Short communication

Spread of vancomycin-resistant Enterococcus faecium in two haematological centres in Russia

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**Abstract**

This paper describes the clonal diversity of vancomycin-resistant Enterococcus faecium isolated from patients with haematological malignancies in Russia. Pulsed-field gel electrophoresis (PFGE) typing of 129 vanA-positive E. faecium strains revealed 23 independent restriction profiles with two predominant clonal types. Multilocus sequence typing (MLST) of 16 strains selected from two predominant PFGE types showed that they belong to the epidemic clonal complex (CC) 17. Tn1546-like elements of isolates were compared with the prototype element from E. faecium BM4147 by polymerase chain reaction (PCR). Four different Tn1546 types were distinguished according to structural alterations. Polymorphism in the orf1 and vanSH genes was detected. However, a significant prevalence of the prototype Tn1546 was revealed. Tn1546-like elements with the same structures were observed in strains of different PFGE types. The virulence genes esp, gelE and hyl were detected by PCR in 118 isolates (91%), 87 isolates (67%) and 35 isolates (27%), respectively. In contrast, agg and cylA genes were not found. The detection frequency of esp was higher in epidemic strains than in sporadic ones (100% vs. 56%; \( P < 0.05 \)). This study describes a genetically variable population of vancomycin-resistant E. faecium in two Russian haematological centres. The spread of vancomycin resistance was mostly due to the distribution of the two subclones of E. faecium CC17, enriched with the virulence marker esp. At the same time, dissemination of an altered Tn1546 also occurred.

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1. Introduction

Vancomycin-resistant Enterococcus faecium have been reported with increasing frequency worldwide. Immunocompromised patients, especially those with haematological malignancies, are at high risk for acquisition of vancomycin-resistant enterococci (VRE). Recent data indicate that a vanA genotype, coding for high-level vancomycin resistance, is the most commonly found in VRE. The vanA gene cluster, carried on transposon Tn1546, consists of the genes vanR, vanS, vanH, vanA, vanX, vanY and vanZ responsible for glycopeptide resistance as well as orf1 and orf2 essential for transposition. Acquisition of vancomycin resistance usually involves horizontal transfer of a Tn1546-containing plasmid. It has also been shown that the majority of E. faecium isolates causing hospital outbreaks worldwide belong to a distinct genetic subpopulation designated clonal complex (CC) 17 [1]. Acquisition of potential virulence traits by such strains might increase their fitness in the hospital environment.

The virulence gene esp, coding for enterococcal surface protein, has been detected in E. faecium as part of distinct genetic element. Since the esp gene was prevalent in E. faecium isolates associated with nosocomial outbreaks, it was concluded that this genetic element constitutes a putative pathogenicity island [2]. Several other factors possibly contributing to the pathogenesis of enterococcal infections, such as aggregation substance (Agg), gelatinase (Gel), cytolysin (Cyl) and hyaluronidase (Hyl), have been reported. Moreover, the presence of the virulence genes esp and hyl was shown to be associated with epidemic clones. However, most hospital VRE outbreaks have a complex epidemiology including clonal spread of specific strains and transfer of Tn1546-like elements [3].

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**Keywords:**
- Enterococci
- Vancomycin resistance
- Virulence genes
- Tn1546
- Clonal complex 17
The purpose of the present work was to investigate the molecular characteristics of vancomycin-resistant Enterococcus faecium spreading in two haematological centres in Moscow, Russia.

2. Materials and methods

2.1. Bacterial isolates

Following isolation of the first Enterococcus gallinarum with high-level resistance to vancomycin from a blood culture of a patient hospitalised in a haematological centre in 2002, screening for VRE in two haematological centres in Moscow was performed. Patients hospitalised in the haematological unit and the Intensive Care Unit were included in the study. All strains were identified to species level using a BBL™ Crystal™ Gram-positive (GP) Identification System (BD, Franklin Lakes, NJ). Identification and detection of vanA resistance genes was performed by polymerase chain reaction (PCR) as described by Dutka-Malen et al. [4].

2.2. Pulsed-field gel electrophoresis (PFGE) typing

PFGE typing of vancomycin-resistant E. faecium harbouring vanA genes was performed using a GenePath Group I Reagent kit (Bio-Rad, Marnes-la-Coquette, France). Genetic fingerprinting was performed on a GenePath System (Bio-Rad Laboratories, Hercules, CA). PFGE patterns were compared using GelCompar software (BioNumerics; Applied Maths, Austin, TX) using the Dice coefficient and unweighted pair grouping by mathematical averaging (UPGMA). Isolates showing three or fewer band differences were regarded as a single PFGE type.

2.3. Multilocus sequence typing (MLST)

MLST was performed as described previously [5] using an ABI PRISM® BigDye® Terminator v1.1 Cycle Sequencing Kit on a 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA). The genes atpA, ddl, gdh, purK, gyd, pstS and adk were analysed. Sequence types (STs) were assigned according to the MLST database at http://www.mlst.net.

2.4. Characterisation of Tn1546-like elements

PCR reactions were carried out using a Tercik thermocycler (DNA Technology, Moscow, Russia). Tn1546-like elements were characterised by PCR amplification of internal transposon regions. Primer positions and sequences are shown in Table 1. PCR products were compared with those obtained for the prototype Tn1546 element from E. faecium BM4147. Primers specific for IS1216V were used in combination with primer 2096-2081R to determine the left end of the truncated Tn1546 elements.

2.5. Detection of virulence genes

PCR was used to detect virulence determinants as described previously using DNA primers specific for extracellular surface protein (esp), hyaluronidase (hyl), aggregation substance (agg), gelatinase (gelE) and cytolyisin (cylA) [10].

2.6. Statistical analysis

The prevalence of different PFGE and Tn1546 types as well as virulence genes in various groups of strains was compared by the $\chi^2$ test ($P<0.05$) using the statistical program STATISTICA (Kernel release 5.5).

3. Results

3.1. Bacterial strains

In total, 129 vancomycin-resistant E. faecium isolates carrying vanA genes were recovered from October 2004 to December 2007. Strains were obtained from 129 patients (62 [48%] male and 67 [52%] female) aged between 1.5 years and 87 years. The majority of patients (91%; 118) were carriers of vancomycin-resistant E. faecium, whereas 9 (7.0%) had urinary tract infections and 2 (1.6%) had bloodstream infections. Vancomycin-resistant E. faecium were isolated from the following clinical specimens: faeces (107); urine (9); wound swabs (4); bronchoalveolar lavage fluid (4); blood (2); throat swabs (2); and endocervical swab (1). Only 8 isolates (6.2%) were obtained in 2004, whilst 47 (36%) were isolated in 2005, 64 (52%) in 2006 and 10 (81%) in 2007.

3.2. Pulsed-field gel electrophoresis typing

PFGE analysis of the 129 vanA-positive E. faecium strains revealed 23 different restriction profiles designated PFGE A to W. PFGE types A–G contained two or more clonally related isolates each, whereas the remaining PFGE types H–W included sporadic isolates. The most prevalent clonal PFGE types, A (A1–A30) and F (F1–F8), accounted for 65% (84/129) and 9.3% (12/129) of strains and were considered as epidemic.

3.3. Multilocus sequence typing

A set of 16 isolates from the most widely spread subclones (A1, A3, A8, A10, A16, A26, F1 and F3) was chosen for MLST analysis. It was revealed that all the isolates belonged to the globally disseminated CC17. Three STs were determined: ST202 (n = 11); ST18 (n = 3); and its single locus variant ST262 (n = 2). Interestingly, ST202 and ST18 could have originated from ST17, which is the predicted founder of the hospital population CC17. In particular, ST202

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>ATT TTC CTG ATC AAC TCC TCG</td>
<td>[6]</td>
</tr>
<tr>
<td>TCG GAA AAC AAG GTC AGC TTA GA</td>
<td>[6]</td>
</tr>
<tr>
<td>GGTGTTGACTGTGGAG</td>
<td>This study</td>
</tr>
<tr>
<td>GGGTTACACTGACC</td>
<td>This study</td>
</tr>
<tr>
<td>CAGTCCGATAATCCC</td>
<td>This study</td>
</tr>
<tr>
<td>ACTAGT/GATCTGCGCC</td>
<td>[7]</td>
</tr>
<tr>
<td>GCTGGGCCCATGTTG</td>
<td>This study</td>
</tr>
<tr>
<td>CTTAACGAGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>CTGCAGACTGTCGACC</td>
<td>This study</td>
</tr>
<tr>
<td>CGGAA/GACA/GAGCGGC</td>
<td>This study</td>
</tr>
<tr>
<td>TACTAATGGGCTGTC</td>
<td>[6]</td>
</tr>
<tr>
<td>CATA/TTATACACCTTTCATAT</td>
<td>[8]</td>
</tr>
<tr>
<td>TTAGTTTGATGATGACACCAGT</td>
<td>[7]</td>
</tr>
<tr>
<td>TCGGAGCTAACCACAT</td>
<td>[6]</td>
</tr>
<tr>
<td>ACG/TGTAGGATAGGGAAGG</td>
<td>[7]</td>
</tr>
<tr>
<td>GAT/TCGGCTGTTTGGC</td>
<td>[7]</td>
</tr>
<tr>
<td>GCCCGATAGCTCAGTTG</td>
<td>[7]</td>
</tr>
<tr>
<td>ACCGCGTCTCCTGTCC</td>
<td>[7]</td>
</tr>
<tr>
<td>GCCGTTACACTGACC</td>
<td>[7]</td>
</tr>
<tr>
<td>AC/TCGAG/GACA/GAGCG</td>
<td>[9]</td>
</tr>
<tr>
<td>AGGT/ATATATAGAAAACCCG</td>
<td>[9]</td>
</tr>
</tbody>
</table>

Table 1: Primers used for Tn1546 analysis and detection of virulence genes.
is a single locus variant of ST17, and ST18 is a double locus variant of ST17.

3.4. Characterisation of Tn1546-like elements

Isolates were assigned four different types (1–4) according to structural differences of the Tn1546-like elements (Fig. 1).

Type 1 harboured transposons indistinguishable or closely related to the prototype Tn1546 from the control strain E. faecium BM4147. Type 2 had a \( \text{vanSH} \) amplicon that was ca. 1.4 kb larger than that of BM4147. In type 3, no product corresponding to nucleotides 42–1141 of Tn1546 (gene \( \text{orf1} \)) was observed. These strains also harboured a 1.4 kb \( \text{vanSH} \) insertion and amplified a product of ca. 1.6 kb with primer 650F specific for IS1216V and primer 2096-2081R specific for \( \text{orf1} \). This suggests that the left end of the transposon (\( \text{orf1} \)) has been partially replaced by IS1216V. Type 4 was assigned to the isolate lacking amplicons corresponding to nucleotides 42–3007 of the \( \text{orf1} \) gene. This strain additionally had the 1.4 kb \( \text{vanSH} \) insertion.

Note that the type number in the above classification reflects the time order in which these types were detected, showing the temporal variation of the transposon structure.

Transposon types 1 and 3 were found in isolates of different PFGE types and persisted for long time intervals. The prototype Tn1546 element (type 1), which accounted for the majority of isolates (91%), was first found in isolates of the epidemic clone A and afterwards also in sporadic PFGE types (S–U) and clonal PFGE types (D–G). Interestingly, the prototype Tn1546 element was present in all strains of the major epidemic clone A. Type 3 transposon was initially detected in a sporadic strain of PFGE type V and later in other sporadic strains of PFGE types I and W and in isolates of the PFGE type B.

3.5. Determination of virulence markers

Virulence analysis of 129 E. faecium revealed \( \text{esp} \), \( \text{gelE} \) and \( \text{hyl} \) in 118 isolates (91%), 87 isolates (67%) and 35 isolates (27%), respectively. In contrast, \( \text{agg} \) and \( \text{cylA} \) genes were not detected.

<table>
<thead>
<tr>
<th>PFGE type (n)</th>
<th>Tn1546 type (n)</th>
<th>ST (n)</th>
<th>Virulence genes [n (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>( \text{esp} )</td>
</tr>
<tr>
<td>Clonally related isolates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (84)</td>
<td>Type 1 (84)</td>
<td>ST202 (9); ST18 (3); ST262 (2); ST202 (2)</td>
<td>84 (100)</td>
</tr>
<tr>
<td>F (12); G (5); C (4); E (4); B (2); D (2)</td>
<td>Type 1 (11)</td>
<td>Type 2 (1)</td>
<td>Type 1 (11)</td>
</tr>
<tr>
<td>Sporadic isolates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H–W (16)</td>
<td>Type 1 (12)</td>
<td>Type 3 (3); type 4 (1)</td>
<td>( _* )</td>
</tr>
</tbody>
</table>

\( _* \) Sequence types were not defined.
Different combinations of virulence genes were found. A single esp gene was observed in 11 strains (9%). The simultaneous presence of the two genes esp and gelE was found in 73 isolates (57%), whilst esp and hyl were present in 20 isolates (16%). Three genes (esp, hyl and gelE) were detected in 14 strains (11%). Variable virulence profiles were found in isolates of the same PFGE type (A, B, C, D and G).

The esp gene was present in isolates of the epidemic PFGE types A and F. A significantly lower number of sporadic isolates were esp-positive (100% vs. 56%; P < 0.05) (Table 2). The gelE gene was present in 77% of clonal strains and was not detected among sporadic isolates. At the same time, no significant difference in the presence of hyl was found: it was detected in 27% of clone A isolates, 41% of other clonally related strains and in 31% of sporadic enterococci.

4. Discussion

Recently, VRE has become a global nosocomial problem, however it has not yet been reported in Russia. The present study is the first description of wide dissemination of vancomycin-resistant E. faecium in two haematological centres in Moscow.

PFGE analysis revealed a clonally diverse population of vancomycin-resistant E. faecium, indicating that strains have originated from several different sources. The predominance of two clonal PFGE types (A and F) was detected. Dissemination of particular clones could be due to the acquisition of specific genetic characteristics that enhance their ability to spread and cause infection. We assume that the presence of the vanA gene cluster, combined with virulence genes in the same genome, could have favoured the selected E. faecium clones. As described in the literature, the esp gene is a marker of the highly prevalent nosocomial E. faecium lineage. It has been detected in the majority of outbreak isolates collected from hospitalised patients on different continents [11]. The trends in the occurrence of the esp gene in epidemic strains revealed in our study (Table 2) agree with the published data. Presence of the esp gene varied in strains of the same clonal type, which can be explained by possible acquisition or loss of a pathogenicity island. In this work, presence of the virulence gene hyl was not associated with the epidemiology of the isolates. The gelE gene has been detected with higher frequency than observed previously [12].

Recent studies have revealed a subtype of E. faecium associated with nosocomial outbreaks, characterised by presence of the esp gene [2], ampicillin resistance and genetic clustering of the isolates by MLST. This analysis revealed the spread of the most globally dispersed E. faecium lineage of CC17 in two haematological centres in Moscow. A remarkable polymorphism of E. faecium housekeeping genes has been detected. The strains belonged to three different sequence types, ST202, ST18 and ST262 (Table 2). We assume that ST202 and ST18 have arisen independently from a common progenitor ST17. This fact suggests the possible presence of two independent sources of vancomycin-resistant E. faecium. However, previous circulation of ST17 cannot be excluded. All of the detected STs have already been described for clinical isolates from Europe, Australia and China. Interestingly, one strain of ST18 was isolated from pig faeces in Belgium. This could be due to possible acquisition of VRE via the food chain in this country. It was suggested that the rise in VRE infection rates was caused by the selection and spread of the nosocomial lineage of CC17 and subsequent horizontal transfer of the resistance element Tn1546 [3]. This agrees with our results. The detected clonal diversity of isolates indicated that E. faecium strains have circulated for a long period of time. Interestingly, we revealed that the prototype Tn1546 element (type 1) prevailed in our strains (Table 2). In contrast, elements different from the prototype were usually more common in other countries [6]. Heterogeneity of transposon structures was due to point mutations, deletions and mostly to integration of insertion sequence elements [7]. The vanS/shA gene cluster has a conservative structure, with most of the rearrangements occurring upstream of the vanR gene or downstream of vanX and resulting from the presence of deletions and insertion of sequence elements in genes not essential for glycopeptide resistance (orf1, orf2, vanY and van2) and in the intergenic regions. The structural variations of Tn1546 result from transposition of transposons to a high variety of plasmid molecules, some of which could then be further transmitted by conjugation to other strains. Therefore, the low variety of transposon structures detected in our work could be explained by relatively short periods of circulation of resistance elements. This supports the suggestion that selection of specific E. faecium clones in the hospital environment occurs prior to the acquisition of resistance determinants. However, we detected some rearrangements in the Tn1546 element. Partial deletions in the orf1 region owing to insertion of IS1216V and insertion between vanS and vanH genes were found in different clones, indicating that horizontal transmission and structural rearrangements of the transposons occurred. Rearrangements of orf1 caused by partial replacement of this region by insertion of IS1216V have also been reported previously [13]. The insertion found between vanS and vanH fits the description of the previously reported IS1251, inserted in the vanSH intergenic region [14]. The role of Tn1546 structural rearrangements is still to be elucidated since the data are controversial. Very interesting observations have been made regarding the evolution of these elements. The insertion sequence IS1216V found extensively among European Tn1546-like elements at different positions and orientations was shown to mobilise parts of the transposon. The inverted duplication of these insertion sequences was found in Tn1546-like elements suggesting that IS1216V elements serve as targets as mediators of a replicative transposition event [15].

The results of this study indicate the ongoing evolution of the E. faecium population. Circulation of vancomycin resistance and virulence genes supplements the evolution of the chromosomal DNA of the circulating clones. At the same time, rearrangements of the Tn1546-like elements also take place. The question of whether the virulent epidemic clones of the worldwide disseminated lineage of CC17 evolved in Russia under selection pressure in the hospital environment or have been transmitted from external community sources is still to be elucidated.

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References


