Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

Investigation of the relationship between virulence factors and antibiotic resistance of *Enterococci* isolates

Umut Safiye Say Coskun*

Department of Medical Microbiology, Medicine Faculty, Tokat Gaziosmanpasa University, Tokat, Turkey

Correspondence to: umut.saycoskun@gop.edu.tr

Received January 4, 2019; **Accepted** February 8, 2019; **Published** February 28, 2019

Doi: http://dx.doi.org/10.14715/cmb/2019.65.2.3

Abstract: The aim of this study was to determine the relationship between aggregation factor (*asa1*), enterococcal surface protein (*esp*), cytolysin (*cyl*), gelatinase (*gelE*), hyaluronidase (*hyl*) virulence factors and antibiotic resistance in *Enterococci*. VITEK 2 ID system was used to identify the isolates and determine their antibiotic susceptibility. Virulence genes were investigated by polymerase chain reaction. Of the 93 isolates, 62 (66 %) were *Enterococcus faecium*, 31 (44 %) were *Enterococcus faecalis (E. faecialis)*. *E. faecium* isolates were more resistant to ampicillin, ciprofloxacin, linezolid, teicoplanin and vancomycin than *E. faecalis*. High-level gentamycin *rate were higher in E. faecium than E. faecalis* (p <0.05). The most prevalant virulence genes were *esp* (60.9 %) and *asa1* (25 %) followed by *gelE* (22.8 %), *cyl* (16.3 %) and *hyl* (8.7 %). *Asa1*, *cyl*, *gelE* genes positivity were higer in *E. faecalis* than *E. faecium*. *Hyl* positivity was higher in *E. faecalis* than *E. faecium* isolates. Ampicillin resistance was higher in *gelE* positive *E. faecalis* than *gelE* negative *E. faecalis* (p <0.05). Ciprofloxacin resistance was higher in *gelE* negative *E. faecalis* than *gelE* positive *E. faecalis* (p <0.05). *Asa*, *cyl*, *hyl*, *gelE* positive *E. faecium* isolates were more susceptible to teicoplanin than the isolates that did not have these genes (p <0.05). *Cyl*, *asa*, *gelE* positive *E. faecalis* isolates were more susceptible to vancomycin than *cyl*, *asa*, *gelE* negative *E. faecalis* isoates (p <0.05). *Hyl* positive *E. faecium* isolates were more susceptible to vancomycin than *hyl* negative *E. faecium* isolates (p <0.05). *E. faecalis* isolates that have virulence genes were more susceptible to vancomycin (p <0.05). The resistance to antibiotics in *E. faecalis* should be a concern for the treatment of infectious disease.

Key words: Antibiotic resistance; *E. faecalis*. *E. faecium*; Vancomycin; Virulence genes.

Introduction

Recently *Enterococci* have received much attention as a nosocomial infectious agent in patients undergoing anti-microbial therapy (1). They are intrinsically resistant to many antibiotics. *Enterococci* are able to acquire drug resistance either by chromosome, transfer of plasmid or transposon. Another important feature is the ability to transfer genetic materials to other bacteria (2).

Virulence factors are features or molecules that are produced by pathogens that help these pathogens with colonization and immunoevasion. The production of virulence factors leads to infection. A*ggregation factor (asa1)*, e*nterococcal surface protein (esp)* (4)*, gelatinase (gelE)* (3,5)*, cytolysin (cyl)* (6) and *hyaluronidase* (*hyl)* (7) are among the enterococcal virulence factors.

The purpose of this study was to determine the relationship between virulence factors and antibiotic resistance in *Enterococci* isolates.

Materials and Methods

Bacterial isolates and antimicrobial susceptibility testing

This study included 93 *Enterococci* (62 *E. faecium*, 31 *E. faecalis*) from various specimens (wound (49.5 %), urine (24.7 %), blood (18.3 %), respiratory samples (6.4 %), etc.) sent to the Microbiology Laboratory at Tokat Gaziosmanpasa University between January 2016 and February 2017. Identification of isolates and antibiotic susceptibility were evaluated with the VITEK 2 ID (bioMérieux, France) automated system according to Clinical and Laboratory Standards Institute (8).

DNA isolation and analysis of virulence genes by Polymerase Chain Reaction

DNA isolation was performed according to the supplier's recommendation with MagCore Genomic DNA Bacterial Kit by Magnesia 16 isolation device (Anatolia Geneworks Turkey). *Esp*, *cyl*, *asa1*, *gelE*, *hyl* genes were investigated with Accustart II PCR ToughMix (Quanta Biosciences, Inc. Gaithersburg, MD) kit by conventional polymerase chain reaction (PCR) (Montania 4896 Anatolia Geneworks /Turkey). The primers were used according to the previous studies: *asa1*, *esp*, *cyl* (9), *hyl* (10) ,*gelE* (11). Amplification for PCR products were done as follows: initial denaturation step at 95°C for 2 min followed by 4 cycles consisting of denaturation (95°C for 20 seconds), annealing (36°C for 4 minutes), and extension (58°C for 10 seconds), and extension (72°C for 20 seconds) for 45 cycles, final extension step at 72°C for 5 minutes. All PCR results were analyzed on 1% agarose containing 0.5 µg/mL ethidium bromide and were subsequently visualized under UV light. The gel images of virulence genes are shown in "Figure 1".

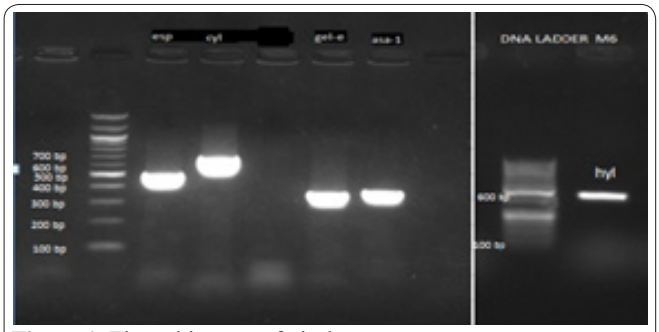

Figure 1. The gel images of virulence genes.

Statistical analysis

Statistical analysis was performed using commercial software IBM SPSS Statistics 20 (SPSS Inc., an IBM Co., Somers, NY).The difference between resistance to antibiotics and virulence genes was investigated with independent samples t-test. The statistical significance level of p was 0.05.

Ethical information

This study was approved by Tokat Gaziosmanpasa University Clinical Research Ethics Committee (18-KAEK-185).

Results

Antibiotic resistance rate of *Enterococci* isolates

Overall, 47.4 % (45/93) of the *Enterococci* was resistant to vancomycin. Of these, 95.6 % (43/45) were *E. faecium* and 4.4 % (2/45) were *E. faecalis*. High-level gentamicin resistance (HLGR) in *E. faecalis* isolates were 19.4 % and in *E. faecium* isolates were 48.4 %. *E. faecium* isolates were more resistant to ampicillin, ciprofloxacin, linezolid, teicoplanin and vancomycin than *E. faecalis*. Also, high-level gentamycin resistance rate was higher in *E. faecium* than *E. faecalis* ($p < 0.05$). Antibiotic resistance rate of *Enterococci* isolates is shown in "Table 1".

Frequency of virulence genes

The virulence genes positivity was *esp* (60.9 %), *asa1* (25 %), *gelE* (22.8 %), *cyl* (16.3 %) and *hyl* (8.7 %). The most prevalant virulence genes were *esp* (60.9 %) and *asa1* (% 25), followed by *gelE* (22.8 %), *cyl* (16.3 %) and *hyl* (8.7 %). *Asa1, cyl, gelE* genes positivity was higer in *E. faecalis* than *E. faecium*. Also, *hyl* genes positivity was higher in *E. faecium* than *E. faecalis* ($p < 0.05$).

Table 1. Antibiotic susceptibility rate of *Enterococci* isolates.

Antibiotics	Isolates	Susceptible (n)	%
Ampicillin	*E. faecium*	7	11.3
	E. faecalis	27	87.1
Ciprofloxacin	*E. faecium*	5	8.1
	E. faecalis	18	58.1
Linezolid	*E. faecium*	54	87.1
	E. faecalis	29	93.5
Teicoplanin	*E. faecium*	19	30.6
	E. faecalis	29	93.5
Vancomycin	*E. faecium*	19	30.6
	E. faecalis	29	93.5

Association of antibiotic resistance and virulence genes

Ampicillin resistance was higher in *gelE* positive *E. faecalis* than *gelE* negative *E. faecalis* ($p < 0.05$). No relationship was found between ampicillin resistance and *asa1*, *esp*, *cyl*, *hyl* genes in any of the isolates ($p > 0.05$). Ciprofloxacin resistance was higher in *gelE* negative *E. faecalis* than *gelE* positive *E. faecalis* ($p < 0.05$) but no relationship with other genes ($p > 0.05$). Ciprofloxacin susceptibility was higher in *esp* positive *E. faecium* than *esp* negative *E. faecium* ($p < 0.05$), but no association was found between ciprofloxacin sensitivity and *asa1*, *cyl*, *hyl*, *gelE* genes ($p > 0.05$). *Asa1*, *cyl*, *hyl* and *gelE* positive *E. faecium* isolates were more susceptible to teicoplanin than the isolates that do not have these genes ($p < 0.05$). However, no relationship was found between teicoplanine resistance and esp ($p > 0.05$). *Cyl*, *asa1*, *gelE* positive *E. faecalis* isolates were more susceptible to vancomycin than *cyl*, *asa1*, *gelE* negative *E. faecalis* isoates ($p < 0.05$). *Hyl* positive *E. faecium* isolates were more susceptible to vancomycin than *hyl* negative *E. faecium* isolates ($p < 0.05$). In *E. faecium* isolates, there was no association between *esp, asa1, hyl, gelE* genes and vancomycin resistance ($p > 0.05$). In addition, there was no relationship between linezolid resistance and virulence genes ($p > 0.05$).

Discussion

High levels of antimicrobial-resistant *Enterococci* remains a global infection control challenge and an important cause of healthcare-associated infections. Vancomycin has been used as the agent of choice in the treatment of *Enterococci* infections. There has been an increase of vancomycin resistant *Enterococci* infection in recent years. This situation has posed a serious problem in the treatment of enterococcal infections. In addition, *Enterococci* can transfer resistant genes horizontally to other vancomycin-susceptible isolates (12).

World Health Organization reported vancomycin-resistant *E. faecium* as a pathogen with high priority in its global priority list of antibiotic-resistant bacteria, drawing attention to the paucity of appropriate and effective treatment options. The percentage for vancomycin resistance in *E. faecium* was 11.8 % in 2016. National percentages ranged from 0 to 46.3 %. However, reported cases of resistance to vancomycin have shown significantly increasing trends fort the last four years (13)

In this study, consistent with the previous studies, *E. faecium* isolates were more resistant to many antibiotics and had more HLGR than *E. faecalis* isolates (13-15). Although the regional differences in vancomycin resistance were observed, the ratio was 6.45-45.1 % (14-18). In this study, 47.4 % (45/93) of the *Enterococci* were resistant to vancomycin. High vancomycin resistance in our region may be caused by the widespread use of vancomycin.

Asa1 contributes conjugation by directing bacterial aggregation, emerging in close cell contact between donor and recipient (3). *Esp* allows *E. faecalis* isolates to colonize in the urinary tract (4). Heidari et al.

detected *asa1* was the most frequently detected gene (100 %) among the isolates followed by *esp* (94.1 %) (18). Baylan et al. indicated *asa1* and *esp* were the most frequent virulence factors, with the rates of 26.7 % and 25.6 % respectively in urinary samples. They have also detected that *asa1* gene positive *E. faecalis* isolates were more resistant to ciprofloxacin, norfloxacin and levofloxacin than *asa1* gene negative isolates; *esp* gene positive *E. faecalis* isolates were more resistant to doxycycline than *esp* gene negative isolates (15). Mete et al. observed the most common virulence genes were *asa1* gene 45 % and *esp* gene 32.3 %. The *esp* gene level in vancomycin resistant *E. faecium* isolates was found to be 24 %, while no *esp* gene was found in vancomycin resistant *E. faecalis* isolates. The existence of *asa1* was negative in both vancomycin resistant *E. faecium* and vancomycin resistant *E. faecalis* isolates (16). In this study, consistent with the previous study, the most common virulence genes were *esp* 60.9%, followed by *asa1* 25 %. Also, *asa1* positivty in isolates of *E. faecalis* was significantly higher than *E. faecium*. The *asa1* positivity in vancomycin susceptible *E. faecalis* was significantly higher than resistant isolates. Also, both vancomycin resistance and susceptible enterococci isolates have *esp* and *asa1* genes.

It was determined gelatinase enzymatic activity is a prerequisite for biofilm formation (19). In previous studies, *gelE* positivity varies between 19.6 % and 80.4 % (14,18,20,21). In this study *gelE* gene positivity was observed at 22.8 %, and ampicilin resistance in *gelE* gene positive *E. faecalis* was significantly higher compared to isolates that did not contain these genes. In addition, *gelE* positive *E. faecalis* isolates *were* significantly more susceptible to ciprofloxacin, teicoplanin and vancomycin than compared to isolates that did not involve these genes.

The other virulence factors are cytolysin and hyaluroidase. Cytolysin lyses macrophages and neutrophils, and causes them to escape immunity (6). Hyaluronidase is a degradative enzyme associated with tissue damage (7). Heidari et al. indicated *cyl* 64.7 % and *hyl* 51% (8), and Triveda et al. detected *hyl* positivity was 36.85 % (5). Mete et al. observed *cyl* positivity was 33.2 %; also, *hyl* was found 42.3 % in *E. faecalis* and 19.3 % in *E. faecium.* In addition, they recorded ciprofloxacin resistance in *cyl* gene positive *E. faecalis* was significantly higher compared to isolates that did not contain these genes. Moreover, *hyl* positive *E. faecium* isolates were significantly more resistant to vancomycin compared to isolates that did not have *hyl* gene (16).

Baylan et al. observed *hyl* activity was higher in *E. faecalis than E. faecium* (15). In this study, teicoplanin susceptibility in *cyl and hyl* genes positive isolates of *E. faecalis* was significantly higher compared to isolates that did not contain these genes. Also, the *cyl* positivity in vancomycin susceptible *E. faecalis* and *hyl* positivity in vancomycin susceptible *E. faecium* were significantly higher than resistant isolates. Consistent with Baylan et al.'s study *cyl* positive *E. faecalis* and *hyl* positive *E. faecium* isolates were more susceptible to vancomycin than negative isolates in this study. It is noteworthy that virulence factors are more prevalent in isolates that are sensitive to antibiotics. According to Beceiro et al., the correlation between antibiotic resistance and virulence follows a Darwinian model. Increased resistance and virulence finally proceed together to confer the bacteria with a selected advantage (22).

In conclusion, *E. faecium* isolates were found to be more resistant to antibiotics than *E. faecalis* isolates. However, *E. faecalis* isolates that have virulence genes were more susceptible to vancomycin. Therefore, in the future, the resistance to vancomycin in *E. faecalis* should be a concern for the treatment of infectious disease.

Acknowledgments

I would like to thank to Yelda Dagcıoglu for her help in the PCR stage of the study.

References

1. Schaberg DR, Culver DH, Gaynes RP. Major trends in microbial aetiology of nosocomial infection. Am J Med 1991; 91(3): 72-5.
2. Mollering RC Jr. Emergence of Enterococcus as significant pathogen. Clin Infect Dis 1992; 14: 1173-8.
3. Olmsted SB, Kao SM, van Putte LJ, Gallo JC, Dunny GM. Role of the pheromone-inducible surface protein Asc10 in mating aggregate formation and conjugal transfer of the Enterococcus faecalis plasmid pcf10. J Bacteriol 1991; 173: 7665–72.
4. Shankar N, Lockatell CV, Baghdayan AS, Drachenberg C, Gilmore MS, Johnson DE. Role of Enterococcus faecalis surface protein Esp in the pathogenesis of ascending urinary tract infection. Infect Immun 2001; 69: 4366–72.
5. Triveda L, Gomathi. Comparative Study for the Detection of Virulence Factors in Clinical and Commensal Isolates of Enterococcus Species. IJHSR 2016; 6(3): 113-7.
6. Miyazaki S, Ohno A, Kobayashi I, Uji T, Yamaguchi K, Goto S. Cytotoxic effect of hemolytic culture supernatant from Enterococcus faecalis on mouse polymorphonuclear neutrophils and macrophages. Microbiol Immunol 1993; 37(4): 265-70.
7. Kayaoglu G, Ørstavik D. Virulence factors of Enterococcus faecalis: relationship to endodontic disease. Crit Rev Oral Biol Med 2004; 15: 308–20.
8. Clinical and Laboratory Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twentyfifth Informational Supplement. Document M100-S23. Clinical and Laboratory Standards Institute, 2013. 2. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 3.1, 2013.
9. Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R et al. 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. J Clin Microbiol 2004; 42(10): 4473-9.
10. Soheili S, Ghafourian S, Sekawi Z, Neela V, Sadeghifard N, Ramli R at al. Wide Distribution of Virulence Genes among Enterococcus faecium and Enterococcus faecalis Clinical Isolates. Scientific World Journal 2014; 2014: 623174. DOI: 10.1155/2014/623174.
11. Al-Talib H, Zuraina N, Kamarudin B, Yean CY. Genotypic Variations of Virulent Genes in Enterococcus faecium and Enterococcus faecalis Isolated from Three Hospitals in Malaysia* Adv Clin Exp Med 2015: 24(1); 121–7.
12. Pearson H. 'Superbug' hurdles key drug barrier. Nature 2002; 418(6897): 469.
13. European Centre for Disease Prevention and Control. Surveillance of antimicrobial resistance in Europe 2016. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm: ECDC; 2017.

14. Shokoohizadeh L, Ekrami A, Labibzadeh M, Ali L, Alavi SM. Antimicrobial resistance patterns and virulence factors of enterococci isolates in hospitalized burn patients. BMC Res Notes 2018; 11(1): 1 DOI:10.1186/s13104-017-3088-5

15. Baylan O, Nazik H, Bektore B, Citil BE, Turan D, Betigul Ongen B at al.The Relationship Between Antibiotic Resistance and Virulence Factors in UrinaryEnterococcus Isolates. Mikrobiyol Bul 2011; 45(3): 430-45.

16. Mete E, Kaleli İ, Cevahir N, Demir M, Akkaya Y, Satılmış OK. Mikrobiyol Bul 2017; 51(2): 101-14.

17. Tuhina B, Anupurba S, Karuna T. Emergence of antimicrobial resistance and virulence factors among the unusual species of enterococci, from North India. Indian J Pathol Microbiol 2016; 59(1): 50-5.

18. Heidari H, Hasanpour S, Ebrahim-Saraie HS, Motamedifar M. High Incidence of Virulence Factors among Clinical Enterococcus faecalis Isolates in Southwestern. Infect Chemother 2017; 49(1): 51-6.

19. Hancock LE, Perego M. Systematic inactivation and phenotypic characterization of two-component signal transduction systems of Enterococcus faecalis V583. J Bacteriol 2004; 186: 7951–8.

20. Saffari F, Dalfardi MS, Mansouri S, Ahmadrajabi R. Survey for Correlation between Biofilm Formation and Virulence Determinants in a Collection of Pathogenic and Fecal Enterococcus faecalis Isolates Infect Chemother 2017; 49(3): 176–83.

21. Kashef M, Alvandi A, Hasanvand B, Azizi M, Abiri R. Virulence Factor and Biofilm Formation in Clinical Enterococcal Isolates of the West of Iran. Jundishapur J Microbiol 2017 ; 10(7): e14379. DOI:10.5812/jjm.14379.

Beceiro A, Tomás M, Bou G. Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world?. Clin Microbiol Rev 2013; 26(2): 185-230.