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Full Length Research Paper

Increasing prevalence of high-level gentamicin resistant enterococci: An emerging clinical problem

Mounir M. Salem-Bekhit[1]*, Ihab Mohamed Ibrahim Moussa[2], Muharram M. M.[3] Ahmed Mahmoud Elsherbini[4] and Salim AlRejaie[5]

[1]Department of Pharmaceutics, Kayyali Chair for Pharmaceutical Industries, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia.
[2]Center of Excellence in Biotechnology Research, King Saud University, P. O. Box 2460, Riyadh 11451, Saudi Arabia.
[3]Faculty of Pharmacy, Prince Salman University, Kingdom of Saudi Arabia.
[4]Department of Clinical Laboratory Sciences. Al-Gad International College for Medical Sciences. Jeddah. Saudi-Arabia.
[5]Department of Pharmacology, College of Pharmacy King Saud University, Saudi Arabia.

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Enterococci have recently been recognized as a causative organism of intractable infections. This study investigated the species prevalence, antimicrobial resistance and characterization of high-level gentamicin resistant (HLGR) enterococci. A total of 163 enterococci were isolated from various clinical samples collected from hospitalized patients in Riyadh, Saudi Arabia. The *Enterococcus faecalis* was the most frequent species (58.9%), followed by *Enterococcus faecium* (26.9%), *Enterococcus avium* (6.1%), *Enterococcus gallinarum* (2.5%), *Enterococcus durans* (1.8%), *Enterococcus hirae* (1.2%), *Enterococcus casseliflavus* (1.2%) and *Enterococcus cecorum* (0.6%). High-level of resistances to gentamicin and streptomycin were detected by the disc diffusion method, in 20.2% of isolates. The prevalence of resistances among β-lactam antibiotics and glycopeptides were very low. All *Enterococcus* isolates were identified phenotypically by conventional methods and genotypically by polymerase chain reaction (PCR) targeting aminoglycoside modifying enzyme (AMEs) genes including *aac*(6`)-*Ie-aph*(2``)-*Ia, aph*(2``)-*Ib, aph*(2``)-*Ic, aph*(2``)-*Id, aph*(3`)-*IIIa* and *ant*(4`)-*Ia*. The genetic diversity of *E. faecalis* presenting HLGR was assessed by pulsed-field gel electrophoresis (PFGE) of chromosomal deoxyribonucleic acid (DNA) after *Sma*I digestion. Most of the high level aminoglycoside resistant isolates contained genes coding for the bifunctional AMEs AAC(6`)-APH(2``), ANT(6`) and APH(3`) but not the ANT(4`). The results demonstrated that the spread of the *aac*(6`)-*Ie-aph*(2``)-*Ia* gene was responsible for HLGR among enterococci isolated in Riyadh hospital.

Key words: Enterococci, resistance, *Enterococcus faecalis,* high-level gentamicin resistant (HLGR).

INTRODUCTION

Over the years, enterococci have changed from being a commensally intestinal organism of little clinical significance to becoming one of the most common nosocomial pathogens associated with significant morbidity and mortality among all age groups worldwide (Cetinkaya et al., 2000; El-Amin and Faidah, 2011). The situation is more complicated when high-level resistance (HLR) to both gentamicin and streptomycin is identified. In such cases, the synergic combination of a cell wall-active agent plus an aminoglycoside cannot be used for the treatment of severe enterococcal infections (Murray, 1990; Dupont et al., 2011).

The prevalence of antibiotic resistance genes is important for treatment and controlling of enterococci infections. Studies have shown increasing resistance of enterococci to antimicrobial agents such as β-lactamase, HLR to aminoglycoside and more recently to glycolpeptides (Kacmaz and Aksoy, 2005). Various antibiotic resistance genes have been identified in enterococci,

*Corresponding author. E-mail: mounirmsalem@yahoo.com or mbekhet@ksu.edu.sa.

especially in *Enterococcus faecium*. High-level aminoglycoside resistance (HLAR) among enterococci first described in 1979 is a significant therapeutic problem, particularly for patients with endocarditis (Patterson and Zervos, 1990). Now, it is increasingly being reported world-wide and became a global problem.

The addition of an agent that interferes with cell wall synthesis such as ampicillin (or vancomycin), markedly increase uptake of the aminoglycoside and greatly enhance the killing of the enterococci (Moellering and Weinberg, 1971). HLAR in enterococci is mediated by aminoglycoside-modifying enzymes (AMEs), which eliminate the synergistic bactericidal effect of combined exposure to a cell wall-active agent such as β-lactams or glycopeptides and virtually all commercially available aminoglycosides, including gentamicin, tobramycin, netilmicin, kanamycin and amikacin (Jesudason et al., 1998). Enterococci have acquired aminoglycoside resistance genes that encode various AMEs, which results in very high resistance to aminoglycosides (minimum inhibitory concentrations (MICs) usually ≥2000 μg/ml^{-1}), thereby eliminating the synergistic killing effect described above. The most common AMEs in *Enterococcus* spp. are the AAC (6`) - APH (2``), which inactivates gentamicin, kanamycin, tobramycin, netilmicin and amikacin; APH (3`), which inactivates kanamycin and amikacin; ANT (4`), which inactivates kanamycin, amikacin and tobramycin; and ANT (6`), which inactivates streptomycin (Chow, 2000). Moreover, all *E. faecium* strains produce a chromosomally encoded aminoglycoside acetyltransferase, AAC (6`)-Ii, which inactivates tobramycin, kanamycin, netilmicin and sisomicin (Chow et al., 2001).

High-level gentamcin resistant (HLGR) (MIC ≥ 512 mg/L) in enterococci is usually mediated by the *aac*(6`-*Ie*-*aph(2``)-Ia* gene, which encodes the bifunctional enzyme AAC(6`)-APH(2``). Three aminoglycoside resistance genes [*aph(2``)-Ib*, *aph(2``)-Ic* and *aph(2``)-Id*] that also mediate resistance to gentamicin, have been detected in enterococci (Chow et al., 2001). Other genes such as the *aph(3`)-IIIa* and *ant(4`)-Ia*, that encode the APH(3`) and the ANT(4`) AMEs and confer resistance to various aminoglycosides but not to gentamicin, have also been identified. During the last decade, the types and distribution of AMEs and genes in enterococci vary in different geographical regions throughout the world (del Campo et al., 2003; Donabedian et al., 2003) and have not yet been reported in Saudi Arabia. The aim of this study was to investigate the prevalence of AMEs genes among clinical isolates of *Enterococcus* spp. isolated from Saudi Arabia.

MATERIALS AND METHODS

Patient specimens and enterococci isolates

A total of one hundred and sixty three (163) *Enterococcus* strains selected for their antimicrobial resistance pattern were included in this study. Strains were isolated from multiple sites or specimens from 514 patients who had been admitted to the King Khalid University (KKU) hospital, (a 680 beds tertiary-care hospital located in Saudi Arabia, Riyadh), between March, 2009 and December, 2010. There were 172 urine, 134 blood, 62 burn swabs, 67 wound swabs, 4 cerebrospinal (CSF) samples, 19 throat swabs, 23 ascitic fluid and 33 catheter tips. Demographic, clinical and laboratory data were prospectively collected, through structured questionnaire on all hospital acquired HLGR enterococci cases, regardless of age, sex and nationality. HLGR was defined as reported by the microbiology laboratory. Cases were classified as infection according to center of disease control and prevention (CDC) criteria.

Specimens were cultivated on bile esculine azide (BEA) agar (Oxoid, England) and 6.5 NaCl media. The isolated enterococci were identified to the species level using test scheme proposed by Facklam and Collins (1989). Confirmatory identification of the strains was performed using the API 20 strep system (BioMerieux S.A, Marcy l'Etoile, France). One strain of *Enterococcus casseliflavus* (isolate 342H), that showed ambiguous identification with API 20 strip, was further identified as *E. faecium* by amplification and sequencing of the 16S ribosomal deoxyribonucleic acid (DNA) performed as described by Angeletti et al. (2001).

Antimicrobial susceptibility testing

Susceptibility to antimicrobial agents was carried out on Mueller–Hinton agar (Difco Laboratories, Detroit, Mich.) by the Kirby–Bauer disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (NCCLS, 2004). Various antibacterial agents tested were ampicillin (10 μg), vancomycin (30 μg), teicoplanin (30 μg), erythromycin (15 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg) and linezolid (30 μg). Aminoglycoside resistance was performed with discs containing amikacin (30 μg), gentamicin (120 μg), kanamycin (200 μg), tobramycin (200 μg) and streptomycin (300 μg), with *Enterococcus faecalis* ATCC 29212 as a control. Inhibition zone was interpreted according to NCCLS guidelines. The resistant isolates (showing zone sizes of < 10 mm for the three antibiotics) were confirmed as HLAR among enterococci using MIC of gentamicin, kanamycin, streptomycin, tobramycin and amikacin. This was performed by the agar dilution method with antibiotic dilutions ranging from 8 to 4000 μg/ml^{-1}. *E. faecalis* strain ATCC 51299 was used as a resistance control for detecting HLAGR.

β-Lactamase production

All enterococci isolates were tested for β-lactamase production with nitrocefin (Oxoid, England) according to the manufacturer's instructions. Nitrocefin solution (5 μl) was dropped onto a loopful of pure overnight growth placed on a filter membrane. The development of a red colour within 60 s. indicated a positive result. *Staphylococcus aureus* strain ATCC 29213 was used as a positive control.

Deoxyribonucleic acid (DNA) isolation and purification

Isolates of enterococci were grown overnight at 37°C on BEA agar. Bacterial cells were lysed to obtain genomic DNA for polymerase chain reaction (PCR) as follows: three to five bacterial colonies were suspended in 25 μl of a 0.25% sodium dodecyl sulfate–0.05 N NaOH solution and boiled for 15 min. Then 200 μl of H_2O was added to the mixture and 5 μl of the diluted mixture was used in the PCR. DNA purification was done according to genomic DNA purification kit (Gentra, USA).

Table 1. Primers used for the amplification of aminoglycoside resistance genes.

Gene	AME	Primer sequence (5` - 3`)	Product size (bp)	Reference
aac(6`)-Ie-aph(2``)-Ia	AAC(6`)-I APH(2``)-Ia	CAGGAATTTATCGAAAATGGTAGAAAAG CACAATCGACTAAAGAGTACCAATC	369	Vakulenko et al. (2003)
aph(2``)-Ib	APH(2``)-Ib	CTTGGACGCTGAGATATATGAGCAC GTTTGTAGCAATTCAGAAACACCCTT	867	Vakulenko et al. (2003)
aph(2``)-Ic	APH(2``)-Ic	CCACAATGATAATGACTCAGTTCCC CCACAGCTTCCGATAGCAAGAG	444	Vakulenko et al. (2003)
aph(2``)-Id	APH(2``)-Id	GTGGTTTTTACAGGAATGCCATC CCCTCTTCATACCAATCCATATAACC	641	Vakulenko et al. (2003)
aph(3`)-IIIa	APH(3`)-III	GGCTAAAATGAGAATATCACCGG CTTTAAAAAATCATACAGCTCGCG	523	Vakulenko et al. (2003)
ant (4`)-Ia	ANT(4`)-I	CAAACTGCTAAATCGGTAGAAGCC GGAAAGTTGACCAGACATTACGAACT	294	Vakulenko et al. (2003)

Amplification of aminoglycoside-modifying enzymes (AMEs) genes

The presence of the genes for the AMEs responsible for HLAR was confirmed by PCR. The studied genes were *aac*(6`)-*Ie*-*aph*(2``)-*Ia, aph*(2``)-*Ib, aph*(2``)-*Ic, aph*(2``)-*Id, aph*(3`)-*IIIa* and *ant*(4`)-*Ia*. PCR reactions were done on bacterial lysates. PCR assays were performed to amplify these genes as described by Vakulenko et al. (2003). The oligonucleotide sequences of primer used for each gene evaluated are shown in Table 1.

The reaction mixture consisted of 45 µl of Supermix (22 mM Tris HCl pH 8.4, 55 mM KCl, 1.65 2 mM MgCl , 220 µM dGPT, 220 µM dATP, 220 µM dCTP, 220 µM dTTP, 22 U recombinant *Taq* DNA polymerase/ml, 3 µl of bacterial DNA and 2 µl of primer solution in a total reaction volume of 50 µl). PCR was performed in a Perkin-Elmer gene amp 2400 thermal cycler. PCR was performed for 32 cycles with the following parameters: denaturation at 94°C for 3 min, annealing at 60°C for 45 s, extension at 72°C for 1 min and final extension at 72°C for 2 min. PCR products were analyzed by electrophoresis at 100 V for 2 h in a 1% agarose gel stained with ethidium bromide and photographed under UV illumination.

Pulsed field gel electrophoresis (PFGE)

For restriction endonuclease digestion of chromosomal DNA, small slices of the agarose plugs were placed into a mixture of 270 µl of distilled water, 30 µl of 10X reaction buffer, and 50 U of *Sma*I (New England BioLabs), and the mixture was incubated at 25°C overnight. After digestion, the plugs were washed for 1 h at room temperature. The slices were placed in wells of a 1.2% SeaPlaque GTG agarose gel (FMC, Rockland, Maine) made with 0.5X Tris-borate- ethylene diamine tetraacetic acid (TBE) (10X TBE is 0.89 M Tris, 0.89 M boric acid and 0.025 M EDTA), and electrophoresed on a CHEF-DRIII apparatus (Bio-Rad) with the following running parameters: initial switch time, 5 s; final switch time, 35 s; start ratio, 1; voltage at 6 V/cm; run time, 20 h; temperature, 14°C. A Lambda ladder PFGE marker (New England BioLabs) was used as the size standard. The gel was stained with ethidium bromide and visualized on a UV transilluminator. DNA banding patterns were compared visually and strains were considered related by PFGE if their patterns differed by one to three bands (Tenover et al., 1995).

Statistical analysis

Univariate analysis was performed using logistic regression and the Fisher exact and Mann-Whitney tests, when appropriate. Variables for which an association with HLGR was suspected (that is, P value ≤0.20) were included in a multivariate stepwise logistic regression model. We considered the P value < 0.05 to be statistically significant.

RESULTS

Distribution of high-level aminoglycoside in enteroccus strains

Of 514 samples tested, a total of 163 antibiotic resistant enterococci were obtained. They were isolated from 89 urine cultures (55%), 26 blood cultures (16%), 19 burn swabs (12%), 14 wound swabs (9%), 11 ascitic fluids (3%) and four catheter tips (2%). The isolates were identified as *E. faecalis* (58.9%), *E. faecium* (26.9%), *Enterococcus avium* (6.1%), *Enterococcus gallinarum* (2.5%), *Enterococcus durans* (1.8%), *Enterococcus hirae* (1.2%), *E. casseliflavus* (1.2%) and *Enterococcus cecorum* (0.6%). The distribution of species and sources are shown in Table 2.

Resistance pattern

The antimicrobial susceptibility patterns of isolated

Table 2. Distribution of *Enterococcus* spp. in various clinical specimens.

Specimen	Total no. of enterococci	No. (%) of *Enterococcus* spp. recovered							
		E. faecalis	*E. faecium*	*E. avium*	*E. durans*	*E. gallinarum*	*E. casseliflavus*	*E. hirae*	*E. cecorum*
Urine	89(55%)	52(58%)	23(26%)	7(8%)	2(2%)	2(2%)	1(1%)	1(1%)	1(1%)
Blood	26(16%)	9(35%)	14(54%)	1(4%)	-	1(4%)	-	1(4%)	-
Burn	19(12%)	15(79%)	2(11%)	-	1(5%)	-	1(5%)	-	-
Wound	14(9%)	8(57%)	3(20%)	1(7%)	-	1(7%)	-	1(7%)	-
Ascitic	11(7%)	9(82%)	2(18%)	-	-	-	-	-	-
Catheter tip	4(2%)	3(75%)	-()	1(25%)	-	-	-	-	-
Total	163	96(59%)	44(27%)	10(6%)	3(2%)	4(2%)	2(1%)	3(2%)	1(1%)

Table 3. Antibiotic resistance of enterococci isolates according to species by disc diffusion method.

Antibiotic	Resistant isolates (%)			
	E. faecalis (n, 96)	*E. faecium* (n, 44)	Other species* (n, 23)	Total (n, 163)
Ampicillin	31(32.3)	16(36.4)	7(30.4)	54(33.1)
Vancomycin	-	2(4.5)	-	2(1.2)
Teicoplanin	-	2(4.5)	-	2(1.2)
Erythromycin	67(69.8)	22(50.0)	11(47.8)	100(61.3)
Ciprofloxacin	58(60.4)	34(77.3)	9(39.1)	101(61.9)
Chloramphenicol	27(28.1)	6(13.6)	4(17.4)	37(22.7)
Amikacin	25(26.0)	15(34.1)	1(4.3)	41(25.2)
Gentamicin [HLR]	20(20.8)	13 (29.5)	1(4.3)	33(20.2)
Kanamycin	20(20.8)	10(22.7)	1(4.3)	31(19.0)
Streptomycin [HLR]	22(22.9)	11(25.0)	-	34(20.9)
Tetracycline	68(70.8)	6(13.6)	6(26.0)	80(49.1)
Linezolid	-	1(2.3%)	-	1(0.6)

*Other species consists of *E. avium* (n, 11), *E. durans* (n, 6), *E. gallinarum* (n, 8), *E. casseliflavus* (n, 4), *E. hirae* (n, 6) and *E. cecorum* (n, 2).

enterococci species by disc diffusion method are summarized in Table 3. They were resistant to erythromycin (61.3%), tetracycline (49.1%) and chloramphenicol (22.7%). HLR to gentamicin (MIC ≥2000 $\mu g/ml^{-1}$), kanamycin (MIC ≥ 1000 $\mu g/ml^{-1}$), amikacin (MIC ≥ 2000 $\mu g/ml^{-1}$) and streptomycin (MIC ≥ 2000 $\mu g/ml^{-1}$) was detected in 20.2, 19.0, 25.2 and 20.9% of the isolates, respectively. The results show that 54 (33.1%) isolates (16 *E. faecium*, 31 *E. faecalis*, 5 *E. avium* and 2 *E. casseliflavus*) were resistant to ampicillin. However, only one strain gave a positive test reaction for production of β-lactamase enzyme from all isolates.

The isolates were tested for their susceptibility to linezolid; a new oxazolidinone antibacterial that has been reported to have activity against Gram-positive cocci, including methicillin resistant *S. aureus* and vancomycin resistant enterococci. They were susceptible to linezolid except one strain of *E. faecium*. Glycopeptide (vancomycin and teicoplanin) resistances were detected in 2 (1.2%) isolates consisting of *E. faecium*.

Detection of genes encoding high-level aminoglycoside resistance enterococci

Multiplex PCR analysis was performed to study the molecular epidemiology of aminoglycoside resistance genes as shown in Figure 1 and Table 4. For the 33 isolates that were known to contain the *aac(6`)-Ie-aph(2``)-Ia* gene, multiplex PCR consistently detected

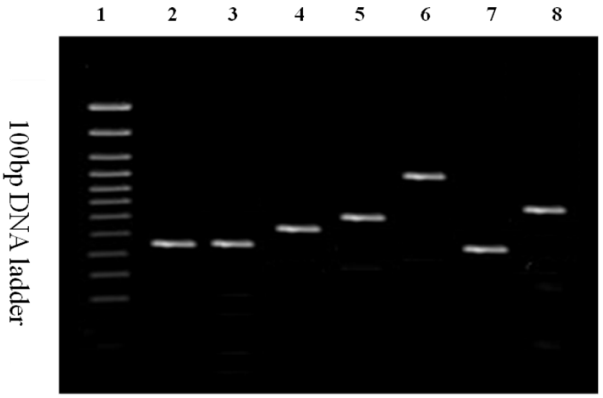

Figure 1. Agarose gel electrophoresis of multiplex PCR products from enterococcal isolates tested for six aminoglycoside resistance genes: *aac(6`)-Ie-aph(2)-Ia* (369 bp), *aph(2)-Ib* (867 bp), *aph(2)-Ic* (444 bp), *aph(2)-Id* (641 bp) and *aph(3')-IIIa* (523 bp). Lane 1, DNA marker; Lane 2 to 7, six representative isolates, respectively that possess one or more of the six genes studied.

Table 4. Profiles of AME genes content among *E. faecalis* and *E. faecium* with intermediate to HLGR.

Enterococcus sp.	No. of Isolate with genes	Presence of AME genes					
		aac(6`)-Ie-aph(2``)-Ia	***aph(2``)-Ib***	***aph(2``)-Ic***	***aph(2``)-Id***	***aph(3`)-IIIa***	***ant (4`)-Ia***
***E. faecium* (n, 20)**	6 (30%)	+	-	+	-	+	-
	1 (5%)	+	+	-	-	+	-
	3 (15%)	+	+	-	-	-	-
	2 (10%)	+	-	+	-	-	-
	2 (10%)	-	+	-	-	+	-
	1 (5%)	-	-	+	-	+	-
	2 (10%)	+	-	+	-	+	-
	3 (15%)	-	+	_	-		-
***E. faecalis* (n, 13)**	3 (23%)	+	-	+	+	+	-
	4 (31%)	+	-	_	+	+	-
	3 (23%)	-	+	_	-	+	-
	1 (8%)	+	-	+	-	+	-
	2 (15%)	+	-	+	-	-	-

this gene in 33 isolates. Multiplex PCR correctly detected the *aph(2')-Ib*, *aph(2')-Ic*, *aph(2')-Id*, *aph(3')-IIIa* in most isolates compared to the conventional PCR method with one pair of primers per reaction for each of the amino-glycoside resistance genes (Table 4). None of the isolates contained *ant(4')-Ia* genes. Coexistance of *aac(6`)-Ie-aph(2``)-Ia* and *aph(3')-IIIa* among *E. faecalis* and *E. faecium* isolates were 27 and 46%, respectively. There was correlation between resistance to tested was examined and we found the existence of 11 isolates with identical profile (clone A). Five (5) isolates proved to be highly correlated to the clone A and were considered aminglycosides and the existence of the *aac(6`)-Ie-aph(2``)-Ia* and *aph(3')-IIIa* genes. Figure 1 shows PCR products in a representative gel electrophoresis photo that includes isolates which together contain the different genes in various strains.

The genetic profile of 20 isolates of HLGR *E. faecalis* to belong to this major clonal group (Figure 2). The other 4 isolates were all unrelated.

DISCUSSION

In the past two decades, the members of the genus

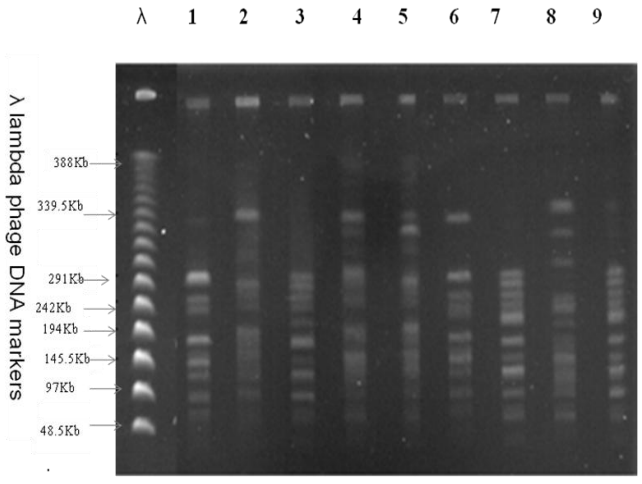

Figure 2. PFGE profile of small-digested chromosomal DNA of HLGR *Enterococcus faecalis* isolates belonging to major clonal group A. λ, Lambda phase DNA markers; Lanes 1 to 4, isolates from urine sample; Lanes 5 to 8, isolates from blood samples; Lane 9, isolates from wound specimen.

Enterococcus have emerged as important nosocomial pathogens worldwide (Murray, 2000). Recent years have witnessed increased interest in enterococci not only because of their ability to cause serious infections but also because of their increasing resistance to many antimicrobial agents (Dupont et al., 2011). The increase of antimicrobial resistance among enterococcal species in Saudi Arabia has presented a serious challenge for the Saudi medical community (El-Amin and Faidah, 2011).

In the present study, we have evaluated the frequency of species, the patterns of antimicrobial resistance and the molecular epidemiology of enterococci in clinical specimens obtained from KKU hospital in Riyadh, Saudi Arabia. Different species of enterococci are currently recognized, but 85 to 95% of enterococcal infections are caused by *E. faecalis*, and 5 to 10% are caused by *E. faecium* (Winn et al., 2006). This study showed that *E. faecalis* (59%) and *E. faecium* (27%) were the most prevalent species. Other species were found only in a minority of cases [*E. avium* (6%), *E. duramce* (2%), *E. gallinarum* (2%), *E. casseliflavus* (2%), *E. hirae* (3%) and *E. cecorum* (1%)]. Although, few studies (Shepard and Gilmore, 2002; Kacmaz and Aksoy, 2005) have documented an increase in the prevalence of *E. faecium*. In this study, the prevalence of this species was considerable low. The prevalence of of *E. faecium* was comparable to the distribution of enterococcal species in some studies (Udo et al., 2003, Helmi et al,, 2008) but contrasted with others, *E. faecium* was the most common isolated species (Wong et al., 2000; Vakulenko et al., 2003). Most of enterococcal isolates were recovered from urine cultures (55%), blood cultures (16%), burn swabs (12%) and wound swabs (9%) which is consistent with reports that enterococci have become the leading cause of urinary tract, surgical wound infections and bacteremia (Toutouza et al., 2001; Helmi et al., 2008).

Antibiotic resistance in enterococci is either intrinsic or acquired. Intrinsic traits expressed by enterococci include resistance to semisynthetic penicillinase resistant penicillins, cephalosporins, low level of aminoglycosides and low level of clindamycin, whereas acquired resistance includes resistance to chloramphenicol, erythromycin, high-level of clindamycin, tetracycline, and high level of aminoglycosides, penicillin, fluroquinolones and vancomycin (Murray, 1990). HLAR in enterococci is usually mediated by AMEs, which eliminate the synergistic bactericidal effect of combined exposure to a cell wall-active agent and an aminoglycoside.

In the present study, 61,9, 61,3, 49.1 and 22,7% were resistant to ciprofloxacin, erythromycin, tetracycline and chloramphenicol, respectively which were similar to levels reported for these antibacterial among enterococci isolated in Germany (Reinert et al., 1999), Kuwait (Udo et al., 2003) and Egypt (Helmi et al., 2008). It is reassuring that 100 and 98.8% of the *E. faecalis* and *E. faecium* in the current work were vancomycin and teicoplanin susceptible, respectively similar to the result obtained in enterococci isolated in different countries (Ozkuyumcu, 1999; Miranda et al., 2001). This result was contrary to the situation in most hospitals in the USA (Perlada et al., 1997) and Europe (Schouten et al., 2000) where high prevalence of vancomycin resistance is common. This finding indicates that vancomycin retains its therapeutic efficacy against most *E. faecalis* infections. Two out of 13 *E. faecium* strains were glycopeptide resistant comparable with other findings that *E. faecium* are usually more resistant than *E. faecalis* (Helmi et al., 2008).

HLR to gentamicin, kanamycin, amikacin and streptomycin was detected in 20.2, 19.0, 25.2 and 20.9% of the isolates, respectively. 41.1% of the enterococci showed HLAR and combined HLGR and high level streptomycin resistance was significantly ($p<0.05$) higher in *E. faecium* (48.8%) than *E. faecalis* (31.7%), as also reported by Gordon et al. (1992). Observations of HLGR in *E. faecalis* and HLAR in E. faecium have also been reported (Udo et al, 2003; Helmi et al., 2008). HLAR was significantly higher among *E. faecium* isolates; an observation which is consistent with that found in previous reports (Bhat et al., 1997). Resistance to aminoglycosides in enterococci is often associated with multidrug resistance (Murray, 1990). In our study, *E. faecalis* and *E. faecium* showed resistance to as many as nine drugs. Concomitant resistance of HLGR strains to the β-lactam antibiotic (ampicillin) was quite high in both the species and it was higher in *E. faecalis* (Table 2). Concomitant HLAR, high-level ampcillin resistance and resistance to vancomycin has been reported in 16% isolates by Helmi et al. (2008) from Egypt. Resistance to ampicillin, which is usually intrinsic, is primarily due to low affinity of the penicillin binding proteins and it results in loss of synergistic effect between β-lactams and

aminoglycosides leading to treatment failures. The reason for higher prevalence of ampicillin resistance in examined hospitals could be because of the set up where chronic cases are prevalent and there is a wider usage of broad spectrum antibiotics. HLAR enterococci have been increasingly reported from hospitalized patients' world-wide (Dupont et al., 2011). In such cases, synergistic combination of a cell wall-active agent plus an amino-glycoside cannot be used for the treatment of severe enterococcal infections.

Different isolates expressing HLR to amikacin, gentamicin, kanamycin, tobramycin and streptomycin were investigated for the presence of genes encoding the AA C(6')-APH (2''), APH (3') and ANT (4') enzymes. The results are summarized in Table 4. The HLAR in enterococci is usually coded by *aac(6')-aph(2'')* genes encoding the AAC (6')-APH (2'') enzymes (Feizabadi et al., 2006). The results of PCR of isolates with gentamicin MIC > 500 µg/ml confirmed that all isolates contained *aac(6')Ie-aph(2'')* gene. However isolates with gentamicin MIC ≤ 500 µg/ml also contained this gene. Detection of *aac(6')Ie-aph(2'')* gene in isolates with gentamicin MIC ≤ 500 µg/ml has also reported in other studies supporting the presence of the *aac(6')Ie-aph(2'')* gene in low level gentamicin resistant enterococci (Udo et al., 2003; Feizabadi et al., 2006). The *aph(3')* genes encoding the APH (3') enzymes were detected in 18 isolates. Importantly, it was found among 31% of *E. faecium* isolates with gentamicin MIC ≤ 500 µg/ml. However, the types and distribution of AME genes in enterococii vary in different geographical region (Gordon et al., 1992; Udo et al., 2003; Helmi et al,, 2008). None of the isolates contained the *ant(4')* genes. One *E. faecalis* isolate that was resistant to kanamycin (MIC > 4000 µg/ml), tobramycin (MIC 2048 µg/ml) and amikacin (MIC 512 µg/ml) gave negative results for all the AME genes tested. It is concluded that there was correlation between resistance to tested aminglycosides and the existence of the *aac(6`)-Ie-aph(2``)-Ia* and *aph(3')-IIIa* genes. It should be regular surveillance of antibacterial susceptibilities to detect resistance and prevent the establishment and spread of HLAR enterococci.

As demonstrated by molecular typing, there was a predominant clone among isolates of HLGR *E. faecalis*. As this clone was obtained from isolates of patients admitted in two wards during a short time-frame period, it is possible to consider that there was an intra and inter-hospital dissemination of *E. faecalis* HLGR. This is the first report of a major clonal *E. faecalis* HLGR spread in KKU hospital. Previously, only a clone of *E. faecalis* HLGR had been reported in other country (D'Azevedo et al., 2006). In fact, we found a considerable genetic diversity among HLGR *E. faecalis* in our study. Noteworthy, clonal dissemination of HLGR *E. faecalis* has not yet been described in the state of Riyadh.

This molecular diversity of *E. faecalis* isolates with similar PFGE patterns, suggesting that patient-to-patient transmission might have occurred at our hospital. The finding of HLGR and non–HLGR isolates with the same PFGE pattern can be explained by the fact that PFGE is based on chromosomal DNA; HLGR phenotype, on the other hand, is associated with plasmids, which can be transferred independently from chromosomal DNA. The identification of a major clonal group in HLGR *E. faecalis*, draws attention to the increasing need for control measures to avoid horizontal transmission, since the enterococci currently represent a serious problem in health institutions, especially because of the possibility of spread from healthy carriers and the lack of effective treatment options.

Drug-resistant enterococci present a challenge for the clinician and the clinical microbiologist because of their increased occurrence in nosocomial infections. However, large differences among hospitals in both antimicrobial use and prevalence of resistance indicate a potential for further improvement of antibiotic policies, and possibly hospital infection control, to maintain the low resistance levels observed in the Saudi hospitals (El-Amin and Faidah, 2011). The situation obligates the physicians should use antibiotics appropriately and comply with the infection-control policies in an effort to prevent further spread of these resistant organisms.

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