The changing epidemiology of VanB *Enterococcus faecium* in Poland

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Received: 23 November 2017 / Accepted: 1 February 2018 / Published online: 13 February 2018 © The Author(s) 2018. This article is an open access publication

Abstract
Increasing prevalence of VanB *Enterococcus faecium* in Polish hospitals reported to National Reference Centre for Susceptibility Testing (NRCST) prompted us to investigate the basis of this phenomenon. Two-hundred seventy-eight *E. faecium* isolates of VanB phenotype from the period 1999 to 2010 obtained by NRCST were investigated by multilocus sequence typing (MLST) and multilocus VNTR analysis (MLVA). Localization, transferability, and partial structure of the vanB-carrying Tn1549 transposon were studied by hybridization, PCR mapping, sequencing, and conjugation. VanB isolates almost exclusively represented hospital-associated *E. faecium*, with a significant shift from representatives of 17/18 lineage to 78 lineage after 2005. The vanB determinant, initially located mostly on transferable plasmids of the pRUM-, pLG1-, and pRE25-replicon types, later on was found almost exclusively on the host chromosome. Fifteen different plasmid and chromosomal insertion sites were identified, typically associated with single transposon coupling sequences, mostly not observed before. Our study demonstrates the significant change in the epidemiology of VanB-*E. faecium* in Poland, associated with the introduction and spread of the lineage 78 of the hospital-adapted *E. faecium*. These data point to the importance of the lineage 78 for the spread of vancomycin-resistance, determined by the vanB gene cluster, resulting in an increasing VRE prevalence in hospitals. This study also supports the scenario, in which representatives of the hospital-associated *E. faecium* independently acquire the vanB determinant de novo and spread within and among hospitals, concomitantly undergoing differentiation.

Keywords Epidemic lineage · Population shift · Transposon · Plasmid · Diversity

Introduction
The importance of enterococci as etiologic agents of hospital-acquired infections (HAIs) is currently increasing [1], and common glycopeptide resistance among these bacteria is especially alarming [2]. Among two most ubiquitous van gene clusters, responsible for this phenotype, vanA confers resistance to both vancomycin and teicoplanin, and vanB typically determines resistance only to vancomycin [3]. The vanB cluster is predominantly associated with the Tn1549-type transposons [4], which may reside either on plasmids or on the bacterial chromosome [4–9]. During the initial steps of conjugative transfer of transposon, the staggered cleavage by the Int recombinase results in the formation of a circular intermediate, joined by a 5- to 6-bp sequence originating from the donor genome, termed a coupling sequence, which, after transposition, is found adjacent to the transposon termini in the recipient [7].

Among the two clinically most important enterococcal species, i.e., *Enterococcus faecalis* and *Enterococcus faecium*, the latter is particularly prone to the acquisition of antimicrobial resistance determinants, including vanA and vanB clusters (vancomycin-resistant *E. faecium*, VREfm), resulting in increasing proportion of VREfm among hospital *E. faecium* [10]. Concomitantly, an increase in the incidence of HAIs caused by *E. faecium* is observed [10, 11], likely due to the selection and worldwide dissemination of successful hospital-adapted clonal complex 17 (CC17) [12] that combines resistance to several antimicrobials with the enrichment in pathogenicity factors and increased epidemic potential. The
Bayesian Analysis of Population Structure (BAPS) of the data obtained by multilocus sequence typing (MLST) demonstrated that CC17 may be divided into two subgroups corresponding to major lineages 17/18 and 78 [13].

The vanB gene was identified in *E. faecalis* at the beginning of the 1990s [14]. In Poland, the first VRE*fm* with vanB2 was isolated in 1999 [15] followed by a growing VanB prevalence in 1999–2005 [16]. A further increase in VanB-VRE*fm* after 2005, noticed by the National Reference Centre for Antimicrobial Resistance and Surveillance (NRCARS), prompted us to investigate these important pathogens to better understand the factors underlying the spread of VanB-*E. faecium* in Poland.

Materials and methods

Bacterial isolates and antimicrobial susceptibility testing

Altogether, 278 non-repetitive isolates with the VanB phenotype received by the NRCARS during 1999–2010 from 36 centers in 22 cities were investigated. Fifty-eight VanB isolates from 1999 to 2005 were partly characterized previously [16]; of these, 56 were available and 222 isolates were received in 2006–2010. Twenty-seven and 48 isolates were obtained from invasive and non-invasive infections, respectively, and 201 from carriage; for two remaining isolates, the source was not reported. Antimicrobial susceptibility was tested using the broth microdilution method [17] and the Etest method for vancomycin, teicoplanin, and daptomycin (bioMérieux, Marcy l’Etoile, France). Results were interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST)-approved breakpoints [18] and the Epidemiological Cut-Offs (ECOFFs) (http://mic.eucast.org/Eucast2/; 6th November 2017, date last accessed).

Detection of *vanB*, IS16 and *esp*, and molecular typing

DNA was purified using the Genomic DNA Prep Plus kit (A&A Biotechnology, Gdynia, Poland) and vanB; IS16 and *esp* were detected by PCR [19–21]. Multilocus VNTR (variable-number tandem repeat) analysis (MLVA) and MLST were performed as described [22, 23]; sequence types (STs) were assigned using the MLST database http://pubmlst.org/efaecium/ (6th November 2017, date last accessed). On the basis of eBURST analysis [24] of the whole MLST database (as of the 21st of April 2015), STs were included into CCs and lineages [13, 25].

Analysis of Tn1549, insertion sites, and coupling sequences

The presence of *int*<sub>Tn1549</sub> and ORF1<sub>Tn1549</sub> was confirmed by PCR, and the *vanY-vanX* sequence in Tn1549 was established using overlapping PCR and sequencing. The Tn1549 insertion sites were identified by inverse-PCR (iPCR) [26] with BspI1431 (Fermentas, Lithuania). Primers targeting sequences adjacent to Tn1549 were designed based on iPCR results. Sequences were analyzed with the Lasergene package (DNASTAR, MD, USA). Primer sequences are available upon request.

Plasmid gene detection, S1 profiles, hybridization, and conjugation

Plasmid rep (rep<sub>pIP501</sub>, rep<sub>pRE25</sub>, rep<sub>pAM373</sub>, rep<sub>pAD1</sub>, rep<sub>pRUM</sub>, rep<sub>pMG1</sub>, rep<sub>pLG1</sub>) and toxin-antitoxin systems (TAS) *axe-txe* and *ω-ζ* were detected by PCR [26–29] with controls from our collection [28, 30]. For profiling of plasmids, DNA in agarose plugs was treated with S1 nuclease (Takara Bio, Japan), separated by pulsed-gel electrophoresis (PFGE) [31] and blotted onto Hybond-N+ (GE Healthcare, Buckinghamshire, UK). Hybridization was carried out using the Amersham ECL System (GE Healthcare). Transferability of vancomycin resistance was examined as described [32] with the recipient *E. faecium* strain 64/3.

Statistics

The differences in distributions were evaluated by the chi-squared test, with a *p* value ≤ 0.05 considered significant.

GenBank accession numbers

New sequences of the *vanY-vanX* region: A1–A6 (KC489780–KC489785), A9–A20 (KT003969–KT003980), B1 (KC489787), B2 (KT003981), D (KC489790), and E (KT003982); rep<sub>pRUM</sub> (KM014783), rep<sub>pRUM</sub>–1 (KM014782), rep<sub>pLG1</sub> (KM014783), and rep<sub>pLG1–2</sub> (KM014784) were submitted to GenBank.

Results

Antimicrobial susceptibility phenotypes and clonal relationships of VanB-VRE*fm* in Poland

All isolates were analyzed by MLVA, yielding 23 different MTs; 13 non-typable isolates repeatedly yielded incomplete MLVA profiles (Table 1). The most prevalent MT159 (186 isolates, 83.0%) was observed solely since 2006. Eighty isolates from 2006 to 2010, representing all hospitals providing
### Table 1  Epidemiological and typing data, *vanY-vanX* region structure and Tn1549 localization among VanB *E. faecium* in Poland, 1999–2010

**A. Plasmid localization of Tn1549**

| Variant name | Centre (n) | Year          | ST (n) | MT (n) | Lineage | vanYvanX (n) | vanB-plasmid representatives approximate size in kb; | plasmid-specific genes | (n) |
|--------------|------------|---------------|--------|--------|----------|--------------|------------------------------------------------|----------------------|-----|
| CS_P1a       | KRA2 (4)   | 2000          | 381 (2) | 264 (2) | 17/18    | A3          | - 100 {rep2}                                           |                      |     |
|              |            | 2000          | 384 (2) | 326 (2) | 17/18    |             | - 160 {rep2}, 320 {rep2, repPGL1, axe-txe}             |                      |     |
|              |            |               |        |        |          |             | - 150 {repPGL1} (2)                                    |                      |     |
| CS_P1b       | WAW1 (4)   | 1999–2000     | 382 (2) | 325 (2) | 17/18    | B1          | - 100 {rep2, axe-txe}                                   |                      |     |
|              |            |               |        |        |          |             | - 80 {rep2, repPGL1, axe-txe}                          |                      |     |
|              |            |               |        |        |          |             | - 100 {rep2}, 240 {rep2, repPGL1, axe-txe}             |                      |     |
|              |            |               |        |        |          |             | - 60 {rep2, rep17, repPGL1, axe-txe}                    |                      |     |
|              |            |               |        |        |          |             | 90 {rep2, rep17, repPGL1, axe-txe}                      |                      |     |
|              |            |               |        |        |          |             | - 150 {repPGL1}                                        |                      |     |
|              | CS_P2      | 2002          | 386     | 4      | 17/18    | C (10)      | - 220 {rep2, repPGL1, axe-txe}                          |                      |     |
|              |            | 2005          | 562     | 375 (4) | 17/18    | A4          | - 220 {rep17, repPGL1, axe-txe}                         |                      |     |
|              |            | 2005          | 260 (2) | 13 (2)  | 17/18    | A4          | - 220 {rep17, repPGL1}                                  |                      |     |
|              |            | 2005          | 920     | 231     | 17/18    | nd          | - 150 {repPGL1}                                        |                      |     |
|              |            | 2005          | 74      | nd      | 17/18    |             | - 150 {repPGL1}                                        |                      |     |
|              |            |               |        |        |          |             | - 150 {repPGL1}                                        |                      |     |
|              |            |               |        |        |          |             | - 150 {repPGL1}                                        |                      |     |
|              | CS_P3      | POZ1 (1)      | 2003    | 202     | 1        | 17/18       | A4          | - 200 {rep2, rep17, repPGL1, axe-txe}                   |                      |     |
|              |            | 2005          | 18      | 1       | 17/18    | A4          | - 150 {repPGL1}                                        |                      |     |
|              |            | 2005          | 440     | 7       | 17/18    | A4          | - 150 {repPGL1}                                        |                      |     |
|              |            | 2006          | 17      | 4       | 17/18    | A4          | - 150 {repPGL1}                                        |                      |     |
|              |            | 2007          | 279     | 3       | 17/18    | nd          | - 150 {repPGL1}                                        |                      |     |
|              | CS_P4      | WAW1 (11)     | 2005    | 17      | 4 (5)    | 17/18       | D (11)      | - 150 {rep2}                                           |                      |     |
|              |            | 2005          | 387 (4) | 50 (4)  | 17/18    | A12         | nd          | - 150 {rep2}                                           |                      |     |
|              |            | 2005          | 384     | 325     | 17/18    |             | - 150 {rep2}                                           |                      |     |
|              |            | 2005          | 384     | 326     | 17/18    |             | - 150 {rep2}                                           |                      |     |
|              | CS_P5      | WAW5 (1)      | 2005    | 387     | 376      | 17/18       | A2          | - 120 {rep2}                                           |                      |     |
|              |            |               |        |        |          |             | - 250 {rep2, rep17, repPGL1}                            |                      |     |
|              | CS_P6a1    | KSZ (2)       | 2010    | 78 (2)  | 159 (2)  | 17/18       | A20         | 3HMW                                                  |                      |     |
|              |            | 2010          | 17      | 11      | 17/18    | A19         | - 250 {rep2, rep17, repPGL1}                            |                      |     |
|              |            | 2010          | 78      | 159     | 17/18    | nd          | - 250 {rep2, rep17, repPGL1}                            |                      |     |
|              |            |               |        |        |          |             | - 250 {rep2, rep17, repPGL1}                            |                      |     |
|              | CS_P6b     | POZ7 (6)      | 2008    | 78 (2)  | 159 (6)  | 17/18       | A15         | - 70 {rep2, rep17, repPGL1, axe-txe}                    |                      |     |
|              |            |               |        |        |          |             | - 70 {rep2, rep17, repPGL1, axe-txe}                    |                      |     |
|              |            |               |        |        |          |             | - 50 {rep2}, 270 {rep17, repPGL1, axe-txe}              |                      |     |
|              | CS_P7      | LUB (3)       | 2009–2010 | 78 (3) | 159 (3) | 17/18       | A15         | - 80 {rep2, rep17, repPGL1, axe-txe}                    |                      |     |
|              |            | 2009          | 192 (2) | 159 (2) | 17/18    |             | - 80 {rep2, rep17, repPGL1, axe-txe}                    |                      |     |

**B. Chromosomal localization of Tn1549**

| Variant name | Centre (n) | Year | ST (n) | MT (n) | Lineage | vanYvanX (n) |
|--------------|------------|------|--------|--------|----------|--------------|
| CS_C1        | KRA1 (1)   | 2003 | 387    | 50     | 17/18    | A1           |
|              | KON (13)   | 2004 | 387 (13)| 50 (3), nt (10) | 17/18 | A1         |
|              | KAL (1)    | 2004 | 387    | 50     | 17/18    | A1           |
|              | POZ1 (1)   | 2004 | 387    | 376    | 17/18    | A1           |
|              | POZ2 (2)   | 2005 | 387 (2)| 50 (2) | 17/18    | A1           |
|              | KRA1 (1)   | 2005 | 387    | 50     | 17/18    | A1           |
|              | KRA1 (1)   | 2005 | 387    | 50     | 17/18    | A1           |
|              | POZ6 (1)   | 2006 | 561    | 50     | 17/18    | A1           |
|              | POZ7 (1)   | 2008 | 202    | 1      | 17/18    | nd           |
| Characteristic | Year 1 | Characteristic | Year 1 | Characteristic | Year 1 | Characteristic | Year 1 |
|---------------|--------|---------------|--------|---------------|--------|---------------|--------|
| CS_C2         | 2005   | 279           | 152    | 17/18         | A8     |
| WAW1 (1)      | 2006   | 78            | 12     | 78            | A2     |
| KKE (1)       | 2007   | 78, 856       | 299, 159 | 78         | A2 (2) |
| BYD (2)       | 2007   | 78            | 159    | 78            | A2     |
| KSC (1)       | 2007   | 78            | 159    | 78            | A2     |
| PLO (1)       | 2007   | 78            | 159    | 78            | A2     |
| GWP (12)      | 2007–2008 | 78 (6), 192 | 159 (12) | 78         | A2     |
| KSZ (56)      | 2007–2010 | 17, 18, 267 | n.t. (3), 7 | 17/18 | A1     |
| POZ1 (20)     | 2007–2009 | 78 (2)       | 159 (51) | 78         | nd     |
| POZ2 (48)     | 2007   | 64, 918 (2)   | n.t. (2) | 17/18 | A2     |
| POZ3 (5)      | 2010   | 78 (3)        | 159 (15); 334 (2) | 78         | A2     |
| POZ4 (7)      | 2008–2009 | 64, 267 (4), 857 | 1 (8), 266 | 17/18 | A2     |
| POZ5 (2)      | 2009   | 78 (7)        | 159 (35), 250, 293 (3) | 78         | A2     |
| POZ6 (5)      | 2010   | 78            | 159 (5) | 78            | A2     |
| ZGO (17)      | 2007–2010 | 78         | 334 (2) | 78            | A2     |
| KIE (1)       | 2008   | 78 (2)        | 159 (5) | 78            | nd     |
| SZC2          | 2008   | 78 (4)        | n.t. (2), 291 | 78         | A11    |
| WAW3 (1)      | 2008   | 382           | 50     | 17/18         | nd     |
| WAW4 (1)      | 2008   | 78 (3)        | 159 (16) | 78         | A1     |
| ZAB2 (1)      | 2008   | 78            | 159    | 78            | A1     |
| KAL (1)       | 2010   | 78 (2)        | 159, 334 | 78         | A2     |
| NWS (1)       | 2010   | 78            | 159    | 78            | C      |
| PRZ (1)       | 2004   | 279           | 231    | 17/18         | A5     |
| PSZ (1)       | 2005   | 561           | 231    | 17/18         | A6     |
| SZC1 (1)      | 2010   | 192           | 159    | 78            | A18    |
| OLS (2)       | 2009   | 78            | 159    | 78            | F (8)  |

*BYD*, Bydgoszcz; *GWP*, Gorzów Wlkp.; *KAL*, Kalisz; *KIE*, Kielce; *KKE*, Kędzierzyn-Koźle; *KON*, Konin; *KRA*, Kraków; *KSC*, Kościerzyna; *KSZ*, Koszalin; *LUB*, Lublin; *NWS*, Nowa Sól; *OLS*, Olsztyn; *PLO*, Plock; *POZ*, Poznań; *PRZ*, Przasnysz; *PSZ*, Pszczyna; *SZC*, Szczecin; *WAW*, Warszawa; *WRO*, Wrocław; *ZAB*, Zabrze; *ZGO*, Zielona Góra; numbers adjacent to these abbreviations indicate hospitals in a city; number of isolates from a hospital given in brackets.

Number of isolates given in brackets if different from one.

HMW, high-molecular weight DNA band, a presumable integration of plasmid into chromosome; *S*, singleton; *n.t.*, non-typable; *nd*, not determined.
isolates and all MTs, were resistant to ciprofloxacin and ampicillin; 88.8 and 88.8% isolates showed high-level resistance to gentamicin (HLGR) and streptomycin (HLSR), respectively; 20.0% of isolates were resistant to tetracycline, which represented a significant decrease \((p = 0.0002)\) after 2005 (61.5% [16]). All isolates were susceptible to linezolid, tigecycline, and daptomycin. STs of 26 VanB isolates from the period 1999–2005 were reported previously [16], and additionally 21 isolates from this group were analyzed by MLST, together with 80 representative isolates from the period 2006–2010, mentioned above, yielding altogether 23 STs, characteristic for 127 isolates. Except for ST74, all isolates belonged to lineages 17/18 and 78, and representatives of 78 lineage were frequently associated with MT159 (Table 1). All isolates carried vanB and IS16; esp was present in 98% isolates from 2006 to 2010, similarly to the earlier period [16]. Based on the combined MLVA and MLST results, no representatives of lineage 78 were observed before 2006; the first VanB isolate from lineage 78 occurred in 2006 and since 2007 isolates from this lineage became much more common, representing 89% of isolates from the period 2006–2010 (\(p < 0.001\)).

**Diversity of the vanY-vanX region in Tn1549**

All isolates were positive for ORF1\(_{\text{Tn1549}}\) and \(\text{int}_{\text{Tn1549}}\). Sequencing of the vanY-vanX region (encompassing genes \(\text{vanY}, \text{vanW}, \text{vanH}, \text{vanB}, \text{vanX}\); Fig. 1a) revealed 26 variants among 57 isolates, representing all centers and STs within a center. The most numerous group included A1-A20 variants, differing only by single-nucleotide polymorphisms (SNPs) at 21 nucleotide positions and highly similar to the corresponding region in *Clostridium* spp. and *Eggerthella lenta* (Fig. 1b). The A-type variants were characteristic for 48 of investigated isolates from 31 centers, and associated with 14 STs and 16 MTs. The B variants differed from the A-type by several SNPs and 6-bp insertion between \(\text{vanS-vanY}\). They were 99% identical to the variant reported for the V583 [33]. An insertion of the IS\(_{\text{Efa11}}\) between \(\text{vanS-vanY}\) in B-type yielded C variants (Fig. 1a). The D-, E-, and F-types represented probable derivatives of an A-type, with a deletion encompassing the nt 12-799 of \(\text{vanW}\), an 11-bp deletion upstream \(\text{vanX}\), and insertion of IS\(_{\text{L3}}\) between \(\text{vanS}\) and \(\text{vanY}\), respectively. All \(\text{vanB}\) genes represented the \(\text{vanB2}\) variant [34].

**Analysis of Tn1549 insertion sites and coupling sequences**

To determine Tn1549 insertion sites, selected isolates were analyzed by iPCR and thus obtained sequences were used to search GenBank and to design primers specific for a genetic neighborhood of Tn1549. These primers were used to screen the whole collection, revealing 15 insertion sites and 14 coupling sequences in total (Table 2). For two isolates, the coupling sequence could not be established due to the fact that sequences resulting from iPCR had no homologs in GenBank. Typically coupling sequences were identical in a given insertion site, with an exception of CS\(_{\text{P1a}}/\text{CS}_{\text{P1b}}\) in *aacA-aphD* and CS\(_{\text{P6a}}/\text{CS}_{\text{P6b}}\) in *citH*. The most prevalent coupling sequence, CS\(_{\text{C3}}\) (198 isolates from 24 centers in 16 cities) was associated with 16 MTs and 14 STs. The first CS\(_{\text{C3}}\) isolate was observed in 2006 (Table 1B).

**Analysis of Tn1549 localization, plasmidome composition, and vanB transferability**

Seventy-eight isolates were analyzed by S1/PFGE-hybridization with the \(\text{vanB}\) probe (Table 1 and Supplementary Fig. 1). These isolates represented all observed variants of coupling sequence and hospital centers; additional isolates from the same center were included in the case of isolates showing plasmid localization of \(\text{vanB}\). In the case of 39 isolates with coupling sequence C1-C8, \(\text{vanB}\) hybridized with a band of high-molecular weight, consistent with transposon insertion within chromosomal sequences and 39 isolates showed hybridization with plasmids from \(\sim30\) to \(\sim310\) kb in size; in five isolates, \(\text{vanB}\) was located on two plasmids. These hybridization studies and iPCR/PCR-based analyses of coupling sequences were consistent with the chromosomal localization of Tn1549 for 227 isolates (81.6%) and plasmid localization for 50 isolates (18.0%); in a single case, a presumable integration of plasmid into chromosome was observed (variant CS\(_{\text{6a1}}\) from WAV2). Isolates with the plasmid localization of \(\text{vanB}\) were much more prevalent among early VRE\(_{\text{fm}}\), i.e., from 1999 to 2005 (61% of these isolates) compared to the isolates collected from 2006 to 2010 (0.7% of these isolates, \(p < 0.001\)). Among isolates with the plasmid localization of \(\text{vanB}\), \(\text{rep17}_{\text{PRUM}}\) was found among 49 isolates, followed by \(\text{rep}_{\text{PGL1}}, \text{rep2}_{\text{P25}}, \text{rep1}_{\text{P501}}, \text{rep}_{\text{PMG1}}, \) and \(\text{rep9}_{\text{BAD}}\) (42, 35, 32, 27, and 5 isolates, respectively). Thirty-one and 12 of these isolates carried \(\text{axe-txe}\) and \(\omega-\text{c-C}\), respectively. S1-PFGE/hybridization analyses revealed that 29, 23, and 22 plasmids hybridized with the \(\text{rep}_{\text{PGL1}}, \text{rep17}_{\text{PRUM}}, \) and \(\text{rep2}_{\text{P25}}\) probes, respectively (Table 1A). In several cases, a single plasmid was associated with two or three \(\text{rep}\) genes. Sixteen \(\text{vanB}\)-plasmids hybridized with the \(\text{axe-txe}\) probe; among them, 13 co-hybridized with \(\text{rep}_{\text{PGL1}}\) and 12 with \(\text{rep17}_{\text{PRUM}}\), respectively. Nine plasmids did not hybridize with any of the four probes used. Sequencing revealed a low variability of \(\text{rep}\) genes within this group that included two, one, three, one, and three variants of \(\text{rep1}_{\text{P501}}, \text{rep2}_{\text{P25}}, \text{rep17}_{\text{PRUM}}, \text{rep}_{\text{PMG1}}, \) and \(\text{rep}_{\text{PGL1}}\), respectively. Among 50 isolates with the plasmid localization of \(\text{vanB}\), 43 isolates (86.0%) were able to transfer vancomycin resistance while conjugation experiments involving 32 representative isolates with various chromosomal localizations of \(\text{vanB}\) were negative in 29 cases.
Discussion

The first VanB-VREfm was detected in Poland in 1999 [15], and our study investigated the VanB epidemiology during the following 12 years. Considering a relatively moderate incidence of VREfm in Poland during this period (e.g., in 2010 amounting to 7.8% of invasive infections [http://ecdc.europa.eu/en/publications/Publications/1111_SUR_AMR_data.pdf; 6th November 2017, date last accessed]), it may be assumed that our collection reasonably well reflected the epidemiological situation in Polish hospitals. Although initially VanA represented the major VREfm phenotype in Poland [16, 35], after 2006, the NRCARS recorded an increasing number of VanB-VREfm, affecting several hospitals. The current global epidemiology of VREfm shows considerable differences, with VanA predominant in Europe and the USA [36], and VanB constituting over 80% of invasive VREfm in Australia [37]. A recent rise of VanB-E. faecium has been reported in Germany [8]. Nearly all isolates in our study belonged to the hospital E. faecium, since 2006 with the predominant role (89%) of lineage 78. VanB-VREfm belonging to this lineage were responsible for recent outbreaks in Germany, Sweden, and Australia [38], and representatives of lineage 78 played a role in vanA dissemination in Polish hospitals [34].

Fig. 1 Diversity of vanY-vanX region among VanB-VREfm in Poland, 1998–2010. a Structure of the region, distribution of single-nucleotide polymorphisms (marked by asterisks) among A-type variants, and localization of deletions and ISs. b Similarity tree of nucleotide sequences of A- and B-type variants and sequences from the V583 strain of E. faecalis and isolates of E. lenta and Clostridium spp.
Table 2  Insertion sites and coupling sequences of Tn1549-type transposons in VanB *E. faecium* in Poland, 1999–2010

| Variant name | Number of isolates | Flanking target sequence (20 bp) | CS | Tn1549 | Flanking target sequence (20 bp) | Insertion region |
|--------------|--------------------|---------------------------------|---|--------|---------------------------------|-----------------|
|              |                    |                                 |   |        | Left end                        | Right end       |                             |
|              |                    |                                 |   |        | AAAA                            | ATAT            | GTTTT                        |
| CS_P1a       | 4                  | TTAGTACTAAATTGGTTTTTTT676       |   |        | AATTIT                          | ATAT            | 675AAAATGTATTTCTATATAC      |
| CS_P1b       | 4                  | TTAGTACTAAATTGGTTTTTTT676       |   |        | AATTIT                          | TAAT            | 675AAAATGTATTTCTATATAC      |
| CS_P2        | 10                 | ATTTATCTTGCTGATTATTTTT          |   |        | AAAA                            | ATAT            | –                            |
|              |                    |                                 |   |        | AAAA                            | TTTT            | 86TTTCTCAAAAAACATACTAA      |
|              |                    |                                 |   |        | AAAA                            | AATTIT          | 90AACAAAAAATTACAGGAGG      |
| CS_P3        | 7                  | GAGAAATGCTAATTTGGTTTTTT         |   |        | AAAA                            | ATAT            | –                            |
|              |                    |                                 |   |        | AAAA                            | AATTIT          | 90AACAAAAAATTACAGGAGG      |
| CS_P4        | 11                 | TTATTAATATATTTTGTATTTT          |   |        | AAAA                            | ATAT            | GGTAGAAATAGCTTACAATAAAT    |
| CS_P5        | 1                  | AATAGCATTATTTATCGTGCA           | nd|        | AAAA                            | ATAT            | –                            |
|              |                    |                                 |   |        | AAAA                            | TTTT            | 913CAATTTTTTGTAATATCAGA    |
| CS_P6a1      | 2                  | GGCTAAATGCTTGTGGTTTTT1912       | GTAAT |        | AAAA                            | ATAT            | –                            |
|              |                    |                                 |   |        | AAAA                            | AATTIT          | 913CAATTTTTTGTAATATCAGA    |
| CS_P6a2      | 1                  | GGCTAAATGCTTGTGGTTTTT1912       | GTAAT |        | AAAA                            | ATAT            | –                            |
|              |                    |                                 |   |        | AAAA                            | AATTIT          | 913CAATTTTTTGTAATATCAGA    |
| CS_P6b       | 6                  | GGCTAAATGCTTGTGGTTTTT1912       | GTAAT |        | AAAA                            | ATAT            | –                            |
|              |                    |                                 |   |        | AAAA                            | AATTIT          | 913CAATTTTTTGTAATATCAGA    |
| CS_P7        | 5                  | CTGTGTGCAAATTTTTAAATA           | – |        | AAAA                            | TTTT            | TTATGAAAAGAAAATATCCTAGG    |
| CS_C1        | 21                 | TTCTAGCAGCTTTTATCGAA            | – |        | AAAA                            | ATAT            | –                            |
|              |                    |                                 |   |        | AAAA                            | AATTIT          | CCAATTTACCTATGCAGCCAGC     |
| CS_C2        | 1                  | ACTTCATGGCTTTAAAAATCC406        | – |        | AAAA                            | TTTT            | –                            |
|              |                    |                                 |   |        | AAAA                            | AATTIT          | CCAATTTACCTATGCAGCCAGC     |
| CS_C3        | 198                | CTAAGAAAAAGCCAGCTTTTT           | TGGCTA |        | AAAA                            | TTTT            | –                            |
|              |                    |                                 |   |        | AAAA                            | AATTIT          | 842TGCTAAAGTTTGGCAGAGG     |
| CS_C4        | 2                  | CCACAAATAGATTAAAATT            | ATCGT |        | AAAA                            | TTTT            | –                            |
|              |                    |                                 |   |        | AAAA                            | AATTIT          | AGAAATTTTTTTAAAAAGG         |
| CS_C5        | 1                  | TGTTATAATGAAAAATATT677          | ATAGAA |        | AAAA                            | TTTT            | –                            |
|              |                    |                                 |   |        | AAAA                            | AATTIT          | 678AAAGAAAATTTTTTGATT      |
| CS_C6        | 1                  | ATAGATGAAAATTTCAAAATT           | nd |        | AAAA                            | TTTT            | –                            |
|              |                    |                                 |   |        | AAAA                            | AATTIT          | –                            |
| CS_C8        | 2                  | GTGGATTTGATGTTAAAAAA           | – |        | AAAA                            | TTTT            | –                            |
|              |                    |                                 |   |        | AAAA                            | AATTIT          | –                            |
| CS_C9        | 1                  | CTCTCTAAAAATTTTCTATT225         | – |        | AAAA                            | TTTT            | CATTGAAAGAAAATACATCTTGCAG  |

Duplicated CS italicized

*nd*, not determined

*a* CS, coupling sequence; *P*, plasmid integration site; *C*, chromosomal integration site

*b* For CS_P5 and CS_C6 sequences adjacent to the transposon termini are provided

*c* Hits corresponding to the DO genome of *E. faecium*

*d* No amplification product in the inverse-PCR
While the structure of Tn1546, harboring vanA shows a high variability [34, 39], the vanY-vanX region in the vanB gene cluster appeared to be less divergent. In the studied collection, the A-type showed the highest prevalence, with variants very similar or identical to those found in E. faecium in Australia, France, and Taiwan [40–42], and in the pMG2200 plasmid of E. faecalis [5]. Importantly, the A variants are also present in gut anaerobes such as Clostridium spp. and E. lenta [26, 40], a presumable reservoir of Tn1549-type transposons. Genomic analyses of VanB-E. faecium and concomitantly isolated vanB-positive gut anaerobes indicated the epidemiological significance of de novo acquisition of Tn1549 by hospital-adapted E. faecium [7, 41]. The B-type characteristic for the first vancomycin-resistant E. faecalis V583 strain [33], to our knowledge, has not been reported in E. faecium so far. The presence of ISs targeting the vanS-vanY intergenic region (resulting in C- and F-types), was observed also elsewhere [43]. Such variability of vanB clusters may be useful in analyses of suspected VRE outbreaks. For example, plasmid-located D-type was found in isolates representing various MTs and STs from the WAW1 hospital (Table 1A). Thus, a spread of a stable ~ 150 kb conjugative plasmid of undetermined replicon type, harboring this specific variant of the vanB cluster was most likely responsible for the outbreak. Similarly, although isolates from SZC2 differed both in the clonal composition and vanB-associated plasmidome, C-type was detected in all these isolates (Table 1A), indicating extensive plasmid recombination during an outbreak. Until now, more detailed knowledge of plasmids carrying vanB in E. faecium remains limited [38]. In our study, vanB-plasmids represented mostly the rep$_{pLG1}$, rep$^{17}_{pRUM}$, and rep$^{2}_{pRE25}$ replicons, similarly to the situation observed for vanA-plasmids in Poland [34]. The original pLG1 contained the complete vanA gene cluster [44] and plasmids with this rep were responsible for an increase of HLGR among E. faecium in Norway [45] but, to our knowledge, vanB-plasmid of the rep$_{pLG1}$ type has not yet been reported. The second observed rep type, rep$^{17}_{pRUM}$ was involved in a multicenter VanB outbreak in Sweden [46] and in the HLGR spread in Norway [45]. The rep$_{pLG1}$ and rep$^{17}_{pRUM}$ genes frequently occurred together and in combination with the axe-txe, characteristic for plasmids with these replicons [34, 45, 46]. Plasmids harboring vanB were typically transferable by conjugation and during outbreaks (e.g., in KRA2, WAW1 and SZC2) were associated with diverse clonal backgrounds. Such plasmid dissemination was additionally accompanied by presumable recombination/co-integration events, resulting in the observed variability of vanB-plasmids. A similar dynamics was observed also for rep$^{17}_{pRUM}$-type vanA-plasmids [30]. Recombination/co-integration likely contributed to the association of vanB with more than a single rep, observed in the current study and characteristic for E. faecium plasmids in general [30, 34, 38, 45]. Two plasmid-located genes, aacA-aphD and citH, showed the integration of Tn1549 with different coupling sequences and might represent transposon integration hotspots. Such hotspots were indeed observed for E. faecium [7].

Isolates with plasmid-borne vanB prevailed until 2006, and later this determinant showed usually a chromosomal localization. This change occurred in parallel with the emergence and spread of lineage 78. The predominantly chromosomal localization of the vanB cluster in lineage 78 was observed recently also in Germany and Australia [7, 8, 40]. Two variants of coupling sequences, CS_C1 and CS_C3, were associated with two most numerous groups of isolates (Table 1). Twenty-one isolates with the CS_C1 variant, present in 17/18 lineage and A1 type of the vanY-vanX region, showed multicenter distribution over 2003–2008. These isolates showed some divergence of their STs/MTs, which may be explained by a transfer of transposon-containing region to a new clonal background [8] and/or exchange of other genomic regions, leading to formation of new STs/MTs [9]. An even more complex epidemiological situation was associated with isolates harboring the CS_C3 variant. This particular group appears to be the main contributor to the increasing proportion of VanB among VREfm and general increase of prevalence of VRE in Poland and was responsible for extensive outbreaks, e.g., in KSZ and POZ2 hospitals. With the exception of a single isolate with CS_C5, which shared a coupling sequence and insertion site with several ST192 isolates from Germany [8], none of the remaining coupling sequences showed identity to coupling sequences described elsewhere [7–9]. This finding is consistent with proposed independent de novo acquisition of Tn1549 [7].

This study provides an analysis of VanB-E. faecium, performed on a country level and over an extensive period of time. We demonstrate a significant change both in the clonal background as well as localization of Tn1549-type transposons, carrying vanB genes. Our study supports the role of lineage 78 of the hospital-adapted E. faecium, presumably acquiring de novo the vanB determinant, followed by spread and differentiation of certain strains as a major factor beyond the current increasing prevalence of VanB-VREfm in Polish hospitals.

Acknowledgements We thank all microbiologists who provided isolates to the National Reference Centre for Antimicrobial Resistance and Surveillance in Warsaw and Dr. Kenneth Van Horn for a critical reading of the manuscript. This publication made use of the Enterococcus faecium MLST website (http://pubmlst.org/efaecium/) sited at the University of Oxford and funded by the Wellcome Trust.

[Parts of this study were presented at the Applied Bioinformatics & Public Health Microbiology Conference, 15-17 May 2013, Cambridge, UK; the Microbiology after genomic revolution: Genomes 2014 EMBO Conference, 24-27 June 2014, Paris, France and the International Conference on the Evolution and Transfer of Antibiotic Resistance, 24-26 June 2015, Amsterdam, The Netherlands.]

Funding This work was supported by grant N N401588540 from the National Science Centre (NCN), Poland, by the MIKROBANK funding from the Ministry of Science and Higher Education, Poland, and by...
statutory funds from the Ministry of Science and Higher Education, Poland.

**Compliance with ethical standards**

Isolates were obtained as a part of routine activity of the NRCARS and were analyzed anonymously in a retrospective manner. Ethical approval and informed consent were thus not required.

**Conflict of interest** The authors declare that they have no conflict of interest.

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