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Virulence factors, biofilm formation and antibiotic resistance pattern in *Enterococcus faecalis* and *Enterococcus faecium* isolated from clinical and commensal human samples in Isfahan, Iran

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Key words: Antibiotic resistance, biofilm formation, Enterococcus faecalis, Enterococcus faecium, virulence gene

Parole chiave: Antibioticoresistenza, formazione di biofilm, Enterococcus faecalis, Enterococcus faecium, geni di virulenza

Abstract

Background. *The aim of this study was to determine and compare antibiotic resistance profile, biofilm formation ability and frequency of agg and ace genes in Enterococcus spp strains isolated from patients and healthy individuals.*

Methods. *A total of 90 non-duplicate Enterococcus spp isolates were isolated from patients and healthy individuals. Antibiotic susceptibility pattern was determined by disk diffusion and E-test method. Virulence genes and two species of enterococci were determined by PCR amplification. The capacity of biofilm formation was also evaluated by microtiter plate technique.*

Results. *E. faecalis was the predominant species among our clinical isolates (80%). The prevalence of agg and ace genes was 37.8% and 73.3% in clinical and 8.9% and 11.1% in "healthy" samples, respectively. The rate of Multiple Drug Resistant strains was 73.3% and 11.1% in clinical and "healthy" isolates, respectively. The ability of biofilm formation was significantly higher in clinical compared to "healthy" isolates (100% vs 75.6%, $P < 0.05$).*

Conclusions. *The frequency of ace and agg genes, antibiotic resistance and biofilm formation ability were significantly higher in clinical than in "healthy" isolates ($P < 0.05$). Existence of agg and ace genes, biofilm formation and antibiotic resistance among the healthy enterococci isolates has a special importance since, in case these strains spread through clinical environments or reach water sources, this issue can be considered as a risk factor for health and sanitation of society.*

Introduction

Enterococci are commonly found in the intestinal tracts of humans and animals and can be responsible for some opportunistic infections such as 10% of bacteremiasa and 15% to 30% of catheter-associated urinary tract infections (CAUTIs) worldwide (1, 2).

In general, *Enterococcus* spp, as coliforms and *E. coli,* as faecal indicator bacteria (FIB) in water, are significant and must be measured; *E. faecalis* and *E. faecium* are the

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most frequent species in human feces and urban sewage, and are clinically associated with multidrug-resistant nosocomial infection important (3, 4). The ability to grow in high salt concentration, the stability over wide ranges of temperature (from 10° C to >45° C), acquiring drug resistance and biofilm formation are some of the characteristics of these opportunistic bacteria (5). Unfortunately, antibiotic resistance has led to a dramatic increase of *Enterococcus* severe infections and it has been declared that they are the second and third causative agent of healthcare-associated bacteremias and nosocomial urinary tracts infections, respectively (6, 7). The ability of biofilm development in genus *Enterococcus* is a main reason for the abundance of these pathogens in hospitals and health facilities. Indeed, biofilm development contributes to 80% chronic infection in human and antibiotic resistance as well as an enhancement in tolerance level under stress and other inappropriate conditions. As an example, a fully developed biofilm can tolerate 1000-folds of antibiotics concentrations compared to planktonic cells. Molecular mechanisms and also nutritional compositions are the main factors in the production and development of bacterial biofilms (8-12). Moreover, these bacteria can produce several virulence factors, such as aggregation substance (encoding by *agg* gene) and collagen binding protein (encoding by *ace* gene) that are associated with pathogenicity of *Enterococcus* strains. *ace* is a microbial surface component recognizing adhesive matrix molecule adhesion of collagen (MSCRAMM), that is a key factor of enterococcal endocarditis. *agg* is an inductive pheromone-protein which facilitates the transmission of plasmids between host and cell receptors. Furthermore, *agg*-coding isolates are capable of conjugating and hence acquiring antibiotic resistance. Besides, the presence of these two genes in *E. faecalis* is greater than that of *E. faecium* (13-16). Therefore, microbial adhesion and biofilm formation can lead to microbial persistence in hospital environments.

There is very limited and unclear information about the prevalence of biofilm-formation, antibiotic-resistance and frequency of virulence genes in strains from healthy people in comparison with clinical *Enterococcus* isolates. Considering the major impact of biofilms on antibiotic resistance development in *Enterococcus* species, as well as the key role of *agg* and *ace* genes in formation of biofilm, the current study was designed to compare and analyze the biofilm formation capacity, antibiotic resistance patterns, and the prevalence of *agg* and *ace* genes in *E. faecalis* and *E. faecium* isolated from both clinical and healthy groups. Moreover, in the present article, the correlation of MDR and biofilm, and MDR and virulence genes and also the correlation of biofilm and virulence genes were studied statistically.

Material and methods

Bacterial isolates. This study was performed on 90 *Enterococcus* (45 clinical samples from patients in Isfahan Al-Zahra hospital as well as 45 fecal isolates from healthy individuals) during March to July 2017. In order to isolate enterococci from healthy individuals, the stool samples were poured into a 6.5% NaCl solutiom as a selective medium, transmitted to the laboratory and incubated 24 h at 42°C. After incubation, a portion of growth medium was streaked on m-enterococcus agar (Oxoid, England), and then the colonies were streaked into blood agar and incubated at 37°C for 24 h.

Identification. The identification of bacteria was performed by standard biochemical analysis such as catalase, growth in 6.5% NaCl, hydrolysis of bile esculin, pyrrolidonyl aminopeptidases (17).

Antibiotic susceptibility testing. The antibiotic susceptibility pattern of isolates was determined by Disk diffusion method (DDM) as described by CLSI 2017 guideline (18) for the following antibiotics: nitrofurantoin (300 µg), ampicillin (10 µg), vancomycin (30 µg), ciprofloxacin (5µg), tetracycline (30 µg), gentamicin (120 µg), linezolid (30 µg), erythromycin (15 µg), chloramphenicol (30 µg), penicillin (10 unit), rifampin (5 µg); subsequently, the Whonet 5.6 software was used to determine the multiple drug resistance (MDR) of the strains. The bacteria which had shown intermediate and resistant patterns for vancomycin disk in DDM were rechecked by Epsilometer-test (BioMérieux, France). According to the CLSI 2017, the isolates were classified according to their MIC (minimum inhibitory concentration): susceptible (MIC ≤ 4µg/ml), intermediate (MIC 8-16 µg/ml), and resistant (MIC ≥ 32 µg/ml).

Biofilm formation assay. Firstly, 200 µl of fresh Trypticase Soy Broth (TSB, Merck, Germany) was added to all polystyrene microtiter plate (Becton Dickinson and co.) wells and then 20 µl TSB containing a 24-hour bacterial culture were added and incubated at 37 °C for 24 h. The plates were washed by phosphate buffered saline (PBS, Sigma®, USA) three times and fixed by 95% methanol for 20 min, then 200 µl crystal violet (1%) was added to each well, and the plate was washed with sterilized distilled water three times to remove the extra crystal violet and 200 µl of alcohol-acetone (80/20%) was added to each well, finally, OD at 570 nm was read by ELISA Reader (Bio-Rad, model 658, USA) (12, 13). The interpretation of biofilm formation test was as follows: (OD < 0.120 negative, 0.120 < OD < 0.240 weak, OD > 0.240 strong. All assays were performed in triplicate. TSB without bacteria and *Staphylococcus epidermidis* ATCC 35984 were used as negative and positive controls, respectively.

DNA extraction. Bacterial DNA was extracted using the Cinnapure TM DNA extraction kit (*Irannano*, Iran) according to the manufacturer's protocol. The concentration (ng/µl) and OD at the wavelength of 260/280 nm of all extracted DNA was quantified using a nanodrop spectrophotometer (WPA, chambering, UK).

PCR detection. PCR amplification was performed in order to confirm *E. faecalis* and *E. faecium* using (*ddl)* genes and to detect *agg* and *ace* genes (19, 20). PCR reactions were carried out using 1.5 µl of the template DNA (1µg/ml), 1 µl of each primer (100 pmol), 15.5 µl of nuclease-free water, and 6 µl of PCR master mix in a total volume of 25 µl. The amplification program for *E. faecalis* and two genes were set as follows: initial denaturation at 94°C/5 minutes, 30 cycles of 94°C/1 minute, 48°C/1 minute, 72°C/1 minute and a final extension step of 72°C/5 minutes. PCR test to confirm *E. faecium* was carried out under the following condition: initial denaturation at 94°C/3 minutes, 35 cycles of 94°C/30 seconds, 54°C/30 seconds, 72°C/1 minute and a final extension step of 72°C/5 minutes (BIO-RAD T100™ Thermal Cycler, USA). The final products were detected by electrophoresis on 1% agarose gel containing DNA green viewer (Parstous, Iran) and the sizes of the PCR products were estimated by the migration pattern of a 100-bp DNA ladder (Sinagene, Iran).

Statistical analysis. Statistical analysis was performed using (SPSS) software (version 18). The results with $P < 0.05$ were considered significant. In order to analyze the data, Chi-square, Mann-Whitney and Fisher's exact test were used.

Results

Study Population. In the present study the clinical isolates were recovered from several sources: urine 64.5%, wound 13.3%, trachea 4.4%, abdominal fluid 4.4%, catheter 4.4%, abscess 4.4%, blood 2.3% and vagina 2.3%.

Antibiotic susceptibility testing.

Table 1 - Comparison of antibiotic susceptibility profile of enterococci from clinical cases and from healthy people

Antimicrobial Agent	Susceptibility profile	Clinical specimens	"Healthy" strains	P-value
		N (%)	N (%)	
Nitrofurantoin	S	26 (92.9)	23 (51.1)	<0.001
	I	2 (7.1)	13 (28.9)	
	R	0 (0)	9 (20)	
Ampicillin	S	27 (60)	42 (93.3)	<0.001
	I	0 (0)	0 (0)	
	R	18 (40)	3 (6.7)	
Vancomycin	S	17 (37.8)	45 (100)	<0.001
	I	15 (33.3)	0 (0)	
	R	13 (28.9)	0 (0)	
Ciprofloxacin	S	2 (7.1)	13 (28.9)	<0.001
	I	6 (21.4)	23 (51.1)	
	R	20 (71.4)	9 (20)	
Tetracycline	S	2 (7.1)	36 (80)	<0.001
	I	0 (0)	0 (0)	
	R	26 (92.9)	9 (20)	
Gentamicin	S	19 (42.2)	43 (95.6)	<0.001
	I	4 (8.9)	0 (0)	
	R	22 (48.9)	2 (4.4)	
Linezolid	S	34 (75.6)	35 (77.8)	0.78
	I	7 (15.6)	7 (15.5)	
	R	4 (8.8)	3 (6.7)	
Erythromycin	S	0 (0)	13 (28.9)	<0.001
	I	6 (13.3)	12 (26.7)	
	R	39 (86.7)	20 (44.4)	
Chloramphenicol	S	33 (73.3)	40 (88.9)	0.04
	I	4 (8.9)	5 (11.1)	
	R	8 (17.8)	0 (0)	
Penicillin	S	26 (57.8)	45 (100)	<0.001
	I	0 (0)	0 (0)	
	R	19 (42.2)	0 (0)	
Rifampin	S	6 (13.3)	10 (22.2)	0.13
	I	0 (0)	2 (4.4)	
	R	39 (86.7)	33 (73.3)	

Antimicrobial susceptibility pattern of all 90 isolates is demonstrated in Table 1. Our results revealed, among clinical specimens, the high rate of antibiotic resistance to tetracycline (92.9%), erythromycin (86.7%), rifampin (86.7%), ciprofloxacin (71.4%), gentamicin (48.9%), penicillin (42.2%), and ampicillin (40%) respectively. On the other hand, nitrofurantoin (92.9%), linezolid (75.6%), chloramphenicol (73.3%), ampicillin (60%), penicillin (57.8%), and gentamicin (42.2%) were the most effective antibiotics. In comparison, the highest resistance among stool isolates from healthy people was related

Table 2 - Correlation between MDR profile and virulence genes of enterococci from clinical cases and from healthy people

Samples	MDR profile	*ace* positive	*ace* negative	P-value	*agg* positive	*agg* negative	P-value
		N (%)	N (%)		N (%)	N (%)	
Clinical specimens	Non MDR	9 (27.3)	3 (25)	0.60	4 (23.5)	8 (28.6)	0.50
	MDR	24 (72.7)	9 (75)		13 (76.5)	20 (71.4)	
Healthy cases	Non MDR	4 (80)	36 (90)	0.46	4 (100)	36 (87.8)	0.61
	MDR	1 (20)	4 (10)		0 (0)	5 (12.2)	

to rifampin (73.3%), erythromycin (44.4%), as well as nitrofurantoin, ciprofloxacin and tetracycline (each one 20%). Antibiotic resistance against other antimicrobial agents was significantly higher in patient group than healthy cases ($P < 0.05$). The rate of MDR in the patients and healthy isolates was 73.3% and 11.1% respectively. However, the prevalence of MDR was 69.4% (25/36) in *E. faecalis* and 88.9% (8/9) in *E. faecium*. Of the total study population, 83.3% of *E. faecium* and 27.3% of *E. faecalis* were resistant to vancomycin (VRE), and all of them were from clinical isolates. VRE was not seen in our healthy specimens.

Molecular assays. According to PCR test for *ddl* gene, the frequency of *E. faecalis* and *E. faecium* in patient group was 80% and 20% respectively, while *E. faecium* was exclusively detected (100%) among the healthy individuals. The prevalence of *agg* and *ace* genes among the clinical isolates was 37.8% (17/45) and 73.3% (33/45), respectively. Interestingly, in healthy samples, the frequency of *agg* and *ace* genes was 8.9% (4/45) and 11.1% (5/45) respectively. Our results showed that the prevalence of these virulence genes was remarkably higher in clinical *E. faecalis* ($p < 0.05$). There was not any significant correlation between virulence genes (*ace* and *agg*) frequency and MDR strains in both pathogenic and commensal isolates ($p > 0.05$) (Table 2).

Biofilm formation analysis. The ability of biofilm formation was notably greater

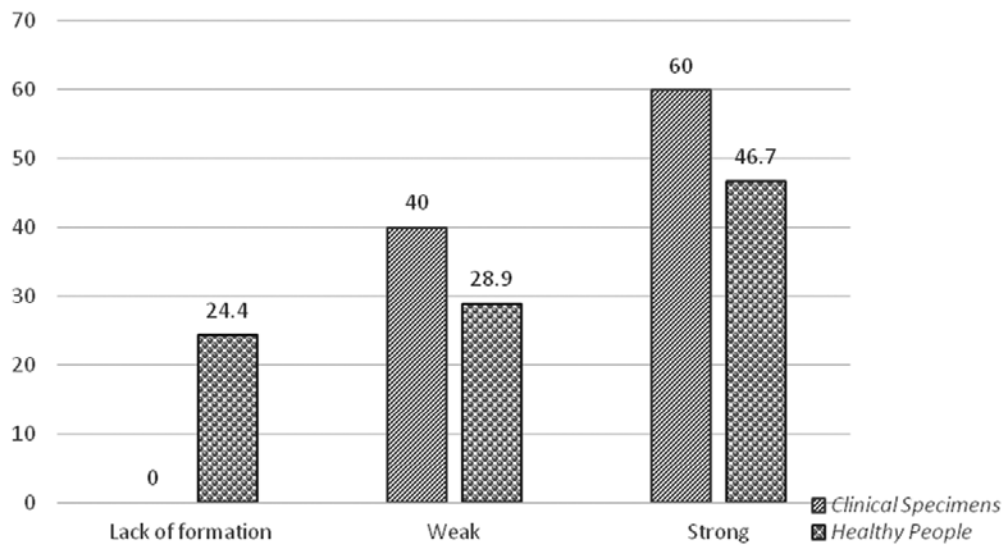

Figure 1 - The frequency of biofilm formation by enterococci from clinical cases and from healthy people

Table 3 - Distribution of biofilm formation among *Enterococcus* species from clinical specimens

Biofilm formation	*E. faecalis* N (%)	*E. faecium* N (%)	P-value
Negative	0 (0)	0 (0)	<0.001
Weak	9 (25)	9 (100)	
Strong	27 (75)	0 (0)	

in pathogenic than commensal isolates ($p < 0.05$) (Fig. 1). Moreover, our results showed that biofilm formation among *E. faecalis* in the patient group was significantly stronger than *E. faecium* isolates ($p < 0.001$) (Table 3) and also, there was no significant relationship between biofilm formation and MDR both in the patient and healthy groups (Table 4). Regarding the present study, the biofilm formation in clinical strains was significantly greater in ace-positive isolates but in the healthy group, there was not any statistically significant relationship between the biofilm formation and ace gene (Table 5). All of the isolates harboring agg gene formed strong biofilm (100%) in the two groups. A positive correlation was seen between biofilm formation and frequency of agg virulence gene in clinical and healthy samples (Table 5).

Discussion

The diffusion of MDR bacteria and, consequently, the increasing weigth of nosocomial infections are major threats for public health. Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae,* as well as vancomycin-resistant enterococci (VRE), are the most frequently isolated MDR bacteria (21). *E. faecium and E. faecalis* are the most clinically important of enterococcal species which are associated with MDR-nosocomial infections and also VRE, especially among

Table 4 - Correlation between MDR profile and biofilm formation of enterococci from clinical cases and from healthy people

Samples	Profile MDR	Negative N (%)	Weak N (%)	Strong N (%)	P-value
Clinical specimens	Non MDR	0 (0)	4 (22.2)	8 (29.6)	0.59
	MDR	0 (0)	14 (77.8)	19 (70.4)	
Healthy cases	Non MDR	10 (90.9)	13 (100)	17 (81)	0.23
	MDR	1 (9.1)	0 (0)	4 (19)	

Table 5 - Correlation between biofilm formation and virulence genes of enterococci from clinical cases and from healthy people

Samples	Biofilm formation	*ace* positive N (%)	*ace* negative N (%)	P-value	*agg* positive N (%)	*agg* negative N (%)	P-value
Clinical specimens	Negative	0 (0)	0 (0)	0.004	0 (0)	0 (0)	0.02
	weak	9 (27.3)	9 (75)		14 (17.6)	15 (53.6)	
	Strong	24 (72.7)	3 (25)		17 (82.4)	13 (46.4)	
Healthy cases	Negative	1 (20)	10 (25)	0.28	0 (0)	11 (26.8)	0.048
	Weak	0 (0)	13 (32.5)		0 (0)	13 (31.7)	
	Strong	4 (80)	17 (42.5)		4 (100)	17 (41.5)	

immunocompromised individuals (4).The aim of the present study was to determine and compare biofilm formation, antibiotic resistance and frequency of selected virulence genes in the two groups: commensal and clinical *E. faecalis*, and *E. faecium*, for the first dissemination of enterococcal opportunistic infections. Our results showed that the distribution of *Enterococcus* species was significantly different between the two study groups. *E. faecalis* was mainly recovered from clinical samples (80%) compared to healthy specimens in which *E. faecium* was isolated predominantly (100%) ($p < 0.001$). Similar to other reports from Iran, *E. faecalis* results as the most prevalent clinical *Enterococcus* species (22). In the current study, *E. faecium* in clinical specimens had greater resistance to penicillin and ampicillin compared to *E. faecalis* ($p < 0.001$), while the resistance to other antibiotics had no significant difference ($p > 0.05$). Antibiotic resistance against rifampin and linezolid had no significant difference between the two groups ($p > 0.05$), while resistance to nitrofurantoin was significantly higher in *E. faecium* isolated from healthy cases than in clinical subjects ($p < 0.001$). Interestingly, we found out that the prevalence of VRE strains was significantly higher in clinical samples in comparison to healthy isolates (39.3% vs 0%) ($p < 0.001$). Several studies showed that the prevalence of VRE was 9.4%, 11.2%, 8.5 to 12.5% and 9% in Iran, Germany, England and Italy respectively (23). According to our results, frequency of VRE in *E. faecium* was significantly higher than in *E. faecalis* in the patient group strains ($P < 0.05$). In addition, 42% of all our isolates were MDR and the frequency of MDR was significantly higher in the patients than the healthy group ($p < 0.001$). Nevertheless, the prevalence of MDR strains in clinical isolates didn't differ between the two species ($p = 0.23$). According to the present study, there was no statistically significant difference between biofilm-forming enterococci and MDR strains in two clinical and commensal isolates ($p = 0.59$ and $p = 0.23$ respectively). Our findings indicated that the frequency of *ace* and *agg* genes in clinical and healthy subjects was 42% and 23%, respectively. Similarly, in a study conducted by Lopez et al. the frequency of *ace* and *agg* genes in clinically isolated *E. faecalis* was 39% and 44%, respectively. In this study, a significant correlation was found between the presence of *ace* gene and resistance to tetracycline as well as the presence of *agg* gene with biofilm formation (24). Our results suggest that the frequency of these genes was significantly higher in the patient group ($P < 0.05$). Moreover, biofilm formation in the patient group was significantly stronger than the healthy cases ($p = 0.03$). In the study performed by Cariolato et al, the *ace* gene was found only in *E. faecalis* and the prevalence of this gene was similar in both human and dairy isolates (76.3%) (25). Niu et al. in China showed that the frequency of virulence genes such as *ace* and *agg* in 30 high-level aminoglycoside resistance (HLAR) isolates was 1 (3.3%) and 0 (0%) respectively (20). Our results revealed that the *ace*-positive pathogenic isolates were significantly more potent for biofilm formation in comparison to *ace*-negative ones as well as all the Enterococcus isolates containing *agg* gene, that had high biofilm development ability compared to *agg*-negative strains. Hubble et al. (26) hypothesized that the products of *ace* gene are involved in bacterial binding to root dentin canal. By studying the *ace*-mutated strains, they found out that there was a significant correlation between the presence of intact gene and the subsequent attachment of *E. faecalis* to dentin. Thus, *agg* and *ace* genes in *E. faecalis* and *E. faecium* can be considered as important virulence factors.

Conclusion

In conclusion, we observed higher frequency of *ace* and *agg* genes, biofilm

formation and antibiotic resistance in clinical specimens, as well as, resistance to ampicillin, penicillin and VRE were higher in *E. faecium* than *E. faecalis* between clinical specimens. Interestingly, the high prevalence of MDR strains, *ace* and *agg* genes and biofilm formation ability in our healthy isolates shows important epidemiological information. The problem with these strains is spreading and persistence of them in clinical environments for a very long time and consequently, they could be as a source of distribution of resistance and virulence traits among the patients. Therefore, new methods are needed for preventing the spread of fecal enterococci of "healthy" origin throughout the hospital environments.

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Conflicts of interest
The authors declare no conflicts of interest.

Riassunto

Fattori di virulenza, formazione di biofilm e profili di antibiotico-resistenza in ceppi di *Enterococcus faecalis* ***ed*** *Eenterococcus faecium* ***isolati da pazienti e da soggetti sani ad Isfahan, Iran***

Premessa. Lo scopo del presente studio è di determinare e confrontare I profili di antibiotico-resistenza, la capacità di formare biofilm e la frequenza dei geni di virulenza *agg* ed *ace* in ceppi di *Enterocuccus* spp isolati sia da pazienti che da individui sani.

Metodi. Novanta isolamenti originali di *Enterococcus* spp sono stati ottenuti in parte da paziente, in parte da sogetti sani, e per tutti sono stati determinati: il profilo di antibioticoresistenza con il metodo della diffusione da dischi e con lo E-test; la presenza eventuale di geni di virulenza con l'amplificazione medisnte PCR; la cspacità di formare biofilm con la tecnica in micropiastra.

Risultati. *Enterococcus faecalis* è risultata la specie più frequente tra i ceppi di isolamento clinico (80%); la prevalenza dei geni *agg* ed *ace* è stata rispettivamente del 37,8% e del 73,3% tra i ceppi di isolamento clinico e dell'8,9% e del 11,1% tra quelli isolati da sani; la quota di multirersistenza agli antibiotici è stata del 73,3% nei ceppi dei isolamento clinico e dell'11,1% nei ceppi isolati da sani; la capacità di formare biofilm è risultata presente nella totalità dei ceppi di origine clinica e nel 75,6% dei casi in quelli isolati da sani ($P < 0,05$).

Conclusioni. Il possesso dei geni *ace* e *agg*, l'antibiotico-resistenza e la capacità di formare biofilm sono risultati significativamente maggiori nei ceppi di isolamento clinico rispetto a quelli isolati dai sani ($P < 0,05$). Ma la presenza, sia pure minoritaria, di queste caratteristiche anche nei ceppi isolati dai sani riveste una certa importanza perché, se questi ceppi si diffondono in ambienti clinici o si infiltrano nelle forniture idriche, ciò può considerarsi un fattore di rischio per la salute e per le garanzie sanitarie della popolazione.

References

1. Coombs GW, Pearson JC, Daley DA, et al. Molecular epidemiology of *enterococcal* bacteremia in Australia. J Clin Microbiol 2014; **52**(3): 897-905.
2. Guiton PS, Hung CS, Hancock LE, Caparon MG, Hultgren SJ. *Enterococcal* biofilm formation and virulence in an optimized murine model of foreign body-associated urinary tract infections. Infect Immun 2010; **78**(10): 4166-75.
3. Van Tyne D, Gilmore MS. Raising the Alarmone: Within-Host Evolution of Antibiotic-Tolerant *Enterococcus faecium*. mBio 2017; **8**(1): e00066-17.
4. Layton BA, Walters SP, Lam LH, Boehm AB. *Enterococcus* species distribution among human and animal hosts using multiplex PCR. J Appl Microbiol 2010; **109**(2): 539-47.
5. Arias CA, Murray BE. The rise of the Enterococcus: beyond vancomycin resistance. Nat Rev Microbiol 2012; **10**(4): 266-78.
6. Higuita NIA, Huycke MM. *Enterococcal* disease, epidemiology, and implications for treatment. *Enterococci*: from commensals to leading causes of drug resistant infection. Boston: Massachusetts Eye and Ear Infirmary, 2014: 67-8.
7. Lewis CM, Zervos M. Clinical manifestations of *enterococcal* infection. Eur J Clin Microbiol Infect Dis 1990; **9**(2): 111-7.
8. Mohamed JA, Huang DB. Biofilm formation by *enterococci.* J Med Microbiol 2007; **56**(12): 1581-8.
9. Talebi M, Moghadam NA, Mamooii Z, et al. Antibiotic resistance and biofilm formation of *Enterococcus faecalis* in patient and environ-

mental samples. Jundishapur J Microbiol 2015; **8**(10): 1-5.
10. Van Acker H, Van Dijck P, Coenye T. Molecular mechanisms of antimicrobial tolerance and resistance in bacterial and fungal biofilms. Trends Microbiol 2014; **22**(6): 326-33.
11. Hall CW, Mah T-F. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. FEMS Microbiol Rev 2017; **4**(3): 276-301.
12. Diani M, Esiyok OG, Ariafar MN, et al. The interactions between esp, fsr, gelE genes and biofilm formation and pfge analysis of clinical *Enterococcus faecium* strains. Afr J Microbiol Res 2014; **8**(2): 129-37.
13. Soares RO, Fedi AC, Reiter KC, Caierão J, d'Azevedo PA. Correlation between biofilm formation and gelE, esp, and agg genes in *Enterococcus* spp. clinical isolates. Virulence 2014; **5**(5): 634-7.
14. Jahan M, Holley RA. Incidence of virulence factors in *enterococci* from raw and fermented meat and biofilm forming capacity at 25 C and 37 C. Int J Food Microbiol 2014; **170**: 65-9.
15. Clewell DB, Francia MV, Flannagan SE, An FY. *Enterococcal* plasmid transfer: sex pheromones, transfer origins, relaxases, and the *Staphylococcus aureus* issue. Plasmid 2002; **48** (3): 193-201.
16. Upadhyaya PG, Ravikumar K, Umapathy B. Review of virulence factors of *enterococcus*: an emerging nosocomial pathogen. Indian J Med Microbiol 2009; **27**(4): 301-5.
17. Mahon CR, Lehman DC, Manuselis G. Textbook of Diagnostic Microbiology-E-Book. 5th ed. United States: Elsevier Health Science, 2015.
18. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing. 27th ed. CLSI Supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute, 2017.
19. Creti R, Imperi M, Bertuccini L, et al. Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. J Med Microbiol 2004; **53**(1): 13-20.
20. Niu H, Yu H, Hu T, et al. The prevalence of aminoglycoside-modifying enzyme and virulence genes a*mong enterococci* with high-level aminoglycoside resistance in Inner Mongolia, China. Braz J Microbiol 2016; **47**(3): 691-6.
21. Fiore M, Maraolo AE, Gentile I, et al. Nosocomial spontaneous bacterial peritonitis antibiotic treatment in the era of multi-drug resistance pathogens: A systematic review. World J Gastroenterol 2017; **23**(25): 4654.
22. Kafil HS, Asgharzadeh M. Vancomycin-resistant *enteroccus faecium* and enterococcus faecalis isolated from education hospital of iran. Maedica 2014; **9**(4): 323-7.
23. Emaneini M, Hosseinkhani F, Jabalameli F, et al. Prevalence of vancomycin-resistant *Enterococcus* in Iran: a systematic review and meta-analysis. Eur J Clin Microbiol Infect 2016; **35** (9): 1387-92.
24. López-Salas P, Llaca-Díaz J, Morfin-Otero R, et al. Virulence and antibiotic resistance of *Enterococcus faecalis* clinical isolates recovered from three states of mexico. Detection of linezolid resistance. Arch Med Res 2013; **44**(6): 422-8.
25. Cariolato D, Andrighetto C, Lombardi A. Occurrence of virulence factors and antibiotic resistances in Enterococcus faecalis and *Enterococcus faecium* collected from dairy and human samples in North Italy. Food Control 2008; **19**(9): 886-92.
26. Hubble T, Hatton J, Nallapareddy S, Murray B, Gillespie M. Influence of *Enterococcus faecalis* proteases and the collagen binding protein, Ace, on adhesion to dentin. Mol Oral Microbiol 2003; **18**(2): 121-6.

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