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Prevalence of class 1 and 2 integrons in *Enterococcus* spp. and their relationship with antimicrobial resistance

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ABSTRACT

A total of 113 clinical isolates of *Enterococcus* were characterized for integron content and for resistance to antibiotics. Class 1 integrons were more frequent than class 2 integrons in *Enterococcus faecalis* and *Enterococcus faecium* isolates. Two different resistance gene arrays were identified among the class 1 integrons. These cassette arrays encode deitheraminogly coside3'-adenyltransferase (*aadA1*cassette) or dihydro folate reductase (*dfrA7*cassette). Isolates were tested against a panel of antibiotics, and most isolates were multiple drugs resistant. This is the first report of class 1 and class 2 integrons in *E.faecalis* and *E.faecium* in Iran.

KEY WORDS: Antibiotic, *Enterococcus,* integron.

1. INTRODUCTION

Over the last two decades enterococci have emergedas one of the leading causes of nosocomial infections the worldover. This is a result of the development of hospital adapted lineages (Andersson and Hughes, 2010), which tend to carry antimicrobial resistance genes and encode virulence factors. Enterococci exhibit intrinsic resistance to several antibiotics, including cephalosporin's, aminoglyco sides and quinolones, and have a propensity to acquire antibiotic resistance genes. They exhibit high-level resistance to most penicillins, chloramphenicol, tetracyclines, aminoglycosides and glycopeptides (mainly vancomycin-VRE) as a result of mutations, or the acquisition of plasmids, transposons or integrons that carry resistance genes (Drahovska, 2004). Some resistance genes are of particular concern. Resistance to vancomycin arises as a result of altered antibiotic binding target in the bacterial peptidogly can pentapeptide precursors, thus preventing peptidoglycan growth and assembly (Nateghian, 2011). Eight resistance genotypes, caused by the presence of *vanA-vanN*genes have been found (Rudy, 2005; Batistao, 2012; Hegstad, 2014). The most frequent is geno type *vanA*, responsible for resistance to vancomycin and teicoplanin, and is mainly found in *Enterococcus faecalis* and *Enterococcus faecium* strains. This resistance often appears after an earlier antibiotic therapy (Hosseini, 2015; Hill, 2010; Eliopoulos and Gold, 2001). Genes encoding antibiotic resistance are also commonly found as components of gene cassettes, associated with integrons. Integrons are a gene capture and expression system known to be responsible for multidrug resistance. They have an important role in the dissemination of antibiotic resistance among pathogenic bacteria. Several classes of integrons, based on differences in the integron-integrase gene, have been described from pathogenic Gram negative bacteria. Of these; the class 1 and class 2 integrons are the most common and widely distributed. In clinical contexts, these integrons can carry single or multiple gene cassettes, each of which encodes resistance to a different antibiotic (Souli, 2010; Khosravi, 2011). Althoughclass 1 integrons are most frequently associated with Gram negative bacteria, they have also recently been observed in Gram-positive bacteria including *Corynebacterium*, *Staphylococcus* and *Aerococcus* (Nandi, 2004). The first report of an integron that carried a gene cassette encoding *aadA* was from *E.faecalis* (Clark, 1999; Hosseini, 2016). Both class1and class 2 integrons have been detected in clinical enterococci in South China (Xu, 2010). In this study, we report class 1 and 2 integron positive enterococci strains isolated in Iran, and characterize their pattern of antimicrobial resistance.

2. MATERIALS AND METHODS

Bacterial isolates: In this study, we report class 1 and 2 integron positive enterococci strains and find a relationship between the patterns of antimicrobial resistance with them. This study included 113 (including 76 *E. faecalis* isolates, 30 *E. faecium* isolates and 7 isolates as other *Enterococcus* spp) clinical specimens submitted for bacterial culture at the microbiology laboratories of three major university hospitals in Hamedan, Iran, from 2014 to 2015. Majority of the samples were urine specimens (89.7%) and the others were isolated from wounds (1%), blood (1%) and other (8.3%). The All isolates were identified by standard biochemical methods. Further confirmation of isolates was done by polymerase chain reaction (PCR) detection of *ddl* gene (Table 1).

Antibiotic resistance screening: Antimicrobial susceptibility testing was performed using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines(Dudley, 2013): Erythromycin (15 µg), Tetracycline (30 µg), Ciprofloxacin (5 µg), Vancomycin (5 µg), Teicoplanin (30 µg), Norfloxacin (10 µg), Nitrofurantoin (100 µg), Quinopristin-Dalfopristin [Synercid (15 µg)], Chloramphenicol (30 µg), Gentamicin (30 µg), Linezolid (10 µg), and Ampicillin (2 µg) (MAST,Merseyside, UK).Isolates shown to be resistant to at least

three different classes of antimicrobial agents were determined to be MDR. In addition, minimum inhibitory concentrations (MIC) of the glycopeptide antibiotics i.e. vancomycin and teicoplanin against the *E. faecalis* and *E. faecium* isolates were determined using microdilution broth method. *E. faecalis* ATCC29212 and *Staphylococcus aureus* ATCC 25923 strains were used for quality control.

Integron characterization and sequencing of resistance encoding gene cassettes: The presence of integrons genes and resistance encoding gene cassettes associated with class 1 was investigated by PCR using specifics primers (Table 1). The total DNA was extracted by DNA extraction kit (Bioneer, Daejeon, Republic of Korea).The PCR was performed in a reaction mixture with total volume of 25 µl, containing 2 µl template DNA; 0.2 mM of each deoxynucleoside triphosphate; 10 pmol of each primers; 10 mM Tris- HCl; 1.5 mM MgCl2; 50 mM KCl; 1.5 U of Taq DNA polymerase. PCR was performed with the Eppendorf and Bioradthermocycler (ASTEC Co., Japan). Amplification was done as follows: initial denaturation step at 94°C for 5 minute followed by 35 cycles consisting of denaturation (94°C for 30 seconds), annealing (55°C, 30 seconds for *intI1*, *intI2* and 5'C Sand 3'CS), and extension (72°C for1minute), followed by a final extension step. The amplified DNA was separated by gel electrophoresis on 1.5% agarose, and visualized under UV transillumination. Sequencing of gene cassettes of class1 integron was performed with the ABI 3730X capillary sequencer (Genfanavaran, Macrogen, Seoul, Korea). Finally, Sequence data were analyzed with Chromas software and aligned with Gen Bank data by using nucleotide Basic Local Alignment Search Tool.

Detection of *vanA* and *vanB* genes by PCR method: The presence of *vanA* and *vanB* genes was detected by the PCR using specifics primers (Table 1). The PCR mixtures with a final volume of 25 ml consisted of 2 ml template DNA; 0.2 mM of each deoxynucleoside triphosphate; 10 pmol of each primer; 10 mM Tris–HCl; 1.5 mM $MgCl_2$; 50 mM KCl; and 1.5 U of Taq DNA polymerase. Amplification involved an initial denaturation at 94°C, 3 minutes followed by 30 cycles of denaturation (94°C, 1 minute), annealing (54°C, 1 minute) and extension (72°C, 1 minute), with a final extension step (72°C, 7 minutes). The following reference strains were used as positive and negative controls: *E. faecalis* ATCC 29212 (Vancomycin sensitive), *E. faecalis* ATCC 51299 (*vanB* positive), *E. faecalis* E 206 (*vanA* positive).

Statistical analysis: The results were analyzed using SSPS version 17.0 software (SPSS, Inc., Chicago, IL, USA) and chisquare test. The statistical significance of the data was determined and a *P* value of <0.05 was considered significant.

3. RESULTS

Analysis of integrons: Among the total 67 isolates of *E.faecalis*, 48 (71.6%) isolates and 4 (6%) isolates carried class 1(*intI1*) or class 2 (*intI2*) integrons, respectively. The frequency ofclass 1 and 2 integrons in *E. faecium* was 86.7% (26/30) and 6.7% (2/30), respectively. Moreover, 5 (7.5%) isolates of *E.faecalis* and 2 (6.7%) isolates of *E. faecium* harbored both *intI1* and *intI2*. Class 1 integrons were more frequent among *E.faecalis* and *E. faecium* isolates in comparison with class 2 integrons (P < 0.001), and the frequency of *intI1* in *E. faecium* was higher than *E.faecalis* isolates. Two different types of gene cassette arrays were found in class 1 integrons. sequence analysis of integron's variable region indicated the presence of aminoglycoside 3'-adenyltransferase (*aadA1*; 1000 bp), dihydrofolate reductase type I (*dfrA7*; 750 bp) gene cassettes among the isolates, which 27% (13/48) and 16.6% (8/48) of *E.faecalis* isolates and 38.5% (10/26) and 23% (6/26) of *E. faecium* isolates harboring *aadA1* and *dfrA7* gene cassettes, respectively.

Susceptibility of *E.faecalis* and *E. faecium* isolates to antimicrobial agents: Antimicrobial susceptibilities of *E.faecalis* and *E. faecium* isolates are presented in Tables 2 and 3. All *E.faecalis* isolates harboring *intI1* or *intI2* were MDR. Resistance to the majority antibiotics except for chloramphenicol and quinopristin-dalfopristin was higher in *E. faecium* isolates compared to *E. faecalis* isolates. All isolates that carried integrons had a higher frequency (P < 0.001) of resistance, except vancomycin and teicoplanin in *E. faecalis* strains, to the entire antibiotics (Table 3). The highest rate of resistance among all *intI1*-positive and *intI1*-negative isolates in *E.faecalis* isolates, showed against quinopristin-dalfopristin and tetracycline. And in *E. faecium* isolates showed against tetracycline in *intI1*-positive strain and *intI1*-negative isolates showed against tetracycline, norfloxacin, quinopristin-dalfopristin, chloramphenicol and gentamicin. All isolates of *E. faecalis* and *E. faecium* were susceptible to linezolid (100%). Fifty percent of studied of *intI1*-positive in E. *faecium* isolates demonstrated high level vancomycin and teicoplanin resistance (MIC ≥ 32). But 1.5 % of *intI1*-positive in *E. faecalis* isolates was resistance to vancomycin and teicoplanin (MIC ≥ 32) table 2, 3 and 4.

Presence of *vanA* and *vanB* genes: According to the PCR assay for van genes, eighteen (60%) of *vanA* gene in *E. faecium*, 15 (83.3%) isolates carried class I integrons. In addition, two isolates with class II integron were positive for *vanA* gene. No *vanB*-positive isolates were detected. The *vanA* and *vanB* genes were not detected in any of the *E. faecalis* isolates.

DISCUSSION

Integrons which are widespread among clinically important Gram negatives have recently been detected in clinical *E. faecium* and *E. faecalis* isolates (Mashouf, 2015; Gu, 2008; Vinue, 2010). It was therefore of great interest to search for intergrons in enterococci, and it was expected to find integron positive isolates in a diverse collection of enterococci. In Asia, the frequency of integron-positive *enterococc* spp has been shown to vary in different countries. The high incidence of antibiotic resistance found in this survey was most probably due to the widespread use of numerous antimicrobial agents in our country. In this study frequency of integrons of class 1, 2 were estimated as 86. 7%, 6.7%, and 71.6%, 6% in clinical *E. faecium* and *E. faecalis* isolates, respectively. The integron prevalence was relatively higher than in other investigations; for example, Xu (2010), reported that class 1 integrons in 8 out of 10 tested enterococci isolates. Furthermore, research by Clark, found integron related aad gene (Clark, 1999). Isolates that carried class 1 integrons were more likely to be resistant to tetracycline, ciprofloxacin, norfloxacin, erythromycin, quinopristin - dalfopristin, chloramphenicol and gentamicin than isolates that did not carry integrons (Table 2, 3). In addition, here we studied the existence and the sizes of variable regions of class 1 and 2 integrons, using their specific primers by PCR technique, as we detected (10/26) and 23% (6/26) *E. faecium* isolates and 27% (13/48) and 16.6% (8/48) *E. faecalis* isolates containing the *aadA1* and *dfrA7* gene cassette, respectively. The aad Al sequence shared 100% identity with that reported for another strain of *E.coli* (Gen Bank accession no. HQ880267.1). The *dfrA7* sequence presented 100% identity with those found in *Klebsiella pneumoniae* strain KD187 (Gen Bank accession no. EU339235.1). This could indicate, the transfer of resistance genes that may occur between Gram-positive and Gram-negative organisms could lead to the construction of diverse resistance to the usual antibiotics (Chen, 2011). Moreover Clark (1999), and published similar results confirming our investigation. They showed the transfer of class 1 integron via plasmid between *E. faecalis*.So, these groups of gene cassettes can be transferred of Gram-negative bacteria to *enterococci* spp, which can be a serious problem. Resistance profiles of clinical enterococci were in agreement with other reports detecting a high frequency of resistance to aminoglycosides, especially in *E. faecium* (Dicuonzo, 2001; Dupre, 2003). No linezolid resistant in *E. faecium*, and no linezolid and Nitrofurantoin resistant in *E. faecalis* isolates were found in this study, and vancomycin and teicoplanin resistance was detected only in two *E. faecalis* isolate. Similar results were previously described in a study performed by Bujdakova, (2004) which is in accordance with the very low occurrence of vancomycin resistance in Slovakia. In our study 92.3% (24 isolates) and 60% (29 isolates) of MDR isolates in *E. faecium* and *E. faecalis*, carried class 1 and 2 integrons, respectively. In other research by Busani (2004), 90% of isolates was associated with MDR. On the other hand, High frequency of resistance to tetracycline and vancomycin was observed in *E. faecium* strains. The resistance vancomycin and teicoplanin was found in *E. faecium*, with 60 % (18 isolates) occurrence, which is more resistant than *E. faecalis* strains. The MIC for vancomycin in all the resistant isolates was ≥512 µg/ml, except for two of the isolates in *E. faecium* which was 256 µg/ml. Furthermore, The MIC for teicoplanin in all the resistant isolates was ≥ 512 µg /ml, except for one of the isolates in *E. faecalis* which was 256 µg/ml. all of strains of enterococc which were sensitive to vancomycin and teicoplanin, demonstrated MIC =2-4 µg/ ml (Tabel 4).Several other reports have indicated the elevated occurrence of antibiotic resistant among enterococc spp (Rudy, 2005; Aarestrup, 2000). This resistance appears often due to excessive use of antibiotics. We also found a significant relationship between integron class 1 and *vanA* in *E. faecium*. *VanA* and *vanB* genes are two of the most common vancomycin resistant genes which *vanA* genes was the most common genotype in this study. We detected the high level of integrons (83.3%) of *vanA* gene in *E. faecium* isolates ($P < 0.01$). Similar to studies conducted in Brazil (Resende, 2014) and India (Praharaj, 2013), *vanA* genes was the most commonly genotypein *E. faecium*.

Table.1.Primers used in this study

Gene targets	Primer sequences (5' to 3')	amplicon / product size (bp)	References
intI1	F:CAGTGGACATAAGCCTGTTC-3' R:CCCGACGCATAGACTGTA-3'	160	(Koeleman, 2001)
intI2	F:TTGCGAGTATCCATAACCTG-3' R:TTACCTGCACTGGATTAAGC-3'	288	(Koeleman, 2001)
3CS,5CS	F:GGCATCCAAGCAGCAAG R: AAG CAG ACT TGA CCT GA	Variable	(Koeleman, 2001)
ddl E. faecalis	F: ATCAAGTACAGTTAGTCTTTATTAG R: ACGATTCAAAGCTAACTGAATCAGT	941	28
ddl E. faecium	F: TTGAGGCAGACCAGATTGACG R: TATGACAGCGACTCCGATTCC	658	28

vanA	F: GGGAAAACGACAATTGC R: GTACAATGCGGCCGTTA	732	(Khair, 2013)
vanB	F: ACCTACCCTGTCTTTGTGAA R: AATGTCTGCTGGAACGATA	300	(28)

Table.2. Antimicrobial susceptibility of integron-positive and integron-negative of *E. faecium*

Antimicrobial agent	**Integron-positive isolates (n = 26)**			**Integronnegative isolates (n = 4)**			**Total (n=30) (*P* value=P<0.001)**		
	R	I	S	R	I	S	R	I	S
Vancomycin	15(50)	0	11(36.7)	3(10)	0	1(3.3)	18(60)	0	12(40)
Teicoplanin	15(50)	0	11(36.7)	3(10)	0	1(3.3)	18(60)	0	12(40)
Ampicillin	10(33.3)	0	16(53.3)	2(6.7)	0	2(6.7)	12(40)	0	18(60)
Tetracycline	25(83.4)	0	1(3.3)	4(13.3)	0	0	28(93.3)	0	2(6.7)
Ciprofloxacin	20(66.7)	4(13.3)	2(6.7)	3(10)	1	3.3	23(76.6)	5(16.6)	2(6.7)
Norfloxacin	19(63.3)	7(23.4)	0	4(13.3)	0	0	23(76.6)	7(23.4)	0
Erythromycin	19(63.3)	5(16.7)	2(6.7)	4(13.3)	0	0	23(76.6)	5(16.7)	2(6.7)
Synercid	18(60)	0	8(26.7)	4(13.3)	0	0	22(73.3)	0	8(16.7)
Chloramphenicol	1(3.3)	5(16.7)	20(66.7)	0	1(3.3)	3(10)	1(3.3)	6(20)	23(76.7)
Gentamicin	24(80)	0	2(6.7)	4(13.3)	0	0	28(93.3)	0	2(6.7)
Nitrofurantoin	3(10)	0	23(76.7)	0	1(3.3)	3(10)	3(10)	1(3.3)	26(86.7)
Linezolid	0	0	26(86.7)	0	0	4(13.3)	0	0	30(100)

Table.3. Antimicrobial susceptibility of integron-positive and integron-negative of *E.faecalis*

Antimicrobial agent	**Integron-positive Isolates (n = 48)(%)**			**Integronnegative Isolates (n = 19)(%)**			**Total (n=67) (*P* value=P<0.001)**		
	R	I	S	R	I	S	R	I	S
Vancomycin	1(1.5)	0	47(70)	1(1.5)	0	18(27)	2(3)	0	65(97)
Teicoplanin	1(1.5)	0	47(70)	1(1.5)	0	18(27)	2(3)	0	65(97)
Ampicillin	2(3)	0	46(68.5)	1(1.5)	0	18(27)	3(4.5)	0	64(95.5)
Tetracycline	45(67)	2(3)	1(1.5)	17(25.5)	0	2(3)	62(92.5)	2(3)	3(4.5)
Ciprofloxacin	16(23.8)	30(44.7)	2(3)	8(12)	9(13.5)	2(3)	24(36)	39(58)	4(6)
Norfloxacin	15(22.3)	31(46.2)	2(3)	8(12)	6(9)	5(7.5)	23(34.3)	37(55.2)	7(10.5)
Erythromycin	27(40.2)	17(25.3)	4(6)	12(18)	6(9)	1(1.5)	39(58.2)	23(34.3)	5(7.5)
Synercid	45(67)	0	3(4.5)	18(27)	0	1(1.5)	64(95.5)	0	3(4.5)
Chloramphenicol	3(4.5)	6(9)	39(58)	2(3)	3(4.5)	14(21)	5(7.5)	9(13.5)	53(69)
Gentamicin	31(46)	7(10.5)	10(15)	12(18)	3(4.5)	4(6)	43(64)	10(15)	14(21)
Nitrofurantoin	0	0	48(71.6)	0	0	19(28.4)	0	0	67(100)
Linezolid	0	0	48(71.6)	0	0	19(28.4)	0	0	67(100)

Table.4.Results of MIC for glycopeptides antibiotics of vancomycin and teicoplanin of intI1-positive in *E. faecium* and *E. faecalis* strains

	***E. faecium* (n=26)**					***E. faecalis* (n=48)**			
Antimicrobial agent	**Teicoplanin**		**Vancomycin**			**Teicoplanin**		**Vancomycin**	
MIC concentration µg/ml	2,4	≥512 512	2,4	-	≥512 256,512	2,4	256	2,4	512
Sensitivity Status	S	R	S	I	R	S	R	S	R
MIC (µg/ml)	≤8	≥32	≤4	-16 8	≥32	≤8	≥32	≤4	≥32
Number	9, 2	2, 13	10, 1	0	2, 4,9	43,4	1	43, 4	1
Percent (%)	34.6, 7.7	7.7, 50	38.5, 3.8	0	7.7, 15.4,34.6	89.6, 8.3	2.1	89.6, 8.3	2.1

CONCLUSION

The results showed that *vanA* gene can be transmitted via integrons, and maybe passed to other bacteria via conjugation. As a result, mobile genetic elements such as integrons can be used as one of the most important mechanisms in the development of antibiotic resistance. This is the first report of class 1 and class 2 integron in *E.faecalis* and *E. faecium* isolates in Iran. Simultaneous presence of *vanA* genes and class 1 integrons enhances possibility of spreading antibiotic resistance via horizontal gene transfer. The widespread occurrence of integrons proved to be a major challenge for the treatment and control of infectious diseases.

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Conflict of Interest: There isn't any conflict of interest in this study.

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