	-	23
Percent (%)	81	-	19	61	-	39	73	-	27

* S: susceptible; I: intermediate-resistant; R: full-resistant

PCR samples were then analyzed for detection of point mutations at Ser83 position of gyrA. The results showed that among the 41 vancomycin resistance isolates, 32 VRE strains (78%) carried the point mutation at Ser83 position of *gyrA* gene. 9 VRE isolates (22%) had no mutation in the sequence areas examined (Fig. 2).

Statistical analysis

Using SPSS software, there were significant correlations between the disk diffusion agar and broth micro serial dilution methods for vancomycin antibiotic ($p \leq 0.001$) and between ARMS-PCR and MIC for vancomycin results ($p \leq 0.001$) in strains of *E. faecium*.

Conclusion

Over the past two decades, enterococci have become significantly important nosocomial bacteria and major healthcare-related urinary tract pathogens (Makino et al., 1986; Huycke et al., 1998; Sood et al., 2008; Fisher and Phillips, 2009). These bacteria are increasingly resistant to a large number of antimicrobial agents and have the potency to rapidly acquire new resistance traits (Huycke et al., 1998; Fisher and Phillips, 2009; Rathnayake et al., 2011; Teymournejad et al., 2011). Vancomycin resistance enterococci were isolated in New York City in 1989. The first occurrence of VRE was reported in September 1989, and resistant strains had been isolated from 360 samples in at least 38 hospitals, 20 food industrials, and 16 environments in 1991 (Ballard et al., 2005; Sood et al., 2008). VRE strains are resistant to different classes of antibiotics simultaneously. Rising high-level resistance to penicillin, ampicillin, glycopeptide, and aminoglycosides has been demonstrated in recent years, especially in vancomycin-resistant *E. faecium* strains and due to intra- and inter hospital spread of this glycopeptides resistant *Enterococcus faecium* many concerns have arisen and limited therapeutic options could be found (Meziane-Cherif et al., 1994; Ballard et al., 2005; Tenover, 2006; Nallapareddy et al., 2011).

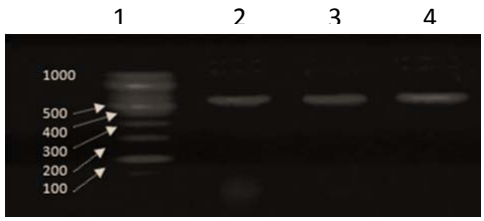

Figure 1. Agarose gel electrophoresis of PCR products of *E. faecium* strains for the detection of the 539bp fragment. PCR products were loaded on 1 % agarose gel. Lane 1; Molecular weight marker, Lane 2, 3, 4; samples from environment, industrial and hospital respectively, all samples were confirmed by the presence of the 539bp fragment.

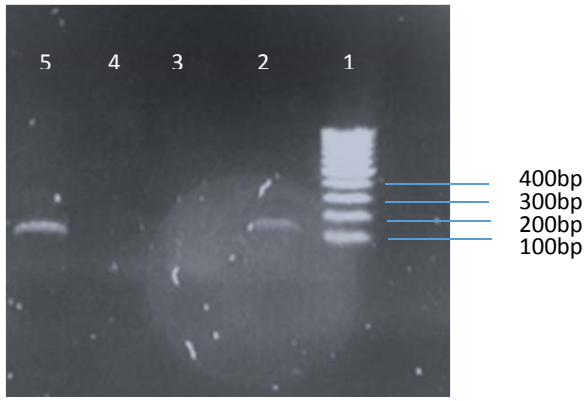

Figure 2. Captured image under U.V. illumination by a video system (Gel DOC 1000 system, BioRad) of PCR; the152bp fragment for the identification point mutation at Ser83 region in vancomycin resistance *E. faecium* strains. PCR products were loaded on 1 % agarose gel. Thermo Scientific GeneRuler 100 bp DNA Ladder (lane 1), sample 1 with mutation (lane 2), sample 2 with no mutation (lane 3), sample 3 with no mutation (lane 4), sample 4 with mutation (lane 5).

The VRE are became increasingly endemic in IRAN, with almost 30% of infections; but, the rates of VRE in European countries and in the United States, is dramatically changing with absence of these species in hospital and healthcare acquired infections (Fatholahzadeh et al., 2006).

In the present study, of 737 samples, 162 isolates were identified as *E. faecium*. The prevalence of *E. faecium* strains were reported 22% in three origins in Tehran, composing 36%, 11%, and 52% in the environment, food industries and hospitals, respectively.

By using designed primers developed from conserved sequence in the gyrA gene, we were able to amplify and identify the existence of corresponding point mutation Ser83 region of the gyrA gene in the strains which were isolated. The ARMS-PCR results showed among the 41 vancomycin resistance isolates, 32 VRE strains (78%) carried the point mutation at Ser83 position of *gyrA* gene which was significantly correlated with the MICs. Ser-83 in the *gyrA* seems to play an important role in the occurrence of resistance to vancomycin in *E. faecium* and it had been showed in the previous study of others.

Almost about 30 out of 121 strains showed point mutation in Ser83, being susceptible to vancomycin, and this may suggest that another mechanism may also be involved in the mechanism of resistance of *E. faecium* strains. It meant that the mutation may be mostly conducted with a second mutation in gyrA or probably a mutation in parC. Our statistical data revealed a correlation between the number of mutations and vancomycin resistance.

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Competing interests

The authors declared that there are no conflicts of interest.

References

Ardebili, A., Lari, A. R., Beheshti, M. and E. R. Lari, (2015). "Association between mutations in gyrA and parC genes of Acinetobacter baumannii clinical isolates and ciprofloxacin resistance." *Iranian Journal of Basic Medical Sciences,* **18**(6): 623-626.

Ballard, S. A., Pertile, K. K., Lim, M., Johnson, P. D. and M. L. Grayson, (2005). "Molecular characterization of vanB elements in naturally occurring gut anaerobes." *Antimicrobial Agents and Chemotherapy,* **49**(5): 1688-1694.

Brinster, S., Posteraro, B., Bierne, H., Alberti, A., Makhzami, S., Sanguinetti M. and P. Serror, (2007). "Enterococcal leucine-rich repeat-containing protein involved in virulence and host inflammatory response." *Infection and Immunity,* **75**(9): 4463-4471.

Cloete, T. E. (2003). "Resistance mechanisms of bacteria to antimicrobial compounds." *International Biodeterioration & Biodegradation*, **51**(4): 277-282.

Dudley, M. N., Ambrose, P. G., Bhavnani, S. M., Craig, W. A., Ferraro, M. J., Jones, R. N., A. S. T. S. o. t. Clinical and L. S. Institute, (2013). "Background and rationale for revised Clinical and Laboratory Standards Institute interpretive criteria (breakpoints) for Enterobacteriaceae and Pseudomonas aeruginosa: I. Cephalosporins and aztreonam." *Clinical Infectious Diseases,* **56**(9): 1301-1309.

Fatholahzadeh, B., Hashemi, F. B., Emaneini, M., Aligholi, M., Nakhjavani F. A. and B. Kazemi, (2006). "Detection of vancomycin resistant enterococci (VRE) isolated from urinary tract infections (UTI) in Tehran, Iran." *DARU Journal of Pharmaceutical Sciences,* **14**(3): 141-145.

Fisher, K. and C. Phillips, (2009). "The ecology, epidemiology and virulence of Enterococcus." *Microbiology,* **155**(6): 1749-1757.

French, G. (1998). "Enterococci and vancomycin resistance." *Clinical Infectious Diseases,* **27**(Supplement_1): S75-S83.

Gardini, F., Martuscelli, M., Caruso, M. C., Galgano, F., Crudele, M. A., Favati, F., Guerzoni, M. E. and G. Suzzi, (2001). "Effects of pH, temperature and NaCl concentration on the growth kinetics, proteolytic activity and biogenic amine production of Enterococcus faecalis." *International Journal of Food Microbiology,* **64**(1): 105-117.

Giedraitienė, A., Vitkauskienė, A., Naginienė, R. and A. Pavilonis, (2011). "Antibiotic resistance mechanisms of clinically important bacteria." *Medicina***, 47**(3): 137-146.

Giraffa, G. (2002). "Enterococci from foods." *FEMS Microbiology Reviews,* **26**(2): 163-171.

Hollenbeck, B. L. and L. B. Rice, (2012). "Intrinsic and acquired resistance mechanisms in enterococcus." *Virulence***, 3**(5): 421-569.

Huycke, M. M., Sahm, D. F. and M. S. Gilmore, (1998). "Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future." *Emerging Infectious Diseases,* **4**(2): 239.

Jorgensen, J. H. and J. D. Turnidge, (2015). "Susceptibility test methods: dilution and disk diffusion methods. " *Manual of Clinical Microbiology, Eleventh Edition*, American Society of Microbiology**:** 1253-1273.

Korten, V., Huang, W. M. and B. E. Murray, (1994). "Analysis by PCR and direct DNA sequencing of gyrA mutations associated with fluoroquinolone resistance in Enterococcus faecalis." *Antimicrobial Agents and Chemotherapy,* **38**(9): 2091-2094.

Lee, W. S. (1972). "Improved procedure for identification of group D enterococci with two new media." *Applied Microbiology,* **24**(1): 1-3.

López, M., Tenorio, C., Del Campo, R., Zarazaga, M. and C. Torres, (2011). "Characterization of the mechanisms of fluoroquinolone resistance in vancomycin-resistant enterococci of different origins." *Journal of Chemotherapy,* **23**(2): 87-91.

Makino, K., Shinagawa, H., Amemura, M. and A. Nakata, (1986). "Nucleotide sequence of the phoB gene, the positive regulatory gene for the phosphate regulon of Escherichia coli K-12." *Journal of Molecular Biology,* **190**(1): 37-44.

Meziane-Cherif, D., Badet-Denisot, M. A., Evers, S., Courvalin, P. and B. Badeta, (1994). "Purification and characterization of the VanB ligase associated with type B vancomycin resistance in Enterococcus faecalis V583." *FEBS Letters,* **354**(2): 140-142.

Michaux, C., Martini, C., Hanin, A., Auffray, Y., Hartke, A. and J.-C. Giard, (2011). "SlyA regulator is involved in bile salts stress response of Enterococcus faecalis." *FEMS Microbiology Letters,* **324**(2): 142-146.

Miller, W. R., Munita, J. M. and C. A. Arias, (2014). "Mechanisms of antibiotic resistance in enterococci." *Expert Review of Anti-Infective Therapy***, 12**(10): 1221-1236.

Morandi, S., Brasca, M., Andrighetto, C., Lombardi, A. and R. Lodi,

(2006). "Technological and molecular characterisation of enterococci isolated from north–west Italian dairy products." *International Dairy Journal,* **16**(8): 867-875.

Moreno, M. F., Sarantinopoulos, P., Tsakalidou, E. and L. De Vuyst, (2006). "The role and application of enterococci in food and health." *International Journal of Food Microbiology,* **106**(1): 1-24.

Murray, B. E. (2000). "Vancomycin-resistant enterococcal infections." *New England Journal of Medicine,* **342**(10): 710-721.

Nallapareddy, S. R., Sillanpää, J., Mitchell, J., Singh, K. V., Chowdhury, S. A., Weinstock, G. M., Sullam, P. M. and B. E. Murray, (2011). "Conservation of Ebp-type pilus genes among Enterococci and demonstration of their role in adherence of Enterococcus faecalis to human platelets." *Infection and Immunity,* **79**(7): 2911-2920.

Ogier, J. C. and P. Serror, (2008). "Safety assessment of dairy microorganisms: the Enterococcus genus." *International Journal of Food Microbiology,* **126**(3): 291-301.

Rathnayake, I., Hargreaves, M. and F. Huygens, (2011). "SNP diversity of Enterococcus faecalis and Enterococcus faecium in a South East Queensland waterway, Australia, and associated antibiotic resistance gene profiles." *BMC Microbiology,* **11**(1): 201.

Rathnayake, I., Hargreaves, M. and F. Huygens, (2012). "Antibiotic resistance and virulence traits in clinical and environmental Enterococcus faecalis and Enterococcus faecium isolates." *Systematic and Applied Microbiology,* **35**(5): 326-333.

Rice, L. B. (2006). "Antimicrobial resistance in gram-positive bacteria." *American Journal of Infection Control,* **34**(5): S11-S19.

Sood, S., Malhotra, M., Das, B. and A. Kapil, (2008). "Enterococcal infections & antimicrobial resistance." *Indian Journal of Medical Research,* **128**(2): 111-121.

Tenover, F. C. (2006). "Mechanisms of antimicrobial resistance in bacteria." *The American Journal of Medicine,* **119**(6): S3-S10.

Teymournejad, O., Mohabati Mobarez, A. and R. Hosseini Doust, (2011). "Epidemiologic evaluation of Vancomycin Resistant genes in Enterococcus spp. isolated from clinical samples." *Journal of Fasa University of Medical Sciences,* **1**(2): 1-6.

Weinstein, R. A. and M. K. Hayden, (2000). "Insights into the epidemiology and control of infection with vancomycin-resistant enterococci." *Clinical Infectious Diseases,* **31**(4): 1058-1065.

Woodford, N. (1998). "Glycopeptide-resistant enterococci: a decade of experience." *Journal of Medical Microbiology***, 47**(10): 849-862.

Wright, G. D. (2011). "Molecular mechanisms of antibiotic resistance." *Chemical Communications,* **47**(14): 4055-4061.