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DIFFERENCES IN GENOTYPE OF *E. FAECALIS* AND *E. FAECIUM* CLINICAL ISOLATES REGARDING CYTOLYSIN AND GELATINASE PRODUCTION IN BULGARIAN PATIENTS

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ABSTRACT

Purpose - compare the phenotype and genotype correlation of cytolysin and gelatinase production in clinical isolates *Enterococcus* spp. Materials and methods - 100 *Enterococcus* strains collected over a period of one year from inpatients of two Bulgarian university hospitals, were tested for phenotype production of cytolysin and gelatinase. Multiplex PCR was performed to screen the presence of *gelE* and *cylA* virulence genes. Results – 17% of the enterococcal isolates demonstrated only cytolysin production phenotypically. Gelatinase activity was found in 21% of the isolates. Only *E. faecalis* showed combined phenotypic production of cytolysin plus gelatinase (21%). Forty-five percent of the tested enterococci were identified negative for both hemolysin and gelatinase activity. *GelE* was the most prevalent virulent gene (48% of the isolates). *CylA* gene was present alone only in four non-invasive *E. faecalis* isolates. Twenty-six percent of the isolates possessed both *cylA* and *gelE* genes and 21% did not harbor any of the virulence factors genotypically. Conclusion - our results prove that it is appropriate to perform both phenotypic and genotypic analysis of the enterococci virulence profile in parallel in order to better characterize the strains, which in turn may serve to develop more effective methods to limit the spread of infections caused by these microorganisms.

Key words: *Enterococcus* spp., virulence factors, virulence genes

INTRODUCTION

Enterococcus are Gram-positive, catalase-negative, non-spore-forming lactic acid bacteria, that are common commensal organisms in the gastrointestinal tract of humans and animals. Moreover, they may cause community-acquired infections and have been reported to be one of the leading causes of nosocomial infections with an increasing mortality rate (1). Although many *Enterococcus* species can cause human infections (2), most frequently isolated species in up to 90% of enterococcal infections is *Enterococcus faecalis* (3), followed by *Enterococcus faecium*, which has shown an increase in prevalence in recent years (4). These microorganisms have been associated with urinary tract infections, bacteremia and sepsis, endocarditis, surgical wound infections and burn wound infections, cholecystitis, peritonitis, neonatal meningitis (2). The therapeutic options of infections caused by *Enterococcus* species are limited because of their intrinsically or acquired via transposons or plasmids resistance to many antibiotics, including to the glycopeptide *Vancomycin* (3). In recent years, there has been a rapid occurrence of multidrug resistant enterococci (5), which pose real therapeutic difficulties.

The numerous virulent determinants that *Enterococcus* possesses and are able to share among other species (2) helps this microorganism to survive in a very crude environment and are considered significantly important in exacerbating diseases caused by them. Therefore, the greater understanding of the virulence traits of circulating *Enterococcus* strains has become more and more important

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and may help to understand better the pathogenesis of these microorganisms.

In this study, we compared the phenotype and genotype correlation of cytolysin and gelatinase production in clinical isolates *Enterococcus* spp.

MATERIALS AND METHODS

Collection and identification of study isolates

A total of 100 *Enterococcus* strains were collected over a period of one year from inpatients of University Hospital "Prof. Dr. Stoyan Kirkovich" AD, Stara Zagora, and University Hospital "St. George" EAD, Plovdiv, Bulgaria. All of the strains were from clinical relevant sites and from various specimens. Most of the enterococcal isolates were recovered from urine (n=45). Other specimens included wound secretions (n=31), blood (n=10), vaginal swabs (n=6), body fluids (n=5) and respiratory samples (n=3).

All the samples were inoculated onto blood agar plates (*HiMedia Laboratories, Mumbai*) and incubated 24 hours at 37℃. *Enterococcus* suspected colonies were identified by standard microbiological methods including Gram staining, catalase test, esculin hydrolysis (6). Species identification was performed according to the Facklam & Collins scheme (7) by a standard conventional biochemical test based on the carbohydrate fermentation of 1% sugar mannitol solution and arabinose, pyruvate hydrolysis (PYR test), arginine decarboxylation, growth of media containing potassium tellurite and via Vitek-MS automated systems (*bioMérieux, France*).

Phenotypic detection of virulence factors

All *Enterococcus* isolates were tested for the presence of virulence determinants cytolysin and gelatinase.

1. Cytolysin production

Cytolysin production was tested by a spot inoculation of a pure culture of the enterococci onto Todd-Hewitt agar, supplemented with 5% human blood and incubated 48 hours at 37℃. A presence of clear zone of β-hemolysis surrounding the colonies was considered as a positive result (8).

2. Gelatinase production

Gelatinase production was determined by a spot inoculation of the *Enterococcus* strains onto Todd-Hewitt agar containing 30 g gelatin per 1 liter. Plates were incubated overnight at 37℃. The appearance of a clear zone/turbid halo around the colonies after the plates were cooled for 2 hours at 4℃ was considered to be a positive indication of gelatinase production (8).

Genotypic detection of virulence genes

For the Polymerase Chain Reaction (PCR) experiments, genomic DNA was extracted using an appropriate DNA extraction kit (*GenneMATRIX Gram Plus & Yeast Genomic DNA Purification Kit, Poland*), according to the manufacturer's instructions and was stored at -80°C until use. The concentration and purity of the DNA samples were measured spectrophotometrically at 260 nm/280 nm using a GeneQuant 1300 spectrophotometer (*GE Healthcare Life Sciences, Switzerland*).

Multiplex PCR was performed to screen the presence of *gelE* and *cylA* (activator of cytolysin) virulence genes. The specific primers were used according to a previous study (9). The primers were supplied by *Biomed Future, Sofia, Bulgaria*. The sequences of primers are shown in **Table 1.**

Table 1. *PCR primers used to detect virulence genes (gelE and cylA) of Enterococcus spp. were selected by Vankerckhoven et al. (2004).*

Target gene	Primer	Oligonucleotide sequence (5'-3')	Product size (bp)
gelE	GEL 11	TATGACAATGCTTTTTGGGAT	213
	GEL 12	AGATGCACCCGAAATAATATA	
cylA	CYT I	ACTCGGGGATTGATAGGC	688
	CYT IIb	GCTGCTAAAGCTGCGCTT	

PCR was applied in a total volume of 20 µl PCR mixture containing 2 µl PCR buffer, 0.8 µl $MgCl_2$, 2 µl dNTP, 0.1µl of Gel 11 and Gel 12 primers, 0.3 µl of Cyt I and Cyt IIb primers, 0.5 µl Taq DNA polymerase, 2 µl of DNA and

adding sterile DNA/RNAse free water up to 20 µl.

The PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR reagents were provided by Thermo Fisher Scientific, USA. Amplification for PCR products were done as follows: initial denaturation at 94℃ for 7 minutes followed by 35 cycles consisting of denaturation (94°C for 1.0 minute), annealing (56.4℃ for 1.0 minute), and extension (72℃ for 1.0 minute), and final extension step at (72℃ for 7 minutes). The resulting PCR amplicons were analyzed by electrophoresis on 2% agarose gel containing ethidium bromide in TBE buffer for 40 minutes at 120 V in the presence of 100 bp DNA ladder.

RESULTS

Totally, among 100 clinical isolates of *Enterococcus* spp. obtained in our study, 85% (n=85) were identified as *E. faecalis* whereas 15% (n=15) harbored *E. faecium*. Most of the patient specimens (45%) were recovered from urine (*E. faecalis*, n=38 and *E.* faecium, n=7), followed by wound secretions (31%) (*E. faecalis*, n=28 and *E. faecium*, n=3). Other specimens included blood (n=10), vaginal swabs (n=6), body fluids (n=5) and respiratory samples (n=3).

The one hundred enterococci were tested for their haemolytic and gelatinase activity by phenotype methods. The presence of genes that encode the both virulence factors cytolysin (*cylA*) and gelatinase (*gelE*) was evaluated by multiplex PCR **(Figure 1a, b).** The virulence determinant content of each isolate under consideration was found both phenotypically and genotypically in varying proportions as reported in **Tables 2 and 3.**

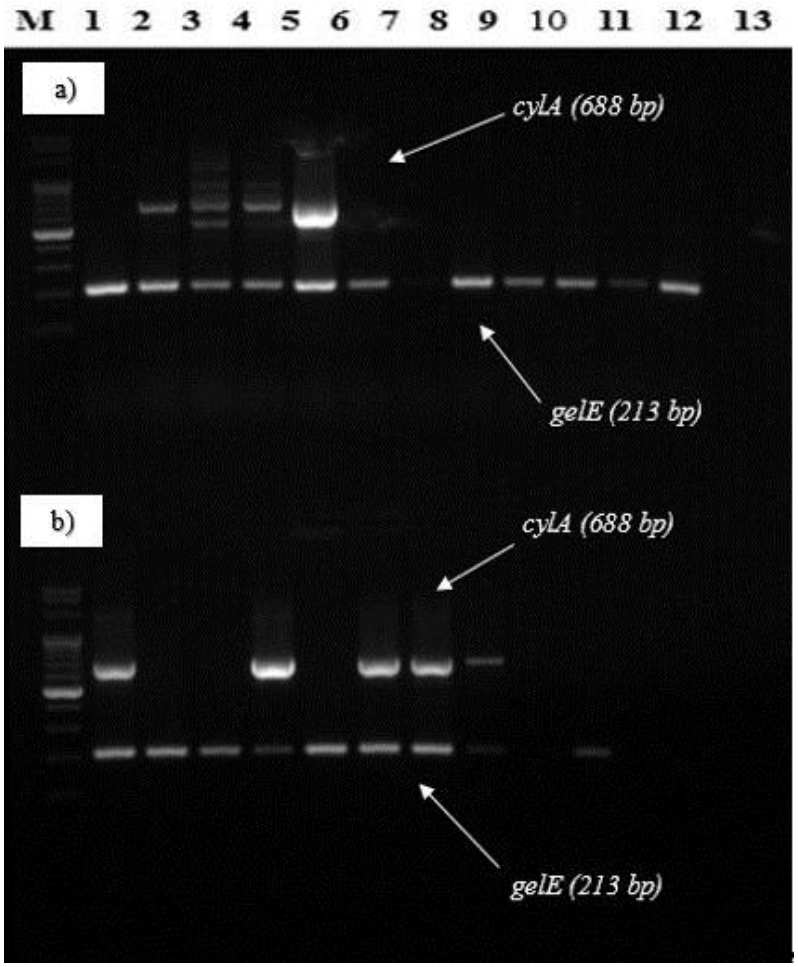

Figure 1 a), b). Gel electrophoresis of multiplex PCR product of amplification of *cylA* and *gelE Enterococcus* spp. virulence genes at 688 and 213 bp, respectively.
Lane M – 100 bp DNA ladder, lanes 1-12 – virulence genes in *Enterococcus* spp., lane 13 - negative control (no DNA added).

Table 2. *Phenotype detection of virulence factors in Enterococcus spp. isolates from clinical samples.*

Species	Cytolysin production N (%)	Gelatinase production N (%)	Both cytolysin and gelatinase production N (%)	Both cytolysin and gelatinase negative N (%)
E. faecalis **N =85**	15 (18%)	20 (23%)	18 (21%)	32 (38%)
E. faecium **N =15**	2 (13%)	1 (7%)	0 (0%)	12 (80%)
Total N=100	17 (17%)	21 (21%)	18 (18%)	44 (44%)

Table 3. *Genotype detection of virulence determinants in clinical isolates of enterococci.*

Species	*CylA+/GelE-* N (%)	*CylA-/GelE+* N (%)	Both *CylA/GelE* positive N (%)	Both *CylA/GelE* negative N (%)
E. faecalis **N =85**	4 (5%)	38 (45%)	25 (29%)	18 (21%)
E. faecium **N =15**	0 (0%)	10 (67%)	1 (7%)	4 (26%)
Total N=100	4 (4%)	48 (48%)	26 (26%)	22 (22%)

"+" – presence of virulence gene tested, "-" – absence of virulence gene tested

Our results determined that 17% (n=17) of 100 *Enterococcus* spp. isolates evaluated, 18% *E. faecalis* (n=15) and 13 % *E. faecium* (n=2) respectively, demonstrate only cytolysin production phenotypically **(Table 2).** Gelatinase activity was only found in 21% (n=21) of the analyzed isolates with a tendency to be present more often in *E. faecalis* (24%, n=20) than *E. faecium* (7%, n=1). Cytolysin and gelatinase activity prevalence was higher in urinary tract and wound secretions enterococcal isolates than from other sources. Eighteen (21%) *E. faecalis* showed combined production of cytolysin plus gelatinase and more than a half of them (56%, n=10) were urine isolates. There were not any *E. faecium* showed both virulence factors. Overall, 44% (n=44) of the *Enterococcus* isolates (37% *E. faecalis*, n=32 and 80% *E. faecium*, n=12) were identified negative for both hemolysin and gelatinase activity.

Among the virulence genes that we tested, *gelE* was the most prevalent one. It was found alone in 45% of *E. faecalis* (n=38) and 67% of *E. faecium* (n=10) respectively as shown in **Table 3.** *GelE* was recovered in higher number isolates from urinary tract (22 strains), followed by isolates from wound secretions (11 strains) but was detected only in three from 10 (30%) invasive isolates. *CylA* gene was present alone only in four *E. faecalis* isolates (three wound secretion isolates and one isolate from urine). It was never detected alone in blood samples. Twenty-six percent of *Enterococcus* isolates (25 *E. faecalis* and only 1 *E. faecium*) possessed both *cylA* and *gelE* genes. However, 21% of the isolates (18 *E. faecalis* and 4 *E. faecium*) did not harbor any of the virulence factors genotypically.

Phenotype and genotype correlation in *Enterococcus* spp. tested isolates is reported in **Table 4.** The highest percentage of correspondence (17%) between the phenotype and the genotype (*cylA* -, *gelE* +) in the studied isolates was observed among the phenotypically gelatinase producing enterococci. In 3 of the strains (3%) demonstrated only cytolysin production phenotypically, there was a match with the genotype (*cylA* +, *gelE* -). In enterococci that demonstrated simultaneous phenotypically production of cytolysin and gelatinase and in those that did not phenotypically showed these two virulence factors, we observed almost the same percentage of genotype correspondence - 15% (15 strains; *cylA* +, *gelE* +) and 14% (14 strains; *cylA* -, *gelE* -), respectively. The differences between phenotype and genotype are also detailed in **Table 4.**

Table 4. *Phenotype and genotype correlation in Enterococcus spp. clinical isolates regarding pathogenicity.*

Correlation between phenotype and genotype	**Cytolysin production, *CylA*+/*GelE*- N (%)**	**Gelatinase production, *CylA*-/*GelE*+ N (%)**	**Both cytolysin and gelatinase positive, both *CylA* and *GelE* positive N (%)**	**Both cytolysin and gelatinase positive, both *CylA* and *GelE* negative N (%)**
PHENOTYPE AND GENOTYPE CORRESPONDENCE	3 (3%)	17 (17%)	15 (%)	14 (%)
PHENOTYPE AND GENOTYPE DIFFERENCE	14 (14%) 3 strains: *CylA* + *GelE* + 3 strains: *CylA* - *GelE* - 8 strains: *CylA* - *GelE* +	5 (5%) 2 strains: *CylA* + *GelE* + 3 strains: *CylA* - *GelE* -	3 (3%) 2 strains: *CylA* - *GelE* – 1 strain: *CylA* - *GelE* +	30 (30%) 6 strains: *CylA* + *GelE* + 23 strains: *CylA* - *GelE* + 1 strain: *CylA* + *GelE* -

“+” – presence of virulence gene tested, “-” – absence of virulence gene tested

DISCUSSION

Enterococci are one of the most important bacterial species, major opportunistic infectious agents that cause a wide range of nosocomial infections, especially in intensive care units worldwide (10). *E. faecalis* and *E. faecium* are known to be the major causative agents of enterococcal infections (11). The virulence factors of enterococci are the main focus in many studies over the past few years. The virulence in this genus is usually considered a multifactorial process that involves several genes and their products and is associated with different factors such as cytolysin and gelatinase production, presence of hyaluronidase, *Enterococcus* surface protein (*Esp*), aggregation substance (*AS*), ability to form biofilm, etc. (12). Their incidence among the strains varied widely from study to another, probably because of their different clinical and geographical origin.

This study evaluated a hundred clinical isolates, of which the prevalence of *E. faecalis* among the clinical isolates was higher than *E. faecium* (85% and 15% respectively). Similar distribution of species was observed in other studies in North and Latin America, some European and Asian countries (13, 14), while other authors reported the opposite trend in India (15).

The cytolysin was the first and the best-studied virulence factor in enterococci. It is a protein toxin capable of lysing human, horse, rabbit, but not sheep erythrocytes (16) and it also has bacteriocin activity against other Gram-positive bacteria (17). The cytolysin is encoded usually on pheromone-responsive plasmids (18) but also can be located in the bacterial chromosome (19). It is encoded by cytolysin operon and several genes are essential for its functional production. The *cylA* gene is responsible for the expression of whole operon (20). *E. faecalis* strains producing cytolysin have been shown to be virulent in animal and nematode models and human infections (21, 22) and have been associated with increased severity of infection (22), and subsequent increased mortality risk.

The prevalence of cytolysin and gelatinase virulence determinants is having been shown to be higher in *E. faecalis* than *E. faecium* isolates even though in some studies in Europe *E. faecium* was reported generally free of virulence factors (23, 24). In our study, phenotype production incidence of cytolysin among *Enterococcus* spp. isolates was 35 % (33% *E. faecalis* and 2% *E. faecium*, respectively). Iran authors (25) reported a similar rate of hemolysin positive isolates, but the rate of the two species was different from the one reported by us (39% *E. faecium* and 27% *E. faecalis*, respectively). Seventeen percent of all the tested enterococci possessed the cytolysin production only. We observed a higher cytolysin activity prevalence in the urinary tract and wound secretions isolates than from other sources. Invasive cytolysin

positive enterococcal isolates were not observed in contrast to the French and American studies (26, 27), which reported a high percentage of invasive isolates that produce cytolysin (40% and 50%, respectively).

The detection of *cylA* by PCR was not strictly correlated with its phenotypic expression. In our work, 35% *Enterococcus* spp. (n=35) showed hemolytic activity on blood agar plates, although *cylA*-positive were 30 of them (*E. faecalis*, n=29 and *E. faecium*, n=1, respectively). These results correlate with the data reported in India and the USA (26, 28). The lack of cytolysin phenotypic/genotypic compatibility may suggest missing genes in the *cyl* operon among *cylA*-positive/haemolysin-negative strains or the presence of the silent *cylA* gene (29). We detected *cylA* gene alone only in four *E. faecalis* isolates (three wound isolates and one isolate from urine) but it was never detected alone in invasive blood isolates. Our results showed a notably lower frequency of *cylA* detection (4%) compared to results reported in South Brazil (54.4%, n=31) (30), and in Italy (26.4%) (31).

Another well-known virulent factor in *Enterococcus* spp. is gelatinase. It is an extracellular zinc metallo-endopeptidase capable to disintegrate gelatin, collagen, casein, hemoglobin and other bioactive peptides (32). Gelatinase is encoded by the chromosomal *gelE* gene (33) and is regulated by the quorum-sensing fsr locus in a cell-density-dependent manner (34). The gelatinase detection was higher present in clinical isolates than in fecal isolates from healthy volunteers (35), which indicate its virulence potential.

Gelatinase production is mediated by *gelE* gene and phenotypically expressed in vitro by liquefaction of a culture medium containing the substrate (36). Similar to the results obtained for the cytolysin activity, the gelatinase activity was present more often in *E. faecalis* (45%, n=38) than *E. faecium* (7%, n=1). A higher rate of *gelE*-positive *E. faecalis* (67%, n=64) than ours result and lack of *gelE* occurrence in *E. faecium* was reported in Iran (25). We observed a lower number enterococcal isolates (n=3) from blood with phenotype gelatinase activity compared to non-invasive isolates (22 isolates from urine and 11 from wound secretions), which is in accordance with other authors who also reported its low prevalence or absence in invasive strains (26).

Because of their ability to remain silent (37) the expression of enterococcus *gelE* genes similar to reported before results for *cylA* genes, vary in-vivo and in-vitro. The presence of the *gelE* gene and the lack of gelatinase activity in *Enterococcus* spp. have been also associated with manipulation in the laboratory and low level expression or down regulation of the *gelE* gene (38). Our results obtained by phenotypic tests always revealed a lower number of isolates that produced gelatinase in phenotypic test, even though *gelE* was detected by PCR - 39 (53%) of total 74 *gelE*-positive strains (63 *E. faecalis* and 11 *E. faecium*). These results correspond to data obtained in Iran where 49,3% of the *E. faecalis* isolates exhibit gelE did not show gelatinase activity (25). In another study, *gelE* genes were not detected among enterococci at all (9). It suggests that geographic features might be the reason for the variation among different rates of the virulence factors. Some authors reported that other genes might be associated with *gelE* expression control and indicated that mutated genes affect *gelE* gene expression and possibly regulate gelatinase production (39). Seventy-five percent of the tested invasive isolates possessed the *gelE* gene, but as it reported previous, only 25% of them expressed the gene. When comparing the results of phenotypic and genotyping analysis it was observed that 72 isolates carried the *gelE* gene and 39 were gelatinase producers, but it is important to highlight that only 18% and 26% of them were positive for phenotypic and genotypic characteristics, respectively.

CONCLUSION

Due to the increasing importance of *Enterococcus* spp. as causative agents of severe infections, including nosocomial infections, the identification of their virulence factors related to the invasiveness and severity of the disease is a subject of great importance and merits particular attention. Due to the existence of silent virulence genes in these microorganisms (*cylA*, *gelE*), in order to better characterize the strains and in order to prevent nosocomial infections, *Enterococcus* spp. must be constantly monitored and phenotypic and genotypic analysis of their virulence pattern need to be performed in parallel. This may serve as a basis for the development of new and more effective strategies to prevent the

spread of enterococcal infections, such as inhibiting the action of the virulence factors and may provide an appropriate treatment and eradication methods.

REFERENCES

1. De Fa´tima, S. L., Ribeiro, M., Abrantes, T., Figueiredo Marques, M., Tenreiro, R. J. J. and Crespo, M. T. B., Antimicrobial resistance profiles of dairy and clinical isolates and type strains of enterococci. *Int J Food Microbiol*, 103:191–198, 2005.
2. Jett, B., Huycke, M. M. and Gilmore, M. S., Virulence of enterococci. *Clin. Microbiol. Rev.*, 7:162–478, 1994.
3. Murray, B. E., The life and times of the enterococcus. *Clin. Microbiol. Rev.*, 3:46–65., 1990.
4. Fisher, K. and Phillips, C., The ecology, epidemiology and virulence of Enterococcus. *Microbiol*, 155:1749-1757, 2009.
5. Poh, C. H., Oh, H. M. L. and Tan, A. L., Epidemiology and clinical outcome of enterococcal bacteraemia in an acute care hospital. *J Infect*, 52:383–386, 2006.
6. Fackland, R., Sham, D. A., Teixeira, L. M., Enterococcus. In: *Manual of Clinical Microbiology* 7th edition. Edited by: Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C., and Yolken, R. H. Washington: American Society of Microbiology, 297-305, 1999.
7. Facklam, R. R. and Collins, M. D., Identification of Enterococcus Species Isolated from Human Infections by a conventional test scheme. *J of Clin Microbiol*, 27:731-4, 1989.
8. Ike, Y. A., Hashimoto, H. A. and Clewell D. B., High incidence of hemolysin production by enterococcus (Streptococcus) faecalis strains associated with human parenteral infections. *J Clinic Microbio.*, 25(8):1524-8, 1987.
9. Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Daniela Jabes and Herman Goossens, H., Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium. *Journal of Clinical Microbiology*, 42(10):4473-4479, 2004.
10. Khan, H., Ahmad, A. and Riffat, M., Nosocomial infections and their control strategies. *Asian Pasific Journal of Tropical Medicine*, vol. 5, issue 7, 509-514, 2015.
11. Shepard, B. D., and Gilmore, M. S., Differential expression of virulence-related genes in Enterococcus faecalis in response to biological cues in serum and urine. *Infect. Immun.*, 70 (8):4344–4352, 2002.
12. Gulhan, T; Aksakal, A., Ekin, I. H., Savasan, S. and Boynukara, B., Virulence factors of Enterococcus faecium and Enterococcus faecalis strains isolated from humans and pets. *Turk. J. Vet. Anim. Sci.*, 30:477-482, 2006.
13. Low, D. E., Keller, N., Barth, A. and Jones R. N., Clinical prevalence, antimicrobial susceptibility, and geographic resistance patterns of enterococci: results from the Sentry Antimicrobial Surveillance Program, 1997-1999. *Clin Infect Dis*, 32 Suppl 2:S133-45, 2001.
14. Fallah, F., Yousef,i M., Pourmand, M. R., Hashemi, A., Nazari, Alam, A. and Afshar, D., Phenotypic and genotypic study of biofilm formation in Enterococci isolated from urinary tract infections. *Microb Pathog.*, 108:85-90, 2017.
15. Kapoor, L., Randhawa, V. And Deb, M., Antimicrobial resistance of enterococcal blood isolates at a pediatric care hospital in India. *Jpn J Infect Dis*, 58(2):101-103, 2005.
16. Masoud A., M., Amirmozafari, N. and Homa, F., Evaluation of Virulence Factors and Plasmid-Related Transmissibility among Different Isolates of Enterococci. *Iranian Biomedical Journal*, 9 (2): 51-55, April 2005.
17. Haas, W. and Gilmore, M. S.: Molecular nature of a novel bacterial toxin: the cytolysin of Enterococcus faecalis. *Med. Microbiol. Immunol.* 187:183–190, 1999.
18. Koch, S., Hufnagel, M., Theilacker, C. and Huebner, J. Enterococcal infections: host response, therapeutic, and prophylactic possibilities. *Vaccine*, 22:822–830, 2004.
19. Coque, T., Patterson, T., Steckelberg, J. and Murray, E., Incidence of hemolysin, gelatinase and aggregative substance among enterococci isolates from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. *J. Infect. Dis.*, 171:1223-1229, 1994.
20. Gilmore, M., Segarra, R., and Booth, M., An HlyB-type function is required for expression of the Enterococcus faecalis hemolysin/bacteriocin. *Infection and immunity*, 58(12), 3914-3923, 1990.
21. Jett, B. D., Jensen, H. G., Atkuri, R. V. and Gilmore, M. S., Evaluation of therapeutic

measures for treating endophthalmitis caused by isogenic toxin-producing and toxin-nonproducing Enterococcus faecalis strains. *Invest Ophthalmol Vis Sci,* 36:9-12, 1995.

22. Garsin, D., Sifri C. D., Mylonakis, E., Qin, X., Singh, K. V., Murray, B. E., et al. (). A simple model host for identifying Gram-positive virulence factors. *Proc Natl Acad Sci USA98*, 10892-10897, 2001.
23. Vankerckhoven V, Huys G, Vancanneyt M, et al., Genotypic diversity, antimicrobial resistance, and virulence factors of human isolates and probiotic cultures constituting two intraspecific groups of Enterococcus faecium isolates. *Appl Environ Microbiol.*, 4(14):4247–4255, 2008.
24. Terkuran, M., Erginkaya, Z., Ünal, E., Güran, M., Kızılyıldırım, S., Ugur, G. and Köksal, F., The relationship between virulence factors and vancomycin resistance among Enterococci collected from food and human samples in Southern Turkey. *Ankara Üniversitesi Veteriner Fakültesi Dergisi,* 61(2):133-140, 2014.
25. Kashef, M., et al., Virulence factor and biofilm formation in clinical Enterococcal isolates of the west of Iran. *Jundishapur J. Microbiol.*, 10 (7), 2017.
26. Archimbaud, C., Shankar, N., Forestier, C., Baghdayan, A., Gilmore, M. S., Charbonne, F., et al., In vitro adhesive properties and virulence factors of Enterococcus faecalis strains. *Res Microbiol*, 153:75-80, 2002.
27. Huycke, M. M. and Gilmore, M. S., Frequency of aggregation substance and cytolysin genes among enterococcal endocarditis isolates. *Plasmid. Sep*, 34(2):152-6, 1995.
28. Banerjee, T. and Shampa, A., Prevalence of Virulence Factors and Drug Resistance in Clinical Isolates of Enterococci: A Study from North India. *Hindawi Publishing Corporation Journal of Pathogens* Volume, Article ID 692612, 7 pages, 2015.
29. Gaspar, F. B., Crespo, M. T. B. and Lopes, M. F. S., Proposal for a reliable enterococcal cytolysin production assay avoiding apparent incongruence between phenotype and genotype. *J Med Microbiol*, 58:1122-1124, 2009.
30. Medeiros, A.W., Pereira, R.I., Oliveira, D.V., et al., Molecular detection of virulence factors among food and clinical Enterococcus faecalis strains in South Brazil. *Braz J Microbiol.*, 45:327–32, 2014.
31. Cosentino, S., Podda, G. S., Corda, A., Fadda, M. E., Deplano, M. and Pisano, M. B., Molecular detection of virulence factors and antibiotic resistance pattern in clinical Enterococcus faecalis strains in Sardinia. *J Prev Med Hyg.*, 51:31–6, 2010.
32. Walters C. M., Antiporta M. H., Murray B. E. and Dunny G. M., Role of the Enterococcus faecalis GelE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. *J Bacteriol*, 185:3613-3623, 2003.
33. Su, Y. A., Sulavik, M. C., He, P., Makinen, K. K., Makinen, P., Fiedler S., Wirth, R. and Clewell, D. B.: Nucleotide sequence of the gelatinase gene (gelE) from Enterococcus faecalis subsp. liquefaciens. *Infect. Immun.*, 59:415–420, 1991.
34. Qin, X., Singh, K. V., Weinstock, G. M. and Murray, B. E., Characterization of fsr, a regulator controlling expression of gelatinase and serine protease in Enterococcus faecalis OG1RF. *J. Bacteriol.*, 183:3372–3382, 2001.
35. Coque, T. M., Patterson, J. E., Steckelberg, J. M. and Murray, B. E., Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalised and community-based persons. *J. Infects. Dis.*, 1715:1223–1229, 1995.
36. Tsikrikonis, G., Maniatis, A. N., Labrou, M., Ntokou, E., Michail, G., Daponte, A, Stathopoulos, C; Tsakris, A and Pournaras, S., Differences in biofilm formation and virulence factors between clinical and fecal enterococcal isolates of human and animal origin. *Microb. Pathog.*, 52:336-343, 2012.
37. Creti, R., Imperi, M., Bertuccini, L., Faretti, F., Orefici, G. and Di Rosa, R., Survey of virulence determinants among Enterococcus faecalis isolated from different sources. *J Med Microbiol*, 53;13–20, 2004.
38. Lopes, M. F. S., Simões, A. N., Tenreiro, R., Marques, J. J. and Crespo M. T. B., Activity and expression of a virulence factor, gelatinase, in dairy enterococci. *Int J Food Microbiol*, 112:208-214, 2006.
39. Lindenstrau, A. G., Pavlovic, M., Bringmann, A., Behr, J., Ehrmann M. A. and Vogel, R. F., Comparison of genotypic and phenotypic cluster analyses of virulence determinants and possible role of CRISPR elements towards their incidence in Enterococcus faecalis and Enterococcus faecium. *Syst Appl Microbiol*, 34:553-560, 2011.