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Faecal carriage of high-level aminoglycoside-resistant and ampicillin-resistant *Enterococcus* species in healthy Iranian children

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ABSTRACT

Objectives: High-level aminoglycoside, ampicillin and vancomycin resistance and virulence genes among enterococcal isolates collected from healthy middle-school children in Ardabil, Iran, during 2016 were investigated.
Methods: Totally, 305 faecal specimens were collected. Isolates underwent antimicrobial susceptibility testing, virulence gene detection and molecular typing.
Results: Totally, 409 enterococcal isolates were collected, comprising *Enterococcus faecium* (235; 57.5%), *Enterococcus faecalis* (56; 13.7%) and other *Enterococcus* spp. (118; 28.9%). Overall, 71 (17.4%), 11 (2.7%) and 10 (2.4%) isolates were identified as high-level streptomycin-resistant (HLSR), high-level gentamicin-resistant (HLGR) and ampicillin-resistant (AR), respectively. Among HLSR isolates, 40 (56.3%), 5 (7.0%) and 26 (36.6%) were *E. faecium*, *E. faecalis* and other *Enterococcus* spp., respectively. Among HLGR isolates 4 (36.4%) and 7 (63.6%) and among AR isolates 7 (70.0%) and 3 (30.0%) were *E. faecium* and other *Enterococcus* spp., respectively. Accordingly, 21.6%, 3.6% and 3.3% of subjects were colonised with HLSR, HLGR and AR *Enterococcus* spp. Carriage of HLGR, HLSR and AR isolates was associated with prior antibiotic consumption ($P \leq 0.05$). Additionally, male sex and antacid consumption were associated with AR enterococcal carriage. Moreover, 69 (97.2%), 10 (90.9%) and 9 (90.0%) of HLSR, HLGR and AR isolates were multidrug-resistant, respectively. No vancomycin-resistant enterococci were detected. ERIC-PCR revealed high genetic diversity among isolates. *gelE* and *asa1* were major virulence genes both in *E. faecalis* and *E. faecium*. Presence of *gelE* was associated with HLSR and HLGR phenotypes ($P \leq 0.05$).
Conclusion: Community intestinal carriage of HLSR enterococci was high; however, carriage of HLGR and AR enterococci was low.

1. Introduction

The genus *Enterococcus* includes several species. *Enterococcus faecalis* and *Enterococcus faecium* are major human pathogens [1] causing a variety of infections, including urinary tract infection, bacteraemia, endocarditis and meningitis [2]. The pathogenesis of enterococcal infections is predominately attributed to their intrinsic resistance to certain classes of antibiotics and their remarkable ability to develop resistance to most commonly used antimicrobial agents [3]. There are several virulence factors in *E. faecalis* and *E. faecium* involved in the pathogenesis of enterococcal infections, including aggregation substances, gelatinase, hyaluronidase, and surface proteins such as collagen adhesin, the adhesin-like *E. faecalis* and *E. faecium* antigen A, and enterococcal surface protein [4].

Treatment of severe invasive enterococcal infections typically includes the combination of a cell-wall-active agent (e.g. ampicillin and vancomycin) and an aminoglycoside (gentamicin or streptomycin). Resistance to these antibiotics weakens the synergistic activity of combination therapy [5]. Resistance to high levels of aminoglycoside antibiotics commonly occurs due to the production of aminoglycoside-modifying enzymes. These enzymes are encoded within mobile genetic elements and are widespread among *Enterococcus* spp., conferring high-level aminoglycoside resistance (HLAR) [6].

Bacteria colonising the gastrointestinal tract are critically important in many opportunistic infections affecting immunocompromised individuals [7]. Enterococci are common intestinal microflora in humans and animals and are also present in

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environments contaminated by animal and human faecal material [1]. People colonised with resistant enterococcal strains are not only at risk of being infected but are also a potential source for the dissemination of micro-organisms to the environment and to other people [7]. It is well known that intestinal colonisation with resistant enterococcal strains is common in hospitalised patients [8]. However, the rate of colonisation in the community setting is not well established.

This study was performed to determine the prevalence of intestinal carriage of high-level streptomycin-resistant (HLSR), high-level gentamicin-resistant (HLGR), vancomycin-resistant and ampicillin-resistant (AR) *Enterococcus* spp. in a community setting in Iran as well as the distribution of their virulence determinants, genetic relatedness between isolates and factors associated with antimicrobial resistance characteristics.

2. Materials and methods

2.1. Subjects and sampling

Subjects were randomly selected students (age 12–14 years) recruited from 19 male/female middle schools in Ardabil city, northwestern Iran. Between May and June 2016, 305 faecal samples were collected. The study was based on informed parental consent of each student and was approved by the regional Ethics Committee of Ardabil University of Medical Sciences (Ardabil, Iran). A questionnaire was completed for each student to record variables including age, sex, stature, weight, number of family members, hospital admission in past 12 months, hospitalisation of a family member in past 12 months, antibiotic consumption in past 3 months, alcohol consumption in past 3 months, antacid consumption in past 3 months, having diarrhoea or constipation in past 3 months, autoimmune diseases, smoking, type of nutrition (all/vegetarian/meat-eater), milk consumption (once/twice or thrice per week), kinds of dairy consumed (pasteurised/non-pasteurised), red meat consumption (regularly/rarely), chicken consumption (regularly/rarely) and hand-washing practices (soap/water/none).

2.2. Isolation and identification of bacteria

Approximately 0.5 g of faecal sample was cultured in 5 mL of brain–heart infusion (BHI) broth (BioMaxima S.A., Lublin, Poland) containing 7.5% NaCl (Merck, Darmstadt, Germany) for 24 h at 35 °C. Then, a 50 μL aliquot of bacterial culture was seeded onto m-*Enterococcus* agar (QUELAB, Montreal, Canada) plates and was further incubated at 37 °C for 24 h. Suspected *Enterococcus* spp. colonies were subjected to catalase test as well as hydrolysis of esculin (Merck) and L-pyrrolidonyl arylamidase (PYR) (HiMedia, Mumbai, India). Definitive identification of isolates as *Enterococcus* spp. was done by targeting the 16S rRNA gene based on a PCR assay as described previously [9]. Genomic DNA was extracted from overnight cultures using a DNA extraction kit (DNP™; Sinaclon, Tehran, Iran). Amplification was performed in a DNA Thermal Cycler (Bio-Rad, Hercules, CA, USA) with an initial denaturation step at 95 °C for 5 min, then 25 cycles of denaturing at 94 °C for 1 min, annealing at 56 °C for 1 min with specific primers (Table 1) and polymerisation at 72 °C for 1 min, followed by a single final extension step at 72 °C for 7 min. Species identification of *E. faecium* and *E. faecalis* was further performed by PCR with primers (Table 1) targeted to the *ddl* genes as described elsewhere [10] with slight modifications as described below. The PCR conditions consisted of a pre-denaturation step at 95 °C for 5 min, followed by 30 cycles of 1 min at 95 °C, 45 s at 45 °C for *ddl* of *E. faecalis* and 47 °C for *ddl* of *E. faecium* and 45 s at 72 °C. A final extension step was performed at 72 °C for 5 min. PCR products were analysed by electrophoresis at

Table 1
Deoxyoligonucleotide primers used in this study.

Gene	Oligonucleotide sequence (5'→3')	Product size (bp)	Reference
16S rRNA	GGA TTA GAT ACC CTG GTA GTC C TCG TTG CGG GAC TTA ACC CAA C	320	[9]
$ddl_{E.\ faecalis}$	ATCAAGTACAGTTAGTCT' ACGATTCAAAGCTAACTG	941	[10]
$ddl_{E.\ faecium}$	TAGAGACATTGAATATGCC TCGAATGTGCTACAATC	550	
vanA	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	
vanB	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	635	
aac(6')-Ie–aph(2")-Ia	GAG CAA TAA GGG CAT ACC AAA GTT CCT ATT TCT TCT TCA CTA TCT TCA	829	[12]
aph(2")-Ib	TCA AAT CCC TGC GGT AGT GTA CGC CAA AAT CAA TAA CTC CAA	428	
aph(2")-Ic	GAG GGC TTT AGG AAT TAC GC ACA CAA CCG ACC AAC AGA GG	125	
aph(2")-Id	TAA TCT GCC GAA GCA ATC TCA TAA TCC CTC TTC ATA CCA ATC C	550	
ant(3")-Ia	ACC GTA AGG CTT GAT GAA ACA GCC GAC TAC CTT GGT GAT CTC	624	
aph(3')-IIIa	GGCTAAAATGAGAATATCACCGG CTTTAAAAAATCATACAGCTCGCG	523	[13]
ant(6')-Ia	GCC CTT GGA AGA GTT AGA TAA TT CGG CAC AAT CCT TTA ATA ACA	198	[14]
asa1	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	375	
gelE	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	213	
cylA	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688	
esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	510	
hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	276	

100 V for 1 h in a 1.5% agarose gel (Sinaclon), were stained with Safe DNA Stain (Sinaclon) and the DNA bands were visualised by ultraviolet illumination (Uvitec Ltd., Cambridge, UK). *Enterococcus faecalis* ATCC 29212 and *E. faecium* ATCC 19434 were used as positive controls, and nuclease-free distilled water was used as a negative control. In addition, representative genes were randomly selected and sequenced to confirm their identity.

Isolates were stored in BHI broth with 15% glycerol (Merck) at −80 °C until further analysis.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the disk diffusion method on Mueller–Hinton agar (BioMaxima) according to Clinical and Laboratory Standards Institute (CLSI) guidelines [11].

The tested antibiotics (Padtan Teb, Tehran, Iran) were ciprofloxacin (5 μg), erythromycin (15 μg), nitrofurantoin (300 μg), tetracycline (30 μg), rifampicin (5 μg), chloramphenicol (30 μg), penicillin G (10 μg) and teicoplanin (30 μg). *Enterococcus faecalis* ATCC 29212 was used as a reference strain for antimicrobial susceptibility testing.

HLAR was determined by the agar-screen method. Briefly, 10 μL of a 0.5 McFarland bacterial suspension was spotted onto a BHI agar (SRL Diagnostics, Mumbai, India) surface containing 500 μg/mL gentamicin and 2000 μg/mL streptomycin separately. Plates were incubated at 35 ± 2 °C for 24–48 h and were inspected for growth (if susceptible at 24 h, plates were re-incubated for an additional 24 h). Growth of >1 colony in a spotted area was considered as HLAR.

The minimum inhibitory concentration (MIC) of ampicillin ((**Bio Basic**, Ontario, **Canada**)) was determined by the standard agar dilution method (concentration range, 0.12–512 μg/mL). Resistance to ampicillin was defined as an MIC ≥ 16 μg/mL [11].

BHI agar containing 6 μg/mL vancomycin ((**Bio Basic**, Ontario, **Canada**)) was used for detection of vancomycin-resistant isolates. The MICs of isolates growing on BHI–vancomycin screening agar were determined by the agar dilution method (concentration range, 0.12–512 μg/mL). Resistance to vancomycin was defined as an MIC ≥ 32 μg/mL [11].

All susceptibility tests were performed and interpreted according to the guidelines of the CLSI. *Enterococcus faecalis* ATCC 29212 was used as a negative control strain.

2.4. PCR amplification of high-level aminoglycoside resistance genes

The presence of the high-level gentamicin resistance-encoding genes *aac(6′)-Ie–aph(2″)-Ia*, *aph(2″)-Ib*, *aph(2″)-Ic* and *aph(2″)-Id* and the high-level streptomycin resistance-encoding genes *ant(3″)-Ia* and *ant(6′)-Ia* were investigated by multiplex PCR using specific primers listed in Table 1. Multiplex PCR was performed in 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min, followed by one cycle at 72 °C for 10 min [12]. The *aph(3″)-IIIa* gene amplification was performed as described above with a distinct annealing temperature of 58 °C [13]. PCR products were analysed as described earlier in this text. Representative genes were randomly selected and were sequenced to confirm their identity. Genes encoding vancomycin resistance (*vanA* and *vanB*) were identified according to previous reports [10].

Genes encoding five common enterococcal virulence determinants, including aggregation substance (*asa1*), cytolysin (*cylA*), enterococcal surface protein (*esp*), gelatinase (*gelE*) and hyaluronidase (*hyl*), were detected using specific primers (Table 1) in a multiplex PCR reaction as described previously [14]. Briefly, PCR was performed with an initial denaturation at 94 °C for 4 min, then 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were size-fractionated by agarose gel electrophoresis and were visualised as described above. A representative PCR product for each virulence gene was randomly selected and sequenced to confirm its identity.

2.5. Enterobacterial repetitive intergenic consensus (ERIC)-PCR

ERIC-PCR was performed for genotyping of the isolates as described previously [15]. Reactions were performed in a total volume of 25 μL containing 12.5 μL of PCR Master Mix, 1 μL of template DNA, 2.5 μL of ERIC1-R primer (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and 9 μL of distilled deionised water. Amplifications were performed with a cycling programme consisting of an initial denaturation step at 94 °C for 3 min, then 35 cycles of 94 °C for 30 s, 48 °C for 60 s and 72 °C for 5 min, and a final extension step at 72 °C for 7 min. Amplicons were size-fractionated by agarose gel electrophoresis at 80 V for 2 h through 1.5% agarose gels, were stained with Safe DNA Stain and were visualised and photographed as described earlier. ERIC patterns were analysed using BioNumerics II software 7.0 trial version (Applied Maths, Kortrijk, Belgium), and similarities among ERIC-PCR profiles were determined using the Dice coefficient and unweighted pair-group method with arithmetic mean (UPGMA). Isolates with an 80% level of similarity were grouped in the same cluster and were considered as clonally related.

2.6. Statistical analyses

SPSS software v.11.5 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Association of risk factors with antimicrobial resistance was calculated using the χ^2 test. A *P*-value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Identification of bacterial isolates

A total of 409 enterococcal isolates were collected from 305 faecal samples obtained from healthy children [176 (57.7%) males and 129 (42.3%) females]. Genotypic identification of 409 enterococcal isolates showed that 235 (57.5%) were *E. faecium*, 56 (13.7%) were *E. faecalis* and 118 (28.9%) were other *Enterococcus* spp. Faecal samples from four children did not have any enterococcal colonies on m-*Enterococcus* agar. The 301 remaining children were all colonised by one to two different enterococcal types. In total, 215 (70.5%), 56 (18.4%) and 103 (33.8%) of the 305 subjects were colonised by *E. faecium*, *E. faecalis* and other *Enterococcus* spp., respectively. Among them, 135 (44.3%), 16 (5.2%) and 41 (13.4%) were colonised with only *E. faecium*, *E. faecalis* or other *Enterococcus* spp., respectively; 27 (8.9%), 33 (10.8%) and 13 (4.3%) were colonised by a combination of *E. faecium* + *E. faecalis*, *E. faecium* + other *Enterococcus* spp. and *E. faecalis* + other *Enterococcus* spp., respectively; and 20 (6.6%) and 16 (5.2%) were colonised by a combination of *E. faecium* + *E. faecium* and other *Enterococcus* spp. + other *Enterococcus* spp., respectively.

3.2. Antimicrobial susceptibility testing

The susceptibility patterns of the isolates are presented in Table 2. Overall, teicoplanin (0% resistant) and rifampicin (83.4% resistant) were the most and least active antibiotics, respectively, against the enterococcal isolates. The rates of antibiotic non-susceptibility (intermediate and resistant) for all antibiotics tested (except for chloramphenicol, rifampicin and tetracycline) were higher in *E. faecium* isolates compared with *E. faecalis* isolates.

Table 2
Antimicrobial susceptibility profiles determined by the disk diffusion method of *Enterococcus* spp. isolated from healthy children in Iran.

Antimicrobial agent	Species/susceptibility category											
	E. faecalis (*n* = 56) [*n* (%)]			*E. faecium* (*N* = 235) [*n* (%)]			Other *Enterococcus* spp. (*N* = 118) [*n* (%)]			Total (*N* = 409) [*n* (%)]		
	R	I	S	R	I	S	R	I	S	R	I	S
Ciprofloxacin	1 (1.8)	20 (35.7)	35 (62.5)	48 (20.4)	86 (36.6)	101 (43.0)	16 (13.6)	34 (28.8)	68 (57.6)	65 (15.9)	140 (34.2)	204 (49.9)
Chloramphenicol	6 (10.7)	5 (8.9)	45 (80.4)	13 (5.5)	11 (4.7)	211 (89.8)	8 (6.8)	16 (13.6)	94 (79.7)	27 (6.6)	32 (7.8)	350 (85.6)
Erythromycin	19 (33.9)	29 (51.8)	8 (14.3)	96 (40.9)	122 (51.9)	17 (7.2)	54 (45.8)	57 (48.3)	7 (5.9)	169 (41.3)	208 (50.9)	32 (7.8)
Nitrofurantoin	3 (5.4)	8 (14.3)	45 (80.4)	61 (26.0)	46 (19.6)	128 (54.5)	15 (12.7)	14 (11.9)	89 (75.4)	79 (19.3)	68 (16.6)	262 (64.1)
Penicillin G	15 (26.8)	–	41 (73.2)	112 (47.7)	–	123 (52.3)	42 (35.6)	–	76 (64.4)	169 (41.3)	–	240 (58.7)
Ampicillin[a]	–	–	56 (100)	7 (3.0)	–	228 (97.0)	3 (2.5)	–	115 (97.5)	10 (2.4)	–	399 (97.6)
Rifampicin	47 (83.9)	5 (8.9)	4 (7.1)	203 (86.4)	6 (2.6)	26 (11.1)	91 (77.1)	11 (9.3)	16 (13.6)	341 (83.4)	22 (5.4)	46 (11.2)
Tetracycline	45 (80.4)	2 (3.6)	9 (16.1)	146 (62.1)	14 (6.0)	75 (31.9)	82 (69.5)	6 (5.1)	30 (25.4)	273 (66.7)	22 (5.4)	114 (27.9)
Teicoplanin	–	1 (1.8)	55 (98.2)	–	–	235 (100)	–	–	118 (100)	–	1 (0.2)	408 (99.8)

R, resistant; I, intermediate-resistant; S, susceptible.
[a] Susceptibility profile was determined by the agar dilution method.

Rifampicin and erythromycin showed the lowest activity against *E. faecalis* and *E. faecium* isolates, respectively. Vancomycin, teicoplanin and ampicillin showed the greatest activity against both species.

Using BHI–vancomycin screening agar (6 μg/mL), 23 (5.6%) of the 409 enterococcal isolates, including 14 (60.9%) *E. faecium*, 1 (4.3%) *E. faecalis* and 8 (34.8%) other *Enterococcus* spp. showed growth. However, in MIC testing 2/235 (0.9%) of the *E. faecium* isolates were confirmed as vancomycin-intermediate (MIC = 8 μg/mL). No *vanA* or *vanB* genes were found in isolates with intermediate vancomycin resistance.

The MIC_{50} (MIC required to inhibit 50% of isolates) of ampicillin was 1 μg/mL for all species. Of the 409 isolates, 10 (2.4%) were found to be resistant to ampicillin (MIC ≥ 16 μg/mL), including 7 (70.0%) *E. faecium* and 3 (30.0%) other *Enterococcus* spp. The MICs of ampicillin ranged between 16 μg/mL and 128 μg/mL in AR isolates (Table 3). In total, 10 (3.3%) of the 305 subjects were colonised with AR enterococci, including 7 (2.3%) and 3 (1.0%) with AR *E. faecium* and other AR *Enterococcus* spp., respectively. Ampicillin resistance was positively associated with sex as well as antibiotic and antacid consumption ($P \leq 0.05$) (Table 4). Of the 10 AR isolates, 9 (90.0%) were multidrug-resistant (MDR) (resistant to at least three antibiotic classes) (Table 5).

High-level gentamicin and streptomycin resistance were detected in 11 (2.7%) and 71 (17.4%) of the 409 isolates, respectively. Among the 71 HLSR isolates, 40 (56.3%), 5 (7.0%) and 26 (36.6%) were *E. faecium*, *E. faecalis* and other *Enterococcus* spp., respectively. Among the 11 HLGR isolates, 4 (36.4%) and 7 (63.6%) were *E. faecium* and other *Enterococcus* spp., respectively (Table 6).

The HLGR isolates included 4 (1.7%) of 235 *E. faecium* and 7 (5.9%) of 118 other *Enterococcus* spp. isolates. The HLSR isolates included 5 (8.9%) of 56 *E. faecalis*, 40 (16.9%) of 235 *E. faecium* and 26 (22.0%) of 118 other *Enterococcus* spp. In addition, combined resistance profiles of HLSR + AR and HLSR + HLGR + AR were each observed in 1 (0.4%) of 235 *E. faecium* isolates. Eight (2.0%) isolates showed a combined HLSR + HLGR profile, including 1/235 (0.47%) *E. faecium* and 7/118 (5.9%) other *Enterococcus* spp. Accordingly, overall 21.6%, 3.6% and 3.3% of subjects were found to be colonised with HLSR, HLGR and AR enterococci. HLSR, HLGR and AR *E. faecium* and other *Enterococcus* spp. carriage was detected in 13.8%, 1.3% and 2.3% and in 8.2%, 2.3% and 1.0% of subjects, respectively. Moreover, 1.3% of subjects were colonised with HLGR *E. faecalis* isolates and 0.32% of subjects were colonised with *E. faecium* isolates showing a combined resistance profile of HLSR + AR and HLSR + HLGR + AR. Eight subjects were colonised with organisms showing a combined profile of HLGR + HLSR, including 0.32% and 6.0% with *E. faecium* and other *Enterococcus* spp., respectively.

The aminoglycoside-modifying enzyme-encoding genes *aac(6')-Ie–aph(2")-Ia* and *aph(3')-IIIa* were found in 8 (72.7%) and 6 (54.5%) of the 11 HLGR isolates. The *ant(6')-Ia* gene encoding streptomycin resistance was detected in 35 (49.3%) of the 71 HLSR isolates.

Antibiotic consumption was found to be a risk factor for carriage both of HLGR and HLSR *Enterococcus* spp. ($P \leq 0.05$). Number of students in class was associated with carriage of HLSR *Enterococcus* spp. ($P \leq 0.05$) (Table 4). In this study, 69 (97.2%) and 10 (90.9%) of the HLSR and HLGR isolates were MDR (resistant to at least three antibiotic classes), respectively (Table 5).

3.3. Detection of virulence genes

Among the 56 *E. faecalis* isolates, 34 (60.7%) were positive for *gelE*, 30 (53.6%) for *asa1*, 18 (32.1%) for *esp*, 7 (12.5%) for *cylA* and 3 (5.4%) for *hyl*. Among the 235 *E. faecium* isolates, 107 (45.5%) were positive for *gelE*, 73 (31.1%) for *asa1*, 32 (13.6%) for *esp*, 13 (5.5%) for *hyl* and 7 (3.0%) for *cylA* (Table 7). Virulence gene profile analyses showed that of the 56 *E. faecalis* isolates, 50 (89.3%) contained at least one virulence factor gene. Collectively, 1 (1.8%) isolate contained four genes and 10 (17.9%), 19 (33.9%) and 20 (35.7%) isolates harboured three, two and one genes, respectively. Of the 235 *E. faecium* isolates, 139 (59.1%) possessed at least one virulence determinant gene. Collectively, 1 (0.4%) isolate contained five genes and 2 (0.9%), 16 (6.8%), 51 (21.7%) and 69 (29.4%) isolates contained four, three, two and one genes, respectively (Table 7). Statistical analyses showed a positive correlation between HLAR phenotype and the presence of the gelatinase encoding gene *gelE* ($P \leq 0.05$) (Table 8).

Table 3
Distribution of ampicillin minimum inhibitory concentrations (MICs) determined by the agar dilution method of enterococcal isolates collected from healthy children in Iran.

Species	No. (%) at MIC (μg/mL) of:											MIC (μg/mL)	
	<0.12	0.25	0.5	1	2	4	8	16	32	64	128	MIC_{50}	MIC_{90}
Total *Enterococcus* spp. (*N* = 409)	6 (1.5)	4 (1)	67 (16.4)	247 (60.4)	56 (13.7)	16 (3.9)	3 (0.7)	7 (1.7)	1 (0.2)	–	2 (0.5)	1	2
E. faecium (*N* = 235)	6 (2.6)	1 (0.4)	27 (11.5)	146 (62.1)	34 (14.5)	12 (5.1)	2 (0.9)	5 (2.1)	1 (0.4)	–	1 (0.4)	1	2
E. faecalis (*N* = 56)	-	1 (1.8)	15 (26.8)	35 (62.5)	4 (7.1)	1 (1.8)	–	–	–	–	–	1	1
Other *Enterococcus* spp. (*N* = 118)	-	2 (1.7)	25 (21.2)	66 (55.9)	18 (15.3)	3 (2.5)	1 (0.8)	2 (1.7)	–	–	1 (0.8)	1	2

$MIC_{50/90}$, MIC required to inhibit 50% and 90% of the isolates, respectively.

Table 4
Factors associated with HLSR, HLGR and AR enterococcal carriage in healthy children in Iran.

Risk factor	Subjects [*n* (%)]								
	HLSR (*N* = 66)	Non-HLSR controls (*N* = 239)	*P*-value	HLGR (*N* = 11)	Non-HLGR controls (*N* = 294)	*P*-value	AR (*N* = 10)	Non-AR controls (*N* = 295)	*P*-value
Sex M/F [*n* (%)]	43 (62.5)/23 (34.8)	133 (55.6)/106 (44.4)	0.16	8 (72.7)/3 (27.3)	168 (57.1)/126 (42.9)	0.30	10 (100)/0	166 (56.3)/129 (43.7)	0.006*
Mean stature (cm)	155.2 ± 13.38	157.8 ± 8.55	0.09	163.27 ± 14.82	157.0 ± 9.5	0.04*	161.4 ± 7.64	157.1 ± 9.9	0.18
Mean weight (kg)	50.27 ± 11.42	50.67 ± 11.3	0.80	59.55 ± 9.43	50.24 ± 11.25	0.07	53.70 ± 15.7	50.47 ± 11.5	0.37
Mean no. of family members	4.45 ± 0.96	4.31 ± 1.5	0.46	4.73 ± 1.19	4.33 ± 1.4	0.36	4.20 ± 1.03	4.3 ± 1.4	0.75
Mean no. of students in class	32.15 ± 3.75	30.31 ± 3.9	0.001*	31.36 ± 4.17	30.68 ± 3.94	0.57	31.70 ± 5.25	30.67 ± 3.9	0.41
Hospital admission[a]	5 (7.6)	12 (5.0)	0.42	2 (18.2)	15 (5.1)	0.06	0	17 (5.8)	0.43
Hospitalisation of a family member[a]	12 (18.2)	42 (17.6)	0.90	2 (18.2)	52 (17.7)	0.96	3 (30.0)	51 (17.3)	0.30
Antibiotic consumption[b]	20 (30.3)	46 (19.2)	0.05*	5 (45.5)	61 (20.7)	0.05*	7 (70.0)	59 (20.0)	0.000*
Antacid consumption[b]	5 (7.6)	11 (4.6)	0.5	1 (9.1)	15 (5.1)	0.55	2 (20.0)	14 (4.7)	0.02*
Having diarrhoea or constipation[b]	11 (16.7)	28 (11.7)	0.26	1 (9.1)	38 (12.9)	0.70	2 (20.0)	37 (12.5)	0.48
Autoimmune disease	2 (3.0)	5 (2.1)	0.79	0	7 (2.4)	0.60	0	295 (100)	0.60
Smoking	0	239 (100)	0	0	294 (100)	0.07	0	295 (100)	0
Alcohol consumption[b]	1 (1.5)	6 (2.5)	0.63	0	7 (2.4)	0.36	0	295 (100)	0.6
Type of nutrition (all/vegetarian/meat-eater)	61 (92.4)/1 (1.5)/4 (6.1)	225 (94.1)/4 (1.7)/10 (4.2)	0.81	10 (90.9)/0/1 (9.1)	276 (93.9)/5 (1.7)/13 (4.4)	0.7	10 (100)/0/0	276 (93.6)/5 (1.7)/14 (4.7)	0.7
Milk consumption (once/twice or thrice per week)	38 (57.6)/28 (42.4)	133 (55.6)/106 (44.4)	0.78	8 (72.7)/3 (27.3)	163 (55.4)/131 (44.6)	0.25	6 (60.0)/4 (40.0)	165 (55.9)/130 (44.1)	0.79
Kinds of dairy consumed (pasteurised/non-pasteurised)	37 (56.1)/29 (43.9)	160 (66.9)/79 (33.1)	0.10	8 (72.7)/3 (27.3)	189 (64.3)/105 (35.7)	0.50	7 (70.0)/3 (30.0)	190 (64.4)/105 (35.6)	0.71
Red meat consumption (regularly/rarely)	36 (54.5)/30 (45.5)	112 (46.9)/127 (53.1)	0.26	6 (54.5)/5 (45.5)	142 (48.3)/152 (51.7)	0.68	7 (70.0)/3 (30.0)	141 (47.8)/154 (52.2)	0.27
Chicken consumption (regularly/rarely)	40 (60.6)/26 (39.4)	161 (67.4)/78 (32.6)	0.30	7 (63.6)/4 (36.4)	194 (66.0)/100 (34.0)	0.87	7 (70.0)/3 (30.0)	194 (65.8)/101 (34.2)	0.78
Washing hands (soap/water/none)	58 (87.9)/7 (10.6)/1 (1.5)	195 (81.6)/41 (17.2)/3 (1.3)	0.43		243 (82.7)/47 (16.0)/4 (1.4)	0.75	10 (100)/0/0	243 (82.4)/48 (16.3)/4 (1.4)	0.34

HLSR, high-level streptomycin-resistant; HLGR, high-level gentamicin-resistant; AR, ampicillin-resistant.
[a] In past 12 months.
[b] In past 3 months.
* Statistically significant ($P \leq 0.05$).

Table 5
Antimicrobial non-susceptibility (intermediate-resistant + resistant) profile of HLSR, HLGR and AR *Enterococcus* spp. isolated from healthy children in Iran

Enterococcus spp.	Phenotypic resistance profile	No. (%) of isolates	No. of antibiotic classes	Total [*n* (%)][a]
HLSR (*N* = 71)	PEN, CIP, ERY, TET, NIT, CHL, RIF	1 (1.4)	7	1 (1.4)
	PEN, CIP, ERY, TET, NIT, RIF	10 (14.1)	6	15 (21.1)
	PEN, CIP, ERY, TET, CHL, RIF	5 (7.0)	6	
	CIP, ERY, TET, NIT, RIF	6 (8.5)	5	25 (35.2)
	PEN, CIP, ERY, NIT, RIF	1 (1.4)	5	
	PEN, CIP, ERY, TET, NIT	1 (1.4)	5	
	CIP, ERY, TET, NIT, CHL	1 (1.4)	5	
	CIP, ERY, TET, CHL, RIF	1 (1.4)	5	
	PEN, ERY, TET, NIT, RIF	3 (4.2)	5	
	PEN, CIP, ERY, TET, RIF	6 (8.5)	5	
	PEN, CIP, ERY, TET, RIF	6 (8.5)	5	
	CIP, ERY, NIT, RIF	2 (2.8)	4	13 (18.3)
	CIP, ERY, TET, RIF	6 (8.5)	4	
	ERY, TET, NIT, RIF	1 (1.4)	4	
	PEN, CIP, ERY, RIF	1 (1.4)	4	
	PEN, ERY, NIT, RIF	1 (1.4)	4	
	ERY, TET, CHL, RIF	2 (2.8)	4	
	CIP, TET, NIT	1 (1.4)	3	15 (21.1)
	TET, NIT, RIF	1 (1.4)	3	
	ERY, NIT, RIF	1 (1.4)	3	
	ERY, TET, RIF	8 (11.3)	3	
	CIP, ERY, RIF	2 (2.8)	3	
	PEN, ERY, RIF	1 (1.4)	3	
	PEN, ERY, TET	1 (1.4)	3	
	ERY, RIF	1 (1.4)	2	1 (1.4)
	PEN	1 (1.4)	1	1 (1.4)
AR (*N* = 10)	PEN, CIP, ERY, TET, NIT, CHL, RIF	2 (20.0)	7	2 (20.0)
	PEN, CIP, ERY, TET, NIT, RIF	4 (40.0)	6	4 (40.0)
	PEN, CIP, ERY, TET, RIF	1 (10.0)	5	1 (10.0)
	CIP, ERY, NIT	1 (10.0)	3	2 (20.0)
	PEN, ERY, TET	1 (10.0)	3	
	CIP	1 (10.0)	1	1 (10.0)
HLGR (*N* = 11)	PEN, CIP, ERY, TET, NIT, RIF	2 (18.2)	6	3 (27.3)
	PEN, CIP, ERY, TET, CHL, RIF	1 (9.1)	6	
	CIP, ERY, TET, NIT, RIF	2 (18.2)	5	2 (18.2
	CIP, ERY, TET, RIF	1 (9.1)	4	2 (18.2)
	PEN, CIP, ERY, RIF	1 (9.1)	4	
	ERY, TET, RIF	3 (27.3)	3	3 (27.3)
	ERY	1 (9.1)	1	1 (9.1)

HLSR, high-level streptomycin-resistant; HLGR, high-level gentamicin-resistant; AR, ampicillin-resistant; PEN, penicillin G; CIP, ciprofloxacin; ERY, erythromycin; TET, tetracycline; NIT, nitrofurantoin; CHL, chloramphenicol; RIF, rifampicin.
[a] Total number of isolates resistant to same number of antibiotic classes.

Table 6
Distribution of HLSR, HLGR, AR and VIR phenotypes among *Enterococcus* spp. isolated from healthy children in Iran.

Resistance phenotype	No. (%) of isolates			
	E. faecium	*E. faecalis*	Other *Enterococcus* spp.	Total (*N* = 409)
HLSR	42 (59.2)	4 (5.6)	25 (35.2)	71 (17.4)
HLGR	4 (36.4)	–	7 (63.6)	11 (2.7)
AR	7 (70.0)	–	3 (30.0)	10 (2.4)
VIR	2 (100)	–	–	2 (0.5)
HLSR + AR	1 (100)	–	–	1 (0.2)
HLSR + HLGR + AR	1 (100)	–	–	1 (0.2)
HLSR + HLGR	1 (12.5)	–	7 (87.5)	8 (2.0)

HLSR, high-level streptomycin-resistant; HLGR, high-level gentamicin-resistant; AR, ampicillin-resistant; VIR, vancomycin-intermediate-resistant.

3.4. ERIC-PCR analysis

The ERIC-1R primer in *E. faecium* generated 4–13 amplicons with molecular weights ranging from 100 to 16 000 bp. According to the dendrogram with 80% similarity, 25 different genotypes (subgroups) were observed (Fig. 1). Of the 47 isolates tested, 12 isolates provided unique genotypes, whereas genotype subgroup 5 contained the highest number of isolates (*n* = 5).

The ERIC-1R primer in *E. faecalis* generated 4–11 amplicons with molecular weights ranging from 120 to 18 000 bp. According to the dendrogram with 80% similarity, 10 different genotypes (subgroups) were observed (Fig. 2). Of the 19 isolates tested, 5 isolates provided unique genotypes (2, 3, 6, 8 and 10), whereas genotype 4 contained the highest number of isolates (*n* = 5). The HLSR *E. faecalis* isolates were distributed in subgroups 1, 2 and 3.

4. Discussion

It has previously been documented that *Enterococcus* spp. colonise the gastrointestinal tract of the vast majority of healthy individuals [16]. Similarly, in the current study *Enterococcus* spp.

Table 7
Virulence gene profile of *Enterococcus* spp. isolated from healthy children in Iran.

Species	Virulence genes	Isolates [*n* (%)]	No. of virulence genes	Total [*n* (%)][a]
E. faecium (*N* = 235)	*cylA*, *esp*, *asa1*, *hyl*, *gelE*	1 (0.4)	5	1 (0.4)
	cylA, *esp*, *asa1*, *gelE*	2 (0.8)	4	2 (0.8)
	esp, *asa1*, *gelE*	9 (3.8)	3	16 (6.8)
	asa1, *hyl*, *gelE*	4 (1.7)	3	
	cylA, *esp*, *asa1*	2 (0.9)	3	
	cylA, *asa1*, *gelE*	1 (0.4)	3	
	esp, *gelE*	11 (4.7)	2	51 (21.7)
	asa1, *gelE*	33 (14.0)	2	
	asa1, *hyl*	2 (0.9)	2	
	hyl, *gelE*	1 (0.4)	2	
	esp, *asa1*	1 (0.4)	2	
	esp, *hyl*	3 (1.3)	2	
	gelE	45 (19.1)	1	69 (29.4)
	asa1	18 (7.7)	1	
	esp	3 (1.3)	1	
	cylA	1 (0.4)	1	
	hyl	2 (0.9)	1	
	–	96 (41)	0	96 (40.9)
E. faecalis (*N* = 56)	*cylA*, *esp*, *asa1*, *gelE*	1 (1.8)	4	1 (1.8)
	cylA, *esp*, *gelE*	1 (1.8)	3	10 (17.8)
	esp, *asa1*, *gelE*	8 (14.3)	3	
	cylA, *esp*, *asa1*	1 (1.8)	3	
	asa1, *gelE*	9 (16.1)	2	19 (34)
	esp, *gelE*	5 (8.9)	2	
	cylA, *asa1*	4 (7.1)	2	
	esp, *hyl*	1 (1.8)	2	
	gelE	10 (17.9)	1	20 (35.7)
	esp	1 (1.8)	1	
	hyl	2 (3.6)	1	
	asa1	7 (12.5)	1	
	–	6 (10.7)	0	6 (10.7)

[a] Total number of isolates harbouring the same number of virulence genes.

Table 8
Association of virulence genes with HLSR, HLGR and AR phenotypes in *Enterococcus* spp. isolated from healthy children in Iran.

Virulence gene	E. faecium [n (%)]									E. faecalis [n (%)]		
	HLSR (*N* = 40)	Non-HLSR controls (*N* = 195)	*P*-value	HLGR (*N* = 4)	Non-HLGR controls (*N* = 231)	*P*-value	AR (*N* = 7)	Non-AR controls (*N* = 228)	*P*-value	HLSR (*N* = 5)	Non-HLSR controls (*N* = 51)	*P*-value
cylA	2 (5.0)	5 (2.6)	0.4	0 (0)	7 (3.0)	0.7	1 (14.3)	6 (2.6)	0.07	0 (0)	7 (13.7)	0.4
esp	6 (15.0)	26 (13.3)	0.8	1 (25.0)	31 (13.4)	0.5	0 (0)	32 (14.0)	0.3	1 (20.0)	17 (33.3)	0.5
asa1	15 (37.5)	57 (29.2)	0.3	2 (50.0)	70 (30.3)	0.4	2 (28.6)	70 (30.7)	0.9	1 (20.0)	28 (54.9)	0.1
hyl	3 (7.5)	11 (5.6)	0.6	1 (25.0)	13 (5.6)	0.1	1 (14.3)	13 (5.7)	0.3	1 (20.0)	3 (5.9)	0.2
gelE	24 (60.0)	83 (42.6)	0.04*	4 (100)	103 (44.6)	0.0*	4 (57.1)	103 (45.2)	0.5	2 (40.0)	31 (60.8)	0.4

HLSR, high-level streptomycin-resistant; HLGR, high-Level gentamicin-resistant; AR, ampicillin-resistant.
* Statistically significant ($P \leq 0.05$).

were isolated in nearly all (98.7%) of the faecal samples collected from healthy children. In this study, *E. faecium* (57.5% of isolates) was the most prevalent coloniser of the gastrointestinal tract, followed by other *Enterococcus* spp. (28.9%) and *E. faecalis* (13.7%). These results are in agreement with the findings of Barreto et al. [16] and Poeta et al. [17] which showed that *E. faecium* accounted for >50% of enterococcal isolates recovered from healthy volunteers. Regarding *E. faecalis* and other *Enterococcus* spp., the current results are in contrast to those of the abovementioned reports which showed that *E. faecalis* and other *Enterococcus* spp. accounted for up to 40% and 10% of isolates [16,17]. In contrast to stool samples from healthy people, *E. faecalis* is the most prevalent species isolated from clinical specimens [18].

Despite the fact that there are plenty of studies reporting the frequency of resistant enterococci in clinical specimens and faecal samples from hospitalised patients, scarce data are available on the distribution of resistant *Enterococcus* species in healthy human faeces. Intestinal carriage of resistant enterococci is a significant factor for the development of infection by the same organism and is a potential source of dissemination of the organism in the community [7,19].

High-level gentamicin and streptomycin resistance were detected in 11 (2.7%) and 71 (17.4%) of the 409 enterococcal isolates, respectively. This finding is in contrast to a report by Kuzucu et al. on faecal isolates of enterococci collected from outpatients in Turkey in which 10.0% and 3.0% of isolates were HLGR and HLSR, respectively [20]. In the current study, HLGR and HLSR isolates mainly belonged to *E. faecium*, being found in 4/11 (36.4%) and 40/71 (56.3%) of resistant isolates, respectively. These results are in contrast to a report by Asadian et al. in which no HLAR *E. faecium* was found in faecal specimen from healthy volunteers [21]. However, in another study much higher percentages of HLGR and HLSR enterococci were reported in clinical isolates, with rates of 26.9% and 73.1% in *E. faecalis* and 77.3% and 90.1% in *E. faecium* species, respectively [22]. Since resistance of enterococci to gentamicin and streptomycin occurs by different mechanisms, streptomycin could be used as a surrogate for gentamicin in the treatment of invasive enterococcal infections. Co-existence of HLGR and HLSR limits the therapeutic options of enterococcal infections. This phenomenon was rare in the current study. Co-existence of HLGR + HLSR, HLSR + AR and HLGR + HLSR + AR resistance profiles was observed in one *E. faecium* isolate each.

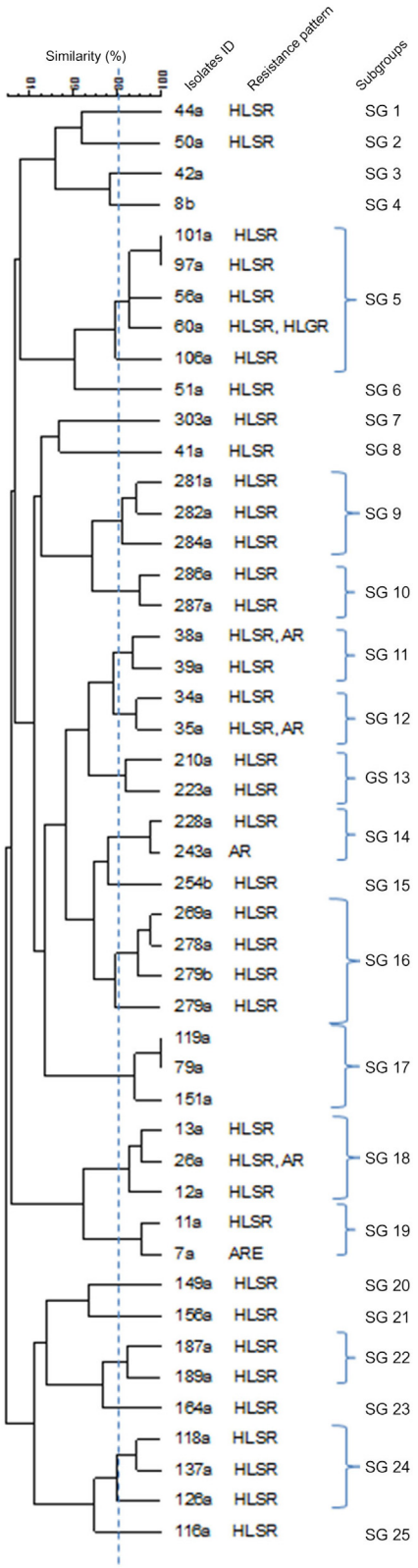

Fig. 1. Dendrogram of ERIC-PCR patterns showing the genetic relationship among 47 *Enterococcus faecium* isolates collected from healthy children in Ardabil, Iran. Similarities >80% were considered for clustering of isolates. ERIC-PCR, enterobacterial repetitive intergenic consensus PCR; SG, subgroup.

Globally, ampicillin resistance is significantly high in clinical enterococcal isolates [23]. Low rates of ampicillin resistance were observed in isolates obtained from healthy humans [21]. Accordingly, in the current study a small numbers of isolates (10/409; 2.4%) were found to be resistant to ampicillin. However, the current results are higher than those from another study in which no AR *Enterococcus* spp. were isolated from healthy people [17], but lower than those from a study by Freitas et al. in which 50% of residents in a long-term care facility in Portugal were colonised with AR *Enterococcus* spp. [24]. The major reservoir of ampicillin resistance was *E. faecium* (70.0%), followed by other *Enterococcus* spp. (30.0%). No ampicillin resistance was observed in *E. faecalis* isolates. This is in accordance with the fact that *E. faecium* is more resistant to ampicillin and penicillin compared with *E. faecalis* [25]. Nowadays, >90.0% of *E. faecium* isolates recovered from healthcare-associated infections in the USA are resistant to ampicillin [23]. In another study conducted in Ardabil, 19.0% and 28.0% of *E. faecalis* and *E. faecium* isolates, respectively, obtained from clinical specimens in 2017 were resistant to ampicillin (authors' unpublished data).

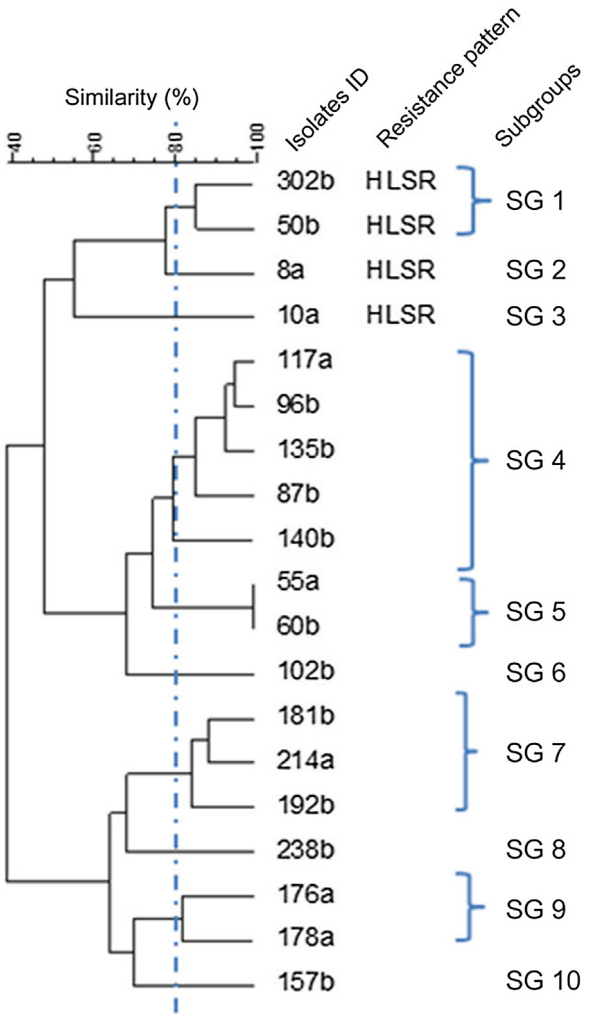

Fig. 2. Dendrogram of ERIC-PCR patterns showing the genetic relationship among 19 *Enterococcus faecalis* isolates collected from healthy children in Ardabil, Iran. Similarities >80% were considered for clustering of isolates. ERIC-PCR, enterobacterial repetitive intergenic consensus PCR; SG, subgroup.

No vancomycin-resistant enterococci (VRE) were found in the faeces of healthy subjects in this study. However, two *E. faecium* isolates were intermediate-resistant to vancomycin. In contrast to these results, intestinal colonisation with VRE in the healthy population is frequently reported around the world. Rates of faecal carriage of VRE in healthy people were recorded as 21.0%, 24.9% and 28.0% in Morocco, Taiwan and Belgium, respectively [26–28]. However, the result of the current study is in agreement with

reports published by others in Iran, which found no VRE in faecal samples from healthy humans [21].

Regarding other routinely used antibiotics, erythromycin, rifampicin and tetracycline were the most non-susceptible antibiotics both against *E. faecalis* and *E. faecium* isolates. Similar results were reported for *Enterococcus* spp. isolated from faecal specimens in Greek healthy infants [29]. Rifampicin resistance was the highest, followed by resistance to tetracycline and erythromycin [29]. Collectively, in this study majority of the HLAR and AR enterococcal isolates obtained from healthy individuals were resistant to multiple classes of antibiotics. Infections by MDR organisms are serious global health problem causing significant mortality [30,31].

Colonisation of healthy people with clinically important MDR *Enterococcus* spp. could act as reservoir for the maintenance and spread of resistant strains in the environment and hospital settings. If factors promoting the acquisition of resistant organisms are identified and controlled, it may be possible to control the incidence of colonisation and thereby clinical infection. Previous reports have indicated an association of colonisation or infection with HLAR and AR enterococci with hospital stay and prior antibiotic usage, especially use of broad-spectrum cephalosporins, ampicillin and aminoglycosides [32–36]. Similarly, the current results showed a positive association between HLAR and AR enterococci intestinal carriage and prior antibiotic treatment. However, in contrast to other studies [33,35], prior hospital stay was not found to be a risk factor for HLAR or AR enterococcal colonisation. Intestinal colonisation with HLSR enterococci was positively associated the mean number of students in the classroom. However, the heterogeneity of ERIC-PCR results among HLAR isolates suggests no clonal dissemination for the spread of these resistant enterococci. Similar to the current findings, a study in Belgium by Schoevaerdts et al. reported antacid use as a risk factor for methicillin-resistant *Staphylococcus aureus* (MRSA) carriage [37].

In this study, all of the *E. faecalis* and *E. faecium* isolates were examined for the presence of *cylA*, *esp*, *asa1*, *hyl* and *gelE* genes encoding cytolysin activator, enterococcal surface protein, aggregation substance, hyaluronidase and gelatinase, respectively. *gelE* and *asa1* were the most prevalent genes detected both in *E. faecalis* and *E. faecium*. These results are in accordance with reports by Shokoohizadeh et al. and Shahraki and Mousavi in which the *gelE* and *asa1* genes were the most prevalent virulence genes in *E. faecalis* and *E. faecium* isolates collected from clinical specimens [38,39]. However, some reports have indicated the absence or low incidence of the *gelE* gene both in *E. faecalis* and *E. faecium* isolates [40,41] and of the *asa1* gene in *E. faecium* isolates [39,42]. In this study, 50/56 (89.3%) and 139/235 (59.1%) *E. faecalis* and *E. faecium* isolates possessed at least one virulence gene and 11 (19.6%) and 19 (8%) isolates contained at least three genes, respectively. This is in contrast to previous studies reporting *E. faecium* isolates devoid of multiple virulence factors [42–44]. The emergence of *E. faecium* with multiple virulence factors along with its MDR characteristic could lead to poor outcomes for enterococcal infection management. Some previous reports showed a significant association between the presence of virulence determinants and antimicrobial resistance in *Enterococcus* spp. [45]. Accordingly, we also found a significant correlation between the presence of *gelE* and HLAR resistance in the isolates in the current study.

In summary, the results of this study show that a significant proportion of *Enterococcus* spp. colonising a heathy population in Iran was resistant to several classes of antibiotics. Moreover, virulence-encoding genes were present in clinically important species. Thus, healthy humans could act as a reservoir for antimicrobial resistance and virulence genes, enabling the distribution of these genes to the environment and community.

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Competing interests

None declared.

Ethical approval

This study was approved by the regional Ethics Committee of Ardabil University of Medical Sciences [IR.ARUMS.REC.1398.487].

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