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The antibiotic resistance, gelatinase production and biofilm formation among *Enterococcus* strains - the correlation analysis using PCR techniques

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Abstract: *Enterococcus* spp. are one of the most frequently detected Gram-positive bacteria in the human intestinal flora. *Enterococcus* strains are known for its resistance to antibiotics and ability to biofilm formation. These features are the cause of its success in colonization of hospital areas. We focused on analysing whether clinical strains of *Enterococcus faecalis* and *Enterococcus faecium* showed resistance to vancomycin and teicoplanin, gelatinase production, and the ability to form biofilm. Methods of classical microbiology, as well as molecular biology techniques were used to determine these features. Our studies determinined the correlation between antibiotic resistance and the *vanA* and *vanB* genes and the co-occurrence of gelatinase production and biofilm formation and *gelE* genes.

Keywords: *Enterococcus spp.*; antibiotic resistance; biofilm; gelatinase; PCR

Citation: Pecyna, P.; Jaworska M.M.; Rosochowicz, M.; Ostapowicz, J.; Lipowicz, J.; Karwacka, M.; Wiacek, P.; Szymanowicz, O.; Gajecka, M. The antibiotic resistance, gelatinase production and biofilm formation among *Enterococcus* strains - the correlation analysis using PCR techniques. *Proceedings* **2021**, *68*, x. https://doi.org/10.3390/xxxxx

Published: date

1. Introduction

Enterococcus spp. are one of the most frequently detected Gram-positive bacteria of the human gut [1]. They are responsible for major cases of healthcare-associated infections (HAIs) and affect mostly elderly patients who have been hospitalized for long periods and received broad-spectrum antibiotics [2].

Enterococcus spp. are the cause of urinary tract infections (UTI), endocarditis, as well as bacteriemia and surgical site infections [3,4]. Although many species have been isolated, *Enterococcus faecalis* and *Enterococcus faecium* are responsible for the majority of infections and both are able to develop resistance to antibiotics - vancomycin, teicoplanin and gentamicin . Enteroccoci also produce gelatinase enzyme and form a biofilm, which increases virulence of those strains [5,6]. The drug resistance is a serious clinical problem and can lead to transmission of antibiotic resistant genes [3]. According to published report patients with VRE (Vancomycin Resistant Enteroccocus) infection had significantly greater risk of mortality than patients with VSE (Vancomycin Susceptible Enteroccocus) infections, moreover time of hospitalization was longer in patients with VRE infections than in those with bacteria without this resistance mechanisms[7]. It is essential to identify genes responsible for virulence factors that help Enteroccocal strains persist and spread in the hospital environment. Current literature focuses on determining the correlation between antibiotic resistance and the *vanA* and *vanB* genes, which was investigated in this experiment [8, 9]. The co-occurrence of

gelatinase production and biofilm formation and *gelE* genes was also tested. The purpose of our research was to analyze presence of resistance to vancomycin and teicoplanin, the gelatinase production and the ability to biofilm formation among clinical strains of *Enterococcus* spp. using both classical microbiology and molecular biology techniques.

2. Materials and methods

Bacterial DNA from rectum smear samples collected from 56 hospitalized patients were isolated using a heat-shock method in the TE buffer. Then, bacterial genetic material was amplified using PCR with appropriately designed primers. The samples were checked for presence of the genes responsible for antibiotics resistance (*vanA*, *vanB*), gelatinase production (*gelE*) and biofilm formation (*esp*, *fsrA*, *fsrB*). Results obtained from molecular biology techniques were compared with results of classical microbiology methods. Statistical comparisons were performed with ANOVA Kruskal-Wallis test and chi-squared test with Fisher's exact. Statistically significant results were indicated by $p<0.05$. All statistical analyses were performed with MedCalc (version 19.8 for Windows; MedCalc Software Ltd, Belgium).

3. Results

Study results showed that 42 of 56 *Enterococcus* strains possessed gene *vanA*+ (75%), 10 were *vanB*+ (18%) strains and four strains were indeterminate with used projected starters (7%). Moreover, in 19 of all strains (34%) genes responsible for biofilm formation were observed. Presence of the rest studied genes among *Enterococcus faecalis* and *Enterococcus faecium* strains are presented on Figure 1.

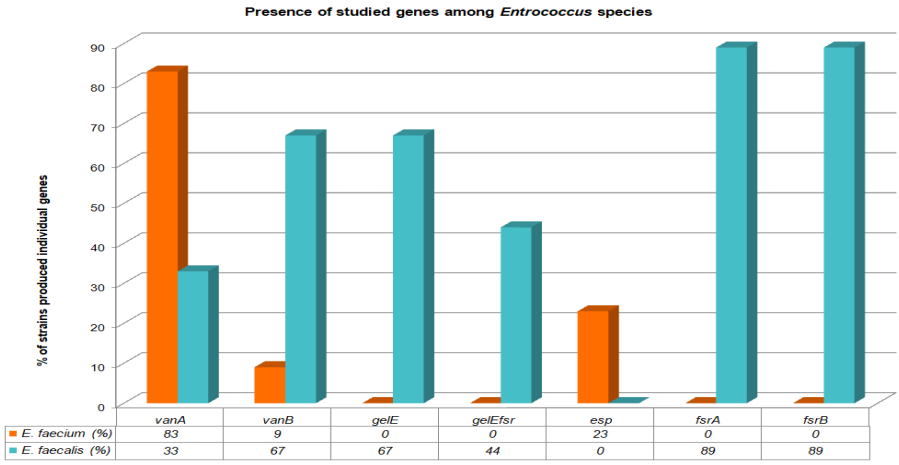

Figure 1. Presence of studied genes among *Enterococcus* species

In all statistical tests performed, a significant relationship between the bacteria resistance, biofilm formation and gelatinase production phenotype and PCR results were found. Among the tested strains, there was a strong correlation between biofilm formation and the presence of genes (*esp*, *fsr)* responsible for production of this virulence factor (Fig. 2).

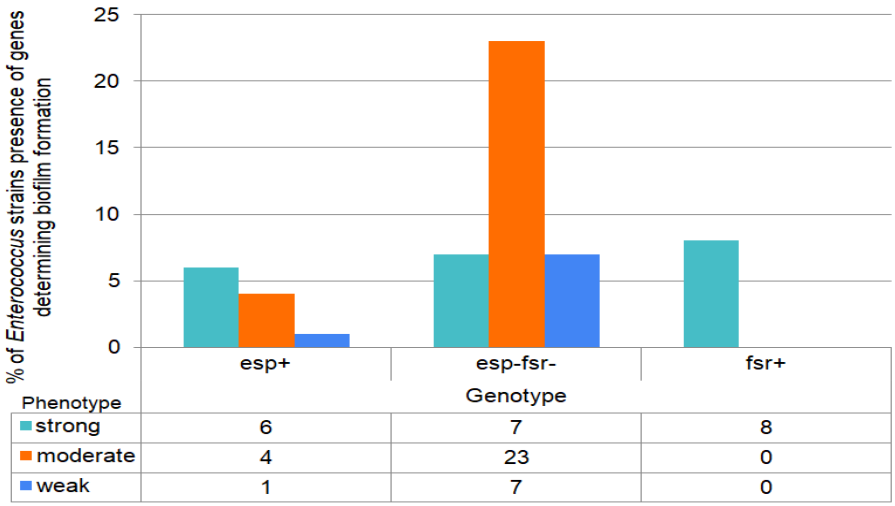

Figure 2. Correlation between biofilm formation genotype and phenotype

What is more, none of the strains tested had simultaneously expressed both of analyzed biofilm genes. In the study bacteria expressed only *fsr* gene, *esp* gene or none of them, suggesting that *fsr* and *esp* genes are mutually exclusive. The strains with *fsr* gene presence, had the ability to form a stronger biofilm structure phenotypically.

Similarly, there was a correlation between the presence of the *gelE* gene and the ability of strains to produce gelatinase (Fig. 3).

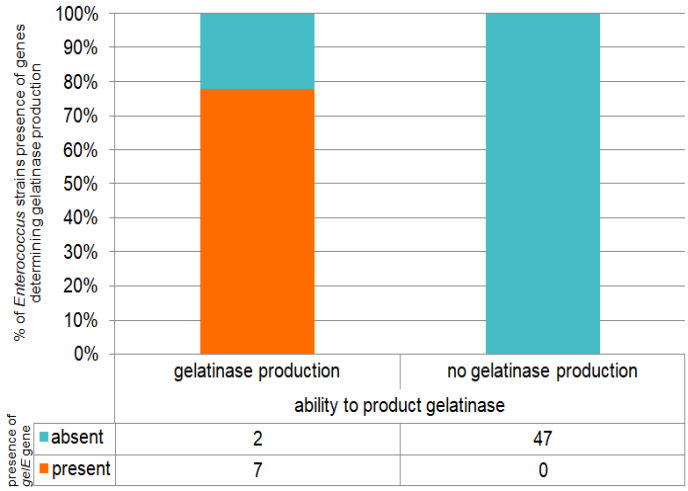

Figure 3. Correlation between ability to produce gelatinase and presence of *gelE* gene among *Enterococcus* strains.

Interestingly, genes responsible for biofilm formation occurred more frequently in *vanB*+ strains (Table 1). Similar result was obtained in case of gelatinase production (Fig. 4).

Table 1. Correlation between biofilm formation and vancomycin and teicoplanin resistance

Genes responsible for biofilm formation	Genes responsible for vancomycin and teicoplanin resistance		Total
	vanA+	*vanB*+	
esp+	9	1	10 (19.2%)
esp-fsr-	31	3	34 (65.4%)
fsr+	2	6	8 (15.4%)
Total	42 (80.8%)	10 (19.2%)	52 (100%)

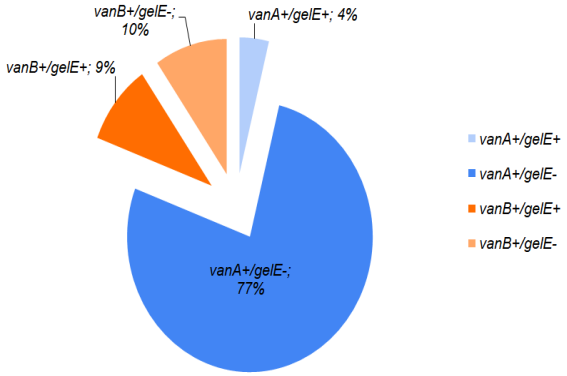

Figure 4. Correlation between gelatinase production and antibiotics resistance

Among the tested strains, gelatinase was more frequently produced by strains that possessed phenotype *vanB*+.

4. Discussion

The aim of this study was to perform analysis of *Enterococcus* spp. clinical strains using both classical and molecular techniques in order to examine their resistance to vancomycin and teicoplanin as well as their ability to produce gelatinase and to form biofilm.

Among 56 strains tested, a substantial majority had a *vanA* phenotype (75%), whereas 18% of the strains were *vanB* positive and only 4 of all strains were classified as indeterminate (7%). Yadav et al. analyzed antibiotic resistance and biofilm formation in *Enterococcal* clinical isolates from urine, pus, blood, genital swabs and other sources. Among 14 VRE isolates, *vanA* type was more frequent (78.5%) and *vanB* was less prominent (21.4%) [10]. In study performed by Das et. al. in 2020 also *vanA* phenotype was prevailing [11]. Among 103 *Enterococcus* spp. strains isolated from the urinary tract, 14 isolates had *vanA* phenotype and only 5 isolates appeared to be *vanB* type [11]. The results of this study are in agreement with those of the study by Farman et al. in 2019 and by Papadimitriou-Olivgeris et al. In 2015 [12,13]. It is worth noting that in our study all isolates were acquired from clinical sources and were classified as vancomycin resistant. However, the proportion of *vanA* and *vanB* genes among VRE strains analyzed in different studies remains similar.

We observed 48 of 56 strains (86%) produced a strong or moderate biofilm *in vitro*, yet only 19 of 56 strains (34%) expressed genes responsible for biofilm formation. This supports previous findings of Biswas et al. and Mohamed et al. that the number of

strains positive for biofilm formation by PCR is smaller than the number of strains showing the ability to form biofilm by phenotypic test [14,15]. However, contrary to our results, Toledo-Arana et al. reported in 2001 that all esp-negative *E. faecalis* strains were unable to produce a biofilm [16].

In all *Enterococcus* spp. strains tested a strong correlation between biofilm forming and the presence of *fsrA* and *fsrB* or *esp* genes was confirmed. In all cases, the *fsrA* gene was expressed concurrently with the *fsrB* gene, which is why we refer to *fsrA* and *fsrB* positive stains as *fsr* positive. In our study most biofilm forming *Enteroccoci* expressed *esp* gene (20%) and *fsr* genes were detected only in 8 strains (14%). Our results are in good agreement with those reported by Goudarzi et al. in 2018 . Among 16 *E. faecium* biofilm-producing isolates from stool, 14 strains were *esp* positive and only 1 strain was *fsr* positive [17].

The vast majority of studied *Enterococcus* spp. strains formed strong or moderate biofilm, 38% and 48% respectively. Interestingly, a stronger biofilm was observed in strains with *fsr* gene expression. Moreover, we found much lower values for weak biofilm producers (14%) to those reported by Haghi et al. in 2019 (86%) [18]. As reported by Das et al., the evidence we found points to the dominance of moderate biofilm producers among *Enterococcus* spp. [11].

As far as we know this is the first time that a negative correlation between *fsr* and *esp* genes was found. In our study bacteria which expressed *fsr* gene were *esp* negative. Though, the possible mutual exclusivity of *esp* and *fsr* genes should be further validated.

Additionally, we confirmed that there is a correlation between the presence of the *gelE* gene and the ability of strains to produce gelatinase. The *gelE* gene was detected in seven out of 56 strains (12,5%) and 9 out of 56 strains (16%) were confirmed to produce gelatinase phenotypically. The values are barely distinguishable from those published by Sun et al. (16.7%) [19]. Gen *fsr* is known to regulate *gelE* expression [15] and in our study all *gelE* positive strains were *fsr* positive (data not shown). However, single strain was *fsr* positive and *gelE* negative. Klibi et al. demonstrated that an intact *fsr* locus and *gelE* gene are both crucial for gelatinase production showing that almost all strains with *gelE+, fsrB-* genotype were not classified phenotypically as gelatinase producers [20]. By using classical methods, the *gelE-,fsr+* strain was confirmed to produce gelatinase, which indicates a possibility of mutation in *gelE* gene and should be additionally examined for instance by sequencing.

5. Conclusion

Study analysis showed a correlation between vancomycin and teicoplanin resistance, ability of biofilm formation and gelatinase production in *Enterococcus* spp., particularly based on results for strains, defined as *vanB+* where biofilm and gelatinase production were more frequent. This relationship is important because it generates a real clinical risk, especially in the context of hospital-acquired infections.

The significance of our study is noticeable as it shows a strong relationship between *Enterococcus* spp. antibiotic resistance and presence of virulence factors such as biofilm formation and gelatinase production on both phenotype and genotype level. However, performing analyses presented herein on a greater group of strains coming from different clinical sources should be considered in the future.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, P.P., M.J., M.R., M.G; methodology, P.P., M.J.; validation, P.P., M.J., M.R., J.O., J.L., M.K, P.W., O.S.; investigation, P.P., M.J., M.R., J.O., J.L., M.K, P.W.; data curation, P.P., M.J., M.R., J.O.; writing—original draft preparation, P.P., M.J., M.R., J.O., J.L., M.K, P.W.; writing—review and editing, P.P., M.J., M.R.; visualization, M.G.; supervision, M.G.; project administration, P.P., M.J.; funding acquisition, P.P., M.J.. All authors have read and agreed to the published

version of the manuscript."Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

Funding: This research was funded by PUMS, grant no. 502-05-33014020-10254

Institutional Review Board Statement: Not applicable

Informed Consent Statement: Not applicable

Data Availability Statement: Not applicable

Acknowledgments: We are very grateful fo Anna Matysiak for collection of clinical strains of *Enterococcus* species.

Conflicts of Interest: The authors declare no conflict of interest.

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