Comparison of antibiotic resistance and virulence in vancomycin-susceptible and vancomycin-resistant Enterococcus faecium strains

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ABSTRACT

Aim. Today, infections caused by vancomycin-resistant Enterococcus faecium (VRE) are a major problem in the healthcare system. The aim of this study was to compare the antibiotic resistance and virulence traits between vancomycin-susceptible E. faecium (VSE) and VRE clinical isolates.

Material and Methods. Studies were performed on 66 E. faecium (32 VRE and 34 VSE) strains. Susceptibility testing and identification were performed, and strains were examined for β-lactamase, hemolysin and biofilm production. Isolates were tested for the presence of 5 van genes, 8 virulence genes and 6 aminoglycoside-modifying enzyme (AME) genes. Obtained amplicons were subjected to electrophoretical separation and DNA sequencing.

Results. Among 32 VRE isolates, 28 were found to have the VanA phenotype, and 4 the VanB. The most frequent resistance and virulence profile among VRE strains was resistance to ampicillin, imipenem, gentamicin, streptomycin, teicoplanin, and vancomycin with enterococcal surface protein (esp), endocarditis antigen (efaA), collagen adhesin (acm), and hialuronidase (hyl) genes; among VSE: resistance to ampicillin, imipenem, gentamicin, streptomycin with esp, efaA, acm, and hyl genes.

Conclusions. Our findings prove that both VRE and VSE strains were well equipped with virulence and resistance genes, although VRE strains were characterized by a greater variety and a higher number of these genes. However, statistical analysis revealed no significant differences between VSE and VRE strains (p > 0.05). Nevertheless, our results suggest that VRE strains may slowly acquire and incorporate resistance and virulence genes, due to their ability to survive in a hospital environment for a long time.

Keywords: Enterococcus faecium, VRE, VSE, resistance, virulence.

Introduction

Enterococcus are nowadays the fourth most common etiological factor in nosocomial infections in Europe [1]. Although these bacteria are natural inhabitants of the normal flora of the gastrointestinal and genitourinary tracts, they can lead to serious infections such as bacteriemia, endocarditis, infections of the urinary tract, and wounds [1–2]. For a long time, the majority of
infections were caused by *Enterococcus faecalis*. In the last few years, *Enterococcus faecium* have evolved as a common nosocomial pathogen and partially replaced *E. faecalis* as a cause of hospital-associated infections [3–4]. This change is related to the fact that *E. faecium* has a number of mechanisms of intrinsic resistance and is also able to acquire resistance by mutations or incorporation of genes located on plasmids, transposons, or integrons [3]. The largest threats are strains resistant to glycopeptides (VRE - vancomycin-resistant *Enterococcus*) [1, 5].

During the past 15 years the knowledge about genetic background and molecular mechanisms responsible for glycopeptide resistance has been increasing [5–6]. *Enterococcus* resistant to glycopeptides produce cell-wall precursors with decreased affinity for the drug, which prevents the antibiotic from blocking cell-wall synthesis [7]. Nowadays, there are ten known types of enterococcal resistance to glycopeptides: VanA, VanB, VanC, VanD, VanE, VanF, VanG, VanL, VanM and VanN [8–10]. VanA and VanB types occur most frequently; VanA is responsible for a high level of resistance to both vancomycin and teicoplanin, whereas VanB confers only a low level of resistance to vancomycin while susceptibility to teicoplanin is conserved. Other types are rarely found in *Enterococcus* species [10], van genes are most often located on transposons (e.g., vanA on Tn1546, vanB on Tn1549/Tn5382) or on plasmids, and they transfer between enterococcal isolates by plasmid conjugation or transposition [5, 7].

In the treatment of enterococcal infections, the use of a cell-wall active agent (β-lactam, glycopeptide) with an aminoglycoside results in synergistic antibacterial activity [11]. *E. faecium* has high-level resistance to many β-lactams as a consequence of modification of penicillin-binding proteins (PBP), or very rarely, by the production of a β-lactamase enzyme [15]. High-level aminoglycoside resistance (HLAR), caused by production of aminoglycoside-modifying enzymes (AMEs), makes therapy with aminoglycosides and β-lactams ineffective [11]. At present, over 70 such enzymes have been discovered; their genes are also located on mobile genetic elements and are widespread among *Enterococcus* [11–12]. Two of the most prevalent AME genes, *aac(6’)-Ie* and *aph(2’’)-Id*, encode a bifunctional 2’-phosphotransferase/6’-acetyltransferase that confers resistance to a broad spectrum of aminoglycosides [13–14]. Recently, new AMEs genes such as *aph(2’’)-Ib, aph(2’’)-Ic*, and *aph(2’’)-Id* have been discovered and they are responsible for high-level gentamicin resistance [11, 14]. Although no single enzyme can inactivate all available aminoglycosides, most VRE strains can produce multiple enzyme types and consequently have the HLAR phenotype [11, 14].

Additionally, *E. faecium* have the abilities to produce several virulence factors and to form strong biofilm structure [4, 15–17]. The most common virulence determinants are: cytolsyn (Cyl), endocarditis antigen (EfaA), enterococcal surface protein (Esp), aggregation substance (As), collagen adhesin (Acm), gelatinase (GeLE) and hialuronidase (Hyl) [4, 18]. Cyl is a toxin, encoded by an operon localized on a plasmid or chromosome, which shows haemolytic and bactericidal activity [17]. As, encoded by a plasmid as gene, causes binding to the host epithelium [19]. Acm (acm) and EfaA (efaA) have been identified as the main virulence factors connected with infective endocarditis [4, 15, 20]. Hyl (hyl) degrades hyaluronic acid and, consequently, is associated with tissue damage [21]. Esp (esp), and GeLE (gelE), a zinc metallaprotease, are involved in the process of biofilm formation [4, 18, 22].

The increasing role of *E. faecium*, especially VRE and HLAR strains, in nosocomial infections calls for constant monitoring of their susceptibility and virulence. Astonishingly, in the literature there are many conflicting reports about the levels of resistance and virulence among VRE isolates in comparison with susceptible strains (VSE - vancomycin-susceptible *Enterococcus*) [6, 13, 21, 23–26]. Moreover, the data about VRE infections in Poland are still very limited [13]. Additionally, it should be noted that many recent studies are based only on phenotypic observations, which have some known limitations and are not fully conclusive [27–29]. This prompted us to perform a study about the exact comparison of resistance and virulence traits between VRE and VSE clinical isolates with the use of molecular and phenotypic methods.

Materials and Methods

Strains
A total of 66 *E. faecium* strains (32 VRE and 34 VSE strains) isolated from November 2012 to May
2014 from hospitalized patients from various departments of the Medical University of Białystok Clinical Hospital, were investigated.

Most of the VRE strains were collected from the haematology (50%) and intensive care units (31.3%), and were isolated mostly from rectal swabs (56%), whereas VSE strains were gathered from intensive care (53%) and surgery (20.7%) units and were isolated from blood (29%) and wound swabs (18%).

Identification and susceptibility testing
The identification and susceptibility testing of study isolates were performed using the VITEK2 system (bioMerieux, France) according to the manufacturer’s guidelines using VITEK2 GP and AST-P516 cards, respectively. E. faecalis ATCC 29212 was used as a reference strain. Later, identification was confirmed by polymerase chain reaction (PCR) with primers targeted to ddl (d-Ala-d-Ala ligase) chromosomal genes [30].

β-lactamase production
Strains were tested for β-lactamase production by a chromogenic cephalosporinase method [31] using nitrocefin discs (OXOID, United Kingdom) as per the manufacturer’s instruction.

Hemolysin production
Hemolysin production was evaluated on Columbia blood agar supplemented with 5% sheep blood (OXOID) [32].

Biofilm production
Biofilm formation was determined using two methods: the tube method and the Congo red agar method as described previously [33–34]. Each experiment was repeated 3 times for each strain. Strains that demonstrated the ability to produce biofilm by both methods were considered as biofilm-positive isolates.

DNA extraction
Genomic DNA was extracted from overnight E. faecium cultures using a commercial kit (Genomic Mini Kit, A&A Biotechnology, Poland).

PCR detection of vancomycin resistance genes, virulence genes, and AME genes
A PCR reactions was used for vanA, vanB, vanC, vanD and vanE detection as described previously [35]. VRE Enterococcus faecalis ATCC 51299 was used as a positive control. Genes encoding virulence factors were investigated as described by Camargo [23], Zou [36] and Ozden Tuncer [37], revealing the presence of gelE, acm, esp, efaA, hyl and cylA, cylII, cylIII. PCR was also used to detect genes encoding the AMEs: aac(6')-le-aph(2'')-Ia, aph(2'')-Ib, aph(2'')-Id, aph(3'')-IIIa, ant(4')-Ia, and aph(2'')-Ic [38].

Sequencing
DNA sequencing was carried out on PCR products by GENOMED S.A. Company, Poland. The sequences were aligned and compared with reference sequences achieved using GenBank with the Basic Local Alignment Search Tool (BLAST) algorithm.

Statistical analysis
STATA 13.1 (StataCorp LP, USA) was used for statistical analysis. Differences in the prevalence of antibiotic resistance and virulence factors between VRE and VSE strains were assessed by the Chi-square test and Fisher’s exact test. Results with $p < 0.05$ were considered significant.

Results
Among 32 VRE strains, 28 were found to be resistant to both vancomycin and teicoplanin; 4 strains were resistant only to vancomycin. Therefore, multiplex PCR for detecting the vancomycin-resistant genes confirmed that those 28 strains had VanA phenotype and 4 strains the VanB phenotype. Both vanA and vanB genes were not detected in any of the tested isolates.

Resistance and virulence patterns among all VRE and VSE strains are shown in Table 1. VSE strains carried 2 or more of the virulence genes, whilst VRE isolates had at least 4 virulence genes. The most frequent antibiotic-resistance profile among VRE strains was AMP® IPM® CN® S® TEC® VA® (resistance to ampicillin, imipenem, gentamicin, streptomycin, teicoplanin, vancomycin), which was detected in 17 (53.1%) strains. Four (12.5%) of these strains had the following virulence genes: esp, efaA, acm, hyl and had the ability to form biofilm and hemolyze. The 2 most frequent resistance and virulence patterns of VSE isolates, which occurred in 7 (20.6%) strains, were
<table>
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AMP<sup>R</sup> IPM<sup>R</sup> CN<sup>R</sup> S<sup>R</sup> and AMP<sup>R</sup> IPM<sup>R</sup> S<sup>R</sup> with esp, efaA, acm, hyl genes. The highest resistance and virulence (resistance to 6 antibiotics and 6 virulence genes) were found in VRE strains. Table 1 shows that VRE isolates were also characterized by a greater variety of resistance and virulence patterns than VSE strains.

The exact comparison of antibiotic susceptibility between VRE and VSE isolates revealed that all (100%) strains showed the highest susceptibility to linezolid and tigecycline. VRE isolates were resistant to β-lactams, whereas, interestingly, two (5.9%) VSE isolates were found to be susceptible to ampicillin and one to imipenem. High-level gentamicin resistance (HLGR) was detected in 2 (5.9%) VSE and 4 (12.5%) VRE strains. High-level streptomycin resistance (HLSR) appeared more frequently in VSE strains (13 (38.2%) and 8 (25%), respectively), while high-level resistance to all aminoglycosides (HLAR) occurred more frequently in VRE strains (16 (47%) and 19 (59.4%), respectively). However, these differences were not statistically significant (p > 0.05).

This study demonstrates that aac(6')-le-aph(2'')-Ia and aph(3')-IIla genes occur more frequently than others (Table 1). The coexistence of these 2 genes was observed in 29 (85.3%) VSE and 29 (90.6%) VRE strains. Interestingly, one VRE strain carried 3 AME genes: aac(6')-le-aph(2'')-la, aph(3')-IIla, and aph(2'')-Ib. One VSE isolate and 3 (9.4%) VRE isolates had only aac(6')-le-aph(2'')-la gene. aph(3')-IIla gene alone was found in one VSE strain, and the remaining 3 (8.8%) VSE strains did not carry any aminoglycoside-resistant genes. However, no statistically significant differences were found between these two groups of E. faecium (p > 0.05). It should also be noted that newer AME genes such as aph(2'')-Ic, aph(2'')-Id, and ant(4')-la were not detected among our study isolates.

Hemolytic activity and biofilm-forming ability were similar between tested groups; α-hemolysis occurred in 29 (90.6%) VRE and 32 (94.1%) VSE strains; biofilm production in 24 (75%) VRE and 25 (73.5%) VSE strains. The ability to produce β-lactamase was not detected in any of the tested isolates. cylA, cylII, cylIIs and gelE genes were only detected in the case of VRE strains; 2 (6.3%) strains carried the Cyl genes and 3 (9.4%) strains - the gelE gene. All (100%) of the VSE and 28 (87.5%) VRE strains had the acm gene. Occurrence of efaA, esp and hyl genes were on similar levels: efaA was detected in 31 (91.2%) VSE and 30 (93.7%) VRE strains, esp in 30 (88.2%) and 26 (81.3%), and hyl in 30 (88.2%) and 25 (78.1%) strains, respectively. These differences were not statistically significant (p > 0.05).

**Discussion**

The present study focused on comparison of antibiotic resistance and virulence traits between VRE and VSE clinical isolates. Comparison of HLAR between VSE and VRE groups showed that more HLAR and HLGR strains were in the VRE group, and more HLSR strains in the VSE group, although these differences were not statistically significant. This is in accordance with Baldir [39] who also did not find a significant difference in HLGR and HLSR rates among VRE and VSE strains, but did find that the HLAR phenotype occurred significantly more often in VRE isolates. In another study [26], all three aminoglycoside-resistant phenotypes occurred significantly more frequently among VRE strains. Likewise, Tripathi [40] revealed that resistance to gentamicin prevailed in VRE isolates. Differences between these results may indicate that the resistance to vancomycin does not always correlate with resistance to aminoglycosides, and determination of HLAR among E. faecium strains must always be performed.

We have demonstrated that the bifunctional enzyme coding gene aac(6')-le-aph(2'')-Ia and aph(3')-IIla gene occurred the most frequently, and none of the tested isolates carried newer AME genes such as aph(2'')-Ic, aph(2'')-Id, and ant(4')-la. Similar results were reported by other authors [37, 41–43]. In our study we observed strains with the (6')-le-aph(2'')-la or aph(3')-IIla gene but without respected phenotypic resistance towards gentamicin. This may be due to low levels or downregulation of these genes’ expression or by inactive gene products.

In our study all of the VRE strains and 94.1% of VSE strains were resistant to ampicillin and imipenem. None of the investigated E. faecium strains showed β-lactamase activity. These results are in agreement with other studies [24–25]. It should be noted that in this study the majority of tested strains that were resistant to β-lactams also had HLAR phenotype. The occurrence of co-resis-
tance between ampicillin and aminoglycosides in VRE isolates is worrisome because it eliminates the synergistic effect between β-lactams and aminoglycosides in the treatment of patients. Moreover, Leavis [44] showed that increasing numbers of β-lactam resistant E. faecium proceeded the growing rates of VRE both in the USA and in Europe. Precisely, high-risk enterococcal clonal complex (CC17), associated with hospital outbreaks of VRE on 5 continents, was strongly correlated with ampicillin resistance. Moreover, ampicillin resistance was followed by resistance to fluoroquinolones and then acquisition of the vanA or vanB gene [44]. Nowadays, Enterococcus spp. isolates resistant to β-lactams, aminoglycosides and glycopeptides are considered as multidrug resistant (MDR), and their increased prevalence and dissemination worldwide cause the necessity of searching for new treatment strategies, including combination therapy [44–46].

The presence of cyl and gelE genes among E. faecium strains is rare. Vankerkhoven [47] did not find any cyl and gelE genes with PCR in 271 E. faecium isolates. In our study, both VSE and VRE isolates were shown to be hemolysin producers (>90%), but only 2 of the VRE strains carried the genes of the cyl operon. This may be due to the expression of other hemolysin genes that are yet not known or not so well studied. Interestingly, these cyl-positive strains also had the gelE gene. A small percentage of strains with the gelE gene has also been reported by other researchers [6, 13, 41] but without the coexistence of cyl genes. A recent study performed by Saba Copur [48] showed that more VSE than VRE strains possessed the gelE (25% and 2.2%, respectively) and cyl (50% and 0%) genes, which is not in concordance with our results. On the other hand, Biswas [49] revealed that 44.4% of VRE and 16.4% of VSE isolates contained the gelE gene (p < 0.001).

We reported that the prevalence of esp and hyl genes among tested strains groups was almost equal and, consequently, these differences among isolates were not statistically significant. Similar proportions and lack of significant differences between VSE and VRE isolates were seen by other researchers [6, 21, 48], but Vankerkhoven [47] and Biswas [49] found that the esp gene was significantly more prevalent among VRE isolates than among VSE strains (77% VRE versus 53% VSE, and 27.8% VRE versus 8.9% VSE, respectively). These contradictory results indicate that the presence of all tested virulence genes cannot be unambiguously correlated with the occurrence of vancomycin resistance among E. faecium strains.

Bacterial ability to form biofilm is considered to be an important factor in the pathogenesis of enterococcal infection [6, 17, 21]. In our study, over 70% of strains from both VRE and VSE groups were found to produce biofilm. These results did not coincide with studies by others [6, 15, 21]. Di Rosa [15] found that VRE strains were able to produce biofilm less frequently (28.8%). Praharaj et al [21] also showed that 13 out of 32 VRE and 70 out of 125 VSE isolates formed biofilm. However, similarly to our results, there was no significant difference when comparing VRE and VSE groups. Collectively, these data suggest that VRE strains do not produce biofilm more often than VSE isolates; nevertheless, potential impact of biofilm forming ability among E. faecium isolates should be taken into account when developing treatment options or infection-control procedures.

VRE was first identified in Europe in 1986, spread rapidly, and is now widespread across Europe [50]. Unfortunately, according to European Centre for Disease Prevention and Control report (2015), resistance to glycopeptides has significantly increased over the last 4 years, especially in Bulgaria, Croatia, Denmark, Hungary, Ireland, Italy, Slovakia and the United Kingdom. The percentage of VRE varied between 0% in Estonia, Finland, Malta, and Iceland to 45.5% in Ireland. A decrease in the prevalence of the VRE strains, compared to previous years, was reported only in France. In Poland, the first VRE strains were reported in 1996 [51]. Nowadays, the percentage of VRE isolates in Poland has also increased and ranges between 8 and 19%. These varieties and changes in VRE epidemiology are a reflection of differences in antibiotic and infection control policies and remain a major challenge throughout Europe.

In conclusion, both VRE and VSE isolates were well equipped with virulence and resistance genes, although VRE strains were characterized by greater variety and a higher number of these genes. This variety, especially when it occur in combination with phenotypic high-resistance level to antimicrobials, might increase the ability to adhere to artificial surfaces, lead
to persistence and spreading in hospital environments, and cause more serious nosocomial infections. Moreover, these results suggest that VRE strains slowly acquire and incorporate resistance and virulence traits, due to their ability to survive in hospital environments for a long time. However, statistical analysis revealed no significant differences in the occurrence of tested features between VSE and VRE strains. This can be related to an insufficient number of isolates that could have compromised the statistical analysis. Studies with a higher number of heterogeneous VRE and VSE strains should be performed to clarify each type of strain’s role in pathogenesis of enterococcal infections. Further studies are also needed on regulation and expression of virulence and resistance genes, how to prevent the spread of MDR enterococcal infections, and on treatment alternatives. Novel pharmacotherapy targeted at specific virulence factors such as anti-adhesins may play preventative or even therapeutic role in the elimination of MDR enterococcal infections.

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Conflict of interest statement
The authors declare no conflict of interest.

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