Clustering of polyclonal VanB-type vancomycin resistant *Enterococcus faecium* in a low-endemic area was associated with CC17-genogroup strains harbouring transferable *vanB2*-Tn*5382* containing pRUM-like plasmids with *axe-txe* plasmid addiction systems

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Running title: Outbreak of *vanB2*-Tn*5382*-pRUM-like plasmid in CC17

ABSTRACT

VanB-type vancomycin-resistant *Enterococcus faecium* (VREfm) isolates (n=17) from 15 patients at the Örebro University hospital in Sweden during 18 months was characterized. All patients had underlying disorders and received broad-spectrum antimicrobial therapy. Pulsed-field gel electrophoresis (PFGE) grouped 14 isolates in three PFGE-types and three isolates in unique PFGE-patterns. All isolates had multi-locus sequence-types (ST17 (n=5); ST18 (n=3); ST125 (n=7); ST262 (n=1); ST460 (n=1)) belonging to the successful hospital adapted clonal complex 17 (CC17), harboured CC17-associated virulence genes, were *vanB2*-positive and expressed diverse vancomycin MICs (8 to >256 mg/L). Isolate 1 had a unique PFGE-type and a chromosomal transferable *vanB2*-Tn*5382* element. Interestingly, the other five PFGE-types had Tn*5382* located on pRUM-like plasmids containing a plasmid addiction system (*axe-txe*) shown by co-hybridization analysis of PFGE-separated S1-nuclease digested total DNA. The resistance-plasmids were mainly of 120-kb and supported intraspecies *vanB*-transfer. In patient 6 both PFGE type III ST17 and later PFGE-type I ST125 were isolated. The PFGE-type I ST125 was subsequently isolated from patients 9 to 11 and 13 to 15. Our observations support the notion that *vanB*-type VREfm can persist in a low-endemic area through successful clones and plasmids with stability functions in hospital patients with known risk factors.

INTRODUCTION

Enterococci are part of the normal bacterial intestinal flora and usually of relatively low virulence. However, they may cause infections in wounds, urinary tract, and abdomen. In addition, they rarely cause more serious infections such as bacteraemia and infective endocarditis. Enterococci display several properties that enable them to colonize and infect patients as well as to persist on inanimate surfaces (1-3), medical equipment (4) and spread in a hospital environment. They have a remarkable ability to resist extreme environments (5) and are able to survive disinfectants such as chlorine, gluteraldehyde and alcohol (6-8). It has been shown that enterococci may survive on a variety of hospital surfaces, including cotton and polyethylene for more than 90 days (2). Moreover, enterococci express intrinsic resistance or reduced susceptibility to important and commonly used antibiotics such as aminoglycosides, cephalosporins, clindamycin, quinolones, trimethoprim and sulphonamides (9). The global

increase in acquired high-level resistance to aminoglycosides and ampicillin in *E. faecium* has compromised their important synergistic bactericidal effect in the treatment of systemic infections paving the way for alternative last resort antibiotics such as vancomycin (10-12).

However, the prevalence of vancomycin-resistant enterococci (VRE) has increased significantly over the years since the first detection in Europe in 1986 (13, 14) (http://www.rivm.nl/earss/Images/EARSS%202007_FINAL_tcm61-55933.pdf). There are nine recognized genotypes of vancomycin resistance in enterococci *vanA-E* and *vanG, vanL* (15), *vanM* (16), and *vanN* (17). Transferable vancomycin resistance in clinical isolates of enterococci is primarily linked to the acquisition of *vanA* or *vanB* gene clusters. The *vanA* cluster is carried on Tn*1546*-like elements which are typically located on conjugative plasmids (18) and mediates high-level resistance to both vancomycin and teicoplanin (VanA-type) (19). The *vanB* cluster can be located on the chromosome or on plasmids (20-25) and mediates low to high level resistance to vancomycin only (VanB-type) (19). The *vanB2* subtype cluster is the most widespread *vanB*-genotype and has been shown to be an integral part of the conjugative transposon Tn*1549*-/Tn*5382*-like (24-27). A majority of transferable vancomycin resistance in hospital associated enterococcal infections has been associated with a specific subpopulation of *E. faecium*, designated clonal complex 17 (CC17) (11, 28).

The Nordic countries have been considered a low-endemic area with respect to human infections with VRE (http://www.rivm.nl/earss/Images/EARSS%202008_final_tcm61-65020.pdf). In Sweden, the first VRE cluster was reported in 1997 in Örebro county comprising four hospitalized patients with VanA-type *E. faecium* (29). In 2002 there was a new cluster of VRE cases observed in Örebro County. From November 2002 to April 2004 a total of 15 hospitalized patients were identified with VanB-type VRE-infections or colonization. Thus, Örebro County reported the highest incidence of VRE in Sweden during that period. Consequently, it was of interest to perform a molecular characterization of the strains. Extensive infection control measures were implemented, and during 2006 only one VRE-case was reported in Örebro County and none during 2005, 2007, 2008, and 2009.

The objectives in this study were to investigate the clustering of vancomycin resistant *E. faecium* at the Örebro University Hospital between 2002 and 2004. We used clinical and demographic data to identify potential risk factors. The strains were thoroughly characterized with regard to clonal relatedness and mobile genetic elements involved.

MATERIALS AND METHODS

Bacterial isolates. During the study period from November 2002 to April 2004 vancomycin resistant *E. faecium* (VRE) isolates were recovered from 29 samples from 15 patients (VRE-cases). Seventeen isolates were selected for molecular analyses.

Clinical and epidemiological data. Essential epidemiological and clinical information on each patient had been collected according to the Swedish Communicable Diseases Act and was available in a county database. This included information about age, gender, and demographic risk factors (referral department, prolonged hospitalization (> 2 weeks), ICU-stay, proximity to a hospitalized patient with VRE). Underlying disorders were searched for as well as prior antimicrobial therapy with vancomycin, cephalosporins, fluoroquinolones, aminoglycosides or metronidazole during the last three month.

Bacterial identification and susceptibility testing. The bacterial strains were isolated and identified using routine diagnostic procedures. Final species identification was confirmed by PCR as previously described (30). The minimum inhibitory concentration (MIC) of vancomycin, teicoplanin and trimethoprim were determined using the Etest (AB Biodisk, Solna, Sweden). The plates were incubated at 36°C and read after 24 and 48h. Clinical breakpoints for antimicrobial susceptibility were according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.escmid.org/research_projects/eu_cast/).

Pulsed-field gel electrophoresis (PFGE). Chromosomal DNA extraction and restriction enzyme digestion for PFGE were prepared as described for the GenePatch Group 1 reagent Kit (Bio Rad, Hercules, CA, USA) with some modifications according to Saeedi *et al.* (31). Briefly, DNA was prepared in agarose plugs and digested by *Sma*I restriction enzyme (Bio Rad) before separation of DNA-fragments using the GenePatch System (Bio Rad), 1% agarose gel (ultra pure DNA grade agarose). The results of the PFGE patterns were processed using Molecular Analyst Fingerprinting software (v. 1,6; Bio-Rad) followed by interpretation according to Carrico *et al.* (32). Larger than 81% threshold similarity value of Dice dendrogram was used to designate type (Capital roman number) and larger than 97% to designate subtype (small letter).

MLST typing and detection of virulence genes. The isolates were investigated for Clonal relationship by Multi Locus Sequence Typing (MLST) using the following primers; adk1n, adk2n, atpA1n, atpA2n, ddl1, ddl2, gdh1, gdh2, gyd-1, gyd2, pstS1n, pstS2, purK1n, and purK2n (33) (http://efaecium.mlst.net/misc/info.asp). Detection of the following *E. faecium* virulence genes were achieved by PCR; *esp* (34), *hyl* (35), *acm* (36), *Efa*A*fm* (primers 5´-GTTCGATAACTTGATGGAAAC-3´ and 5´- CATCTGATAGTAAGAATCTCCTTG-3´), *sgrA*, and *ecbA* (37).

Detection of *van* genes. *vanA* and *vanB* detection were performed using a duplex real-time PCR (LightCycler 2.0; Roche Applied Science, Mannheim, Germany) and oligosequences in accordance with Palladino *et al*. (38). Briefly, total genomic bacterial DNA was used as template for amplification in a PCR mixture containing LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science), 4 mM of $MgCl_2$, 0.7 µM of the forward primers (VanAF and VanBF), 1.0 µM of the reverse primers (VanAR and VanBR), and 0.3 µM of each probe. The cycling parameters were 95°C for 10 min and 40 cycles of 95°C and 53°C for 15 s and 72°C for 25 s. *E. faecium* (CCUG 36804; *vanA*), *E. faecium* (CCUG 33829; *vanB*) and water were used as positive and negative controls.

***vanB* subtyping and linkage to Tn*5382*.** *vanB* gene subtyping, $vanX_B$ and Tn*5382* ORFC linkage, and *pbp5* gene and Tn*5382* linkage were examined as previously described (24, 26, 39).

pRUM replicon detection. Plasmid pRUM replicon detection was performed by PCR (40) using *E. faecium* U37 as positive control (41).

S1-nuclease PFGE, Southern transfer and hybridisation. To expose plasmid-located *vanB* genes and explore their linkage to pRUM replicons agarose plugs containing genomic DNA was digested with S1-nuclease. DNA fragments were separated by PFGE before Southern blot and sequential hybridisation with *vanB*, pRUM and *axe-txe* probes using the DIG-Luminescent Detection Kit (Roche Applied Science) (42). Genomic DNA from *E. faecium* U37 (41) and *E. faecium* TUH2-19 (24, 43) were used as templates for probe synthesis for pRUM *repA*, *axe-txe*,

and *vanB*, respectively. *vanB* consensus primers (39), pRUM-F and pRUM-B (40), as well as *axe-txe*F and *axe-txe*R were used (42). *E. faecium* DO (44) and TUH44-39 (45) were used as positive and negative control for pRUM and *axe-txe*, respectively. *E. faecalis* V583 (46) or *E. faecium* TUH2-19 (24, 43) and *E. faecium* BM4105-RF (47) were used as positive and negative controls respectively, for *vanB* hybridisation.

Conjugative transfer of *vanB*. Selected isolates were investigated for *vanB*-transfer by filter-mating according to Dahl *et al*. (26) with some modifications using *E. faecium* BM4105-RF (47) as recipient strain. The strains were selected to cover all PFGE and ST types present. Briefly, donor and recipient cultures were mixed in a 1:1 ratio to a total volume of 1 ml, centrifuged at 10.000 x g for 10 min and resuspended in 150 µl BHI. Suspensions of 50 µl were transferred to 0.45 µm nitrocellulose filters on BHI agar. Transconjugants were analysed by S1-nuclease (25U, Takara Bio Inc, Shiga, Japan) PFGE and *vanB*-hybridisation.

RESULTS

Patient characteristics. The patients (cases), bacterial isolates and their characteristics are given in Table 1. Briefly, the average age of the 15 patients, 8 male and 7 female, was 60.3 years (range 37-89). Data on prior antimicrobial therapy was unavailable for one patient (Case 1). The patient had been transferred from another hospital after renal transplantation. The mean average time from admission to hospital to the first positive culture yielded VRE was 15.2 days (range 0 – 47 days) excluding two out-patients (case 8 and 11) and case 1 that was already infected with VRE on arrival when transferred from the referral hospital. Almost all patients had underlying diseases. During the last three months all patients had been treated with antimicrobial agents such as vancomycin (n=6), cephalosporins (n=8), fluoroquinolones (n=6), aminoglycosides (n=3) or metronidazole (n=7). Ten patients had received treatment with at least two of those antimicrobial agents. Thirteen patients were considered to have a clinical VRE-infection while faecal colonization was detected in the remaining two patients (case 3 and 4). VRE were isolated from blood (n=3), wounds or abscesses (n=9), urine (n=3), ascites (n=1), a tip from a urinary catheter (n=1), and faeces (n=11) (Table 1). Isolates with significant different vancomycin MICs ($\geq$ 4 fold) were found in 2 patients. Thus, 17 isolates from 15 patients were included in the molecular analyses.

Identification and susceptibility testing of *vanB E. faecium*. All 17 isolates were confirmed as *vanB* positive and *vanA* negative *E. faecium*. Vancomycin MICs varied between 8 to >256 mg/L (Table 1). Thirteen isolates expressed vancomycin MICs between 8 to 48 mg/L. All isolates were susceptible to teicoplanin. Ampicillin MICs varied between 16 and >256 mg/L. Four isolates showed high level ampicillin resistance (>128 mg/L). All isolates except one (case 4) showed high level resistance to ciprofloxacin (>32 mg/L). High level gentamicin resistance was not detected. Four isolates showed *in vitro* susceptibility (0.125 to 0.25 mg/L) to trimethoprim (Table 1).

Clonal relatedness and detection of virulence genes. Fourteen isolates belonged to three PFGE types, I (n=8); III (n=4); V (n=2). Isolates 1, 7, and 8 showed unique PFGE patterns (II, IV, and VI) (Table 1 and Fig. 1). Briefly, isolates 2a, 2b, 5, and 6a, belonged to the same PFGE type III, but showed minor band differences (>81% but <97% similarity) and were thus considered subtypes. Isolates 3 and 4 showed indistinguishable patterns (PFGE type V). Isolates 9 to 15 and 6b belonged to PFGE type I, subtypes a to d; isolates 13 and 14 (subtype Ia): 9, 10, and 15 (subtype Ib); 6b and 11 (subtype Ic); 12 (subtype Id). PFGE subtype Ia and Ib isolates originated from patients who shared ward rooms.

MLST showed that all the VRE isolates belonged to the CC17 genogroup. PFGE types I, V and VI belonged to Sequence Type (ST) 18 or Single Locus Variants (SLVs; ST125 and 262). PFGE types II, III and IV all shared the same ST17 or an SLV (new ST460; isolate 6a) (Table 1 and Fig. 1).

The presence of enterococcal virulence genes including enterococcal surface protein (*esp*), the cell-wall adhesin (*efaAfm*), hyaluronidase (*hyl*), and several genes encoding cell-wall anchored surface proteins that binds to extracellular matrix molecules (*acm*, *sgrA*, *ecbA*) were examined by specific PCRs. All 17 isolates contained *efaAfm* and *sgrA*. Most of them also scored positive for *acm* (n=15) and *ecbA* (n=14). The *ecbA*–positive isolates were of PFGE type I, II, III and IV and belonged to ST17, ST18, ST125, and ST460. The *acm*-gene was present in all PFGE and ST types. The *hyl*-positive isolates (n=6) were of PFGE type I, III and IV and belonged to ST17 (n=3), ST125 (n=2) or ST460 (n=1). The *esp* gene was detected in isolate 8 (PFGE type VI and ST262) only (Table 1).

Detection of *vanB2*-Tn*5382* on transferable pRUM-like plasmids with *axe-txe* plasmid addiction system. All isolates scored positive for *vanB2* as an integral part of Tn*5382*. Linkage between *pbp5* and Tn*5382* was not detected by PCR. Fourteen isolates representing all PFGE types and subtypes were examined for plasmid and/or chromosomal localization of *vanB2*-Tn*5382*. PFGE of S1-nuclease digested total DNA showed that the isolates contained two to eight plasmids in the range of <10 to >300 kb (data not shown). Thirteen isolates (2a, 2b, 3, 4, 5, 6a, 6b, 7, 8, 9, 12, 13 and 15) supported *vanB2*-plasmid hybridization whereas one isolate (isolate 1) did not (Fig. 1). Twelve isolates (2a, 2b, 3, 4, 6a, 6b, 7, 8, 9, 12, 13 and 15) contained similarly sized *vanB2*-positive plasmid bands of approximately 120-130 kb. Several of these isolates supported *vanB2-Tn5382*-hybridization to additional plasmid bands ranging in size from 50 to 320 kb (Fig. 2). This could be due to *vanB*-positive co-integrates or different plasmid forms.

The isolates were further examined for the presence of pRUM-like replicon previously shown to harbour a segregation stability module encoded by a toxin-antitoxin cassette (*axe-txe*) (41). Both PCR and hybridisation analyses showed that all strains (except isolate 1) contained pRUM-like *repA*. *axe-txe* hybridisation was performed on 9 isolates representing all PFGE types (data not shown). Co-hybridization of pRUM *repA* and *axe-txe* probes was observed to all *vanB*-positive plasmid bands. Examples of *vanB2*-pRUM *repA* co-hybridization are given in Fig. 2. Isolate 1 showed positive hybridisation to only a large *vanB2*-Tn*5382* location presumably chromosomal fragment (>650 kb) (Fig. 2, lane 9) and did not support hybridization with pRUM-like *repA* (Fig. 2, lane 9) or *axe-txe* probes (data not shown).

Selected isolates (1, 2a, 2b, 3, 4, 5, 6a, 6b, 7, 8, 9, 12, and 13), representing all PFGE types were all shown to support *vanB* transfer with transfer rates ranging between $2x10^{-3}$ to $9x10^{-11}$ transconjugants per donor (TC/D) (data not shown). Isolates 1, 6b, 12, and 13 showed the lowest transfer frequencies of 10^{-11} TC/D. Transfer rates for isolates 2a, 6a, 3, 4, 7, 8, and 9 varied between 10^{-6} to 10^{-8} TC/D, whereas isolates 2b and 5 both of PFGE type III, supported high transfer frequencies (10^{-3} TC/D). S1-nuclease PFGE and *vanB2*/pRUM *repA* hybridisation analyses confirmed transfer of similar sized *vanB2* pRUM-like plasmids between donors and recipient (examples given in Fig. 2, lanes 1-8). Chromosomal to chromosomal transfer of *vanB2*-Tn*5382* was shown for isolate 1 (Fig. 2, lanes 9 and 10).

DISCUSSION

In the present study we have examined the clustering of *vanB*-type VRE infections and/or colonization in 15 hospitalized patients in a low endemic area in Sweden during an 18 months period from 2002 to 2004. All patients showed underlying diseases or predisposing conditions, such as renal insufficiency, haematological malignancies or other malignancies, immunosuppression, neutropenia and organ transplant recipient. Exposure to vancomycin, cephalosporins, fluroquinolones and/or metronidazole as well as prolonged hospital and ICU stay and exposure to VRE-colonized patients have been shown to be associated with increased risk for acquisition of VRE (9, 48, 49). All patients in this study had received prior antibiotic treatment. The majority of patients (n= 10) had been treated with at least two of the above mentioned antibiotics. All patients were hospitalized for more than 2 weeks before diagnosing VRE-infection or colonization.

PFGE characterization revealed a polyclonal collection with three clusters (PFGE type I, III, and V) and three unique patterns (PFGE type II, IV, and VI). Some isolates with similar PFGE-patterns were isolated from patients within the same department. Type III and V isolates were recovered from patients at the Department of Nephrology and Haemodialysis. Similar associations were confirmed for case 9 and 10 (PFGE type Ib) at the Department of Haematology as well as case 13 and 14 (PFGE type Ia) at the Department of Surgery. For the other patients no clear epidemiological association was observed. Transmission of VRE has previously been shown to occur via contaminated medical equipment and environmental surfaces, and directly via patients or indirectly through health care workers via transiently contaminated hands and clothes (2-4, 9, 50)

The MLST results were in accordance with the PFGE-patterns. All STs clustered within CC17-related strains. Some isolates representing different subtypes within PFGE type I and III displayed SLVs of ST18 and ST17, respectively. Interestingly, ST125 first recovered from case 6, was subsequently the dominant ST and recovered from six additional patients of which five isolates showed minor differences in PFGE-patterns. Population analysis of *E. faecium* has revealed a high rate of recombinations (11). Moreover, high mutation rates have been described in CC17 strains compared to non-CC17 strains (51). Our observation of SLVs of prevalent STs (17 and 18) and corresponding PFGE-subtypes support the notion of local clonal diversification during the 18 months hospital clustering of VRE.

Detection of virulence determinants showed that all isolates contained *EfaAfm* and *sgrA* and most of the isolates harboured *ecbA* and *acm*. This was expected due to their association with CC17. The genes encode proteins experimentally shown to be involved in adhesion and/or biofilm formation that are supposed to be important for spread and persistence within the hospital environment (52). Six isolates were *hyl* gene positive that has been associated with enhanced colonisation of the mouse gastrointestinal tract (53). The *hyl* virulence determinant was initially described in clinical hospital isolates in the U.S. (35, 54) and subsequently in European hospitals (54). Only one isolate (case 8) in our study was shown to contain *esp*, encoding enterococcal surface protein involved in biofilm formation (55). This was somewhat surprising given that this gene is often found in CC17 strains (56) including 65% of the CC17-related isolates described in a recent Swedish report (57). Lack of *esp* and *hyl* has been described in early (around 1982) *E. faecium* outbreaks in the US where as in this study the hospital adapted CC17 isolates were more associated with putative pili or adhesin genes (58).

All VRE isolates were found to be *E. faecium* carrying the *vanB2* subtype as an integral part of the conjugative transposon Tn*5382* which is typical for the *vanB2* subtype (25-27, 59). MIC for vancomycin showed a broad range; 8 to >256 mg/L, with sustained susceptibility for teicoplanin which is characteristic for the VanB-phenotype. Further, all isolates were resistant to ampicillin which is typical for the CC17 hospital adapted genogroup (11, 28). All isolates except case 4 were high-level resistant to ciprofloxacin which is a trait previously shown to be linked to the CC17 genogroup (60).

Plasmids have an important role in the spread and maintenance of antimicrobial resistance determinants in enterococci (61). Recent progress in PCR-based typing methods targeting replicon-specific plasmid DNA has allowed molecular epidemiology studies of R-plasmids in enterococci (42). Interestingly, in this study the *vanB*-Tn*5382* element was shown to be integrated into a pRUM-like plasmid in most of the strains which supported intraspecies transfer of *vanB*. pRUM was originally described as a 25 kb non-conjugative multidrug resistant plasmid in a clinical isolate of *E. faecium* (24). Recently, pRUM-like plasmids were shown to be widely distributed in *E. faecium* strains and even more prevalent in CC17-related strains (42). Many plasmids ensure their stability within the host by different maintenance/addiction systems. In enterococci different TA systems, like ω-ε-ζ in pRE25 and the *axe-txe* in pRUM have been reported (41, 45, 62). The addiction system (*axe-txe*) of pRUM has been shown to support

plasmid stability in *E. faecium* (41). Thus, we speculate that the linkage of *vanB2*-Tn*5382* to the widespread and successful pRUM and *axe-txe* plasmid backbones have contributed to the dissemination and persistence of VRE in this setting. A similar type of enterococcal plasmid persistence encoding *vanA* has been observed in the farm animals exposed to avoparcin (45, 62).

The conjugative properties of Tn*5382* may also have contributed to the transferability of pRUM-like plasmids as well as chromosomal *vanB2*-Tn*5382*-like transfer in isolate 1 at a low frequency. The two isolates supporting the highest *vanB*-transfer rates belonged to PFGE type III ST17 from case 2 and 5. Previous studies suggest that transfer frequencies of *vanA* and *vanB* clusters can be even higher *in vivo* than *in vitro* especially when located on plasmids (63). In case 6 the pRUM-like transferable plasmid of approximately 120 kb containing *vanB2*-Tn*5382*-like was first found in a PFGE type III isolate (6a). The *vanB2*-Tn*5382*-like then appeared in a similar sized *vanB2*-Tn*5382*-like pRUM-like plasmid two months later in case 6 and for the first time during this study in a PFGE type I isolate (6b) suggesting *in vivo* intraspecies *vanB*-transfer. The subsequent dominance of PFGE type I strains during the last 7 months of this VRE-clustering suggested the establishment of a successful combination of a pRUM-like plasmid containing *vanB2*-Tn*5382*-like in a ST125 background.

High rates of faecal *vanB* carriage primarily of the *vanB2* subtype have been described in both community and hospital samples despite the absence of cultivable vancomycin resistant enterococci (64). The *vanB2* subtype seems to be the dominant *vanB* genotype in most studies (5, 15, 24, 26, 27, 65-70). This dominance is presumably related to its integral location in the conjugative transposon Tn*5382*-like. A study by Seville *et al.* (71) revealed that 5 of 6 faecal metagenomes contained a Tn*5382*-like integrase gene. The Tn*5382*-like elements containing *vanB2* have been identified in other bacterial species belonging to the normal intestinal flora such as *Clostridium, Ruminococcus, Eggerthella,* and *Streptococcus* (59, 72, 73). Tn*5382*-like has been transferred from *Clostridium* to *Enterococcus* in the gut of gnotobiotic mice during vancomycin exposure (73). Thus, also in a low endemic area vancomycin should be used with caution to prevent the establishment of VRE from Tn*5382*-like elements already present in the faecal flora.

We observe significant differences in vancomycin MICs between isolates with similar PFGE-types and similarly sized *vanB*-pRUM-like plasmids. From our experience (data not shown) transfer of a *vanB* element conferring high level vancomycin resistance may result in a

transconjugant with a low vancomycin MIC. Thus, in a polyclonal outbreak the vancomycin MIC values may vary considerably between isolates which should be considered when performing VRE-screening.

In summary, the molecular typing of *E. faecium* strains and the recent PCR-based replicon typing of enterococcal plasmids has allowed the identification of *vanB2*-Tn*5382*-like containing pRUM-like plasmids within a polyclonal population of CC17-related strains. Hospital clustering of VanB-type VRE in a low endemic area may involve both clonal spread as well as transfer of *vanB2*-Tn*5382*-like between clones as part of successful pRUM-plasmids containing a stability module enhancing its persistence.

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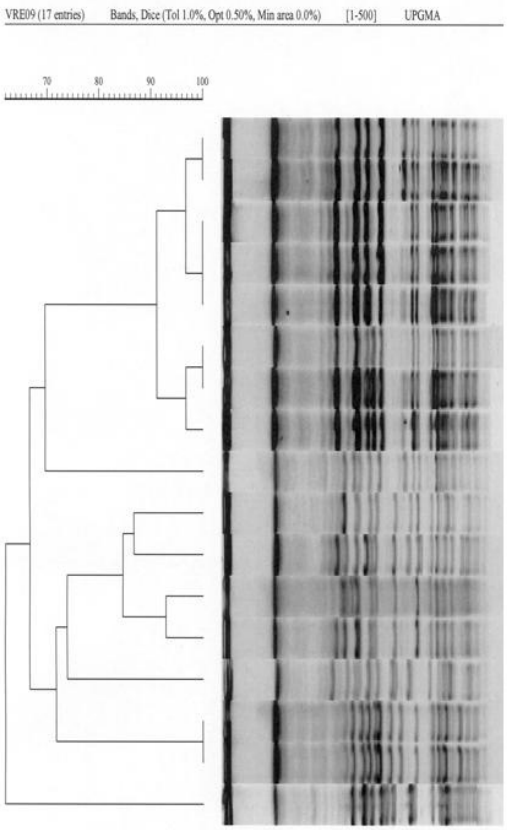

PFGE type	ST	Isolate designation	*vanB2*-Tn*5382* localisation	*axetxe*
Ia	125	13	pRUM-like plasmid	+
Ia	125	14	nd	nd
Ib	125	9	pRUM-like plasmid	+
Ib	125	10	nd	nd
Ib	125	15	pRUM- like plasmid	+
Ic	125	6b	pRUM- like plasmid	nd
Ic	125	11	nd	nd
Id	18	12	pRUM-like plasmid	nd
II	17	1	Chromosomal	-
IIIa	460	6a	pRUM-like plasmid	+
IIIb	17	5	pRUM-like plasmid	nd
IIIc	17	2a	pRUM-like plasmid	nd
IIId	17	2b	pRUM-like plasmid	nd
IV	17	7	pRUM-like plasmid	+
V	18	3	pRUM-like plasmid	+
V	18	4	pRUM-like plasmid	+
VI	262	8	pRUM-like plasmid	+

FIG. 1. PFGE dendrogram and profiles, isolate names, PFGE and sequence types and case numbers of the 17 vancomycin resistant *E. faecium* presented in this paper. Localisation of the *vanB2*-Tn*5382* element conferring vancomycin resistance and confirmed presence of *axe-txe* on pRUM-like plasmids is also shown in this figure. + = positive, - = negative, nd = not determined.

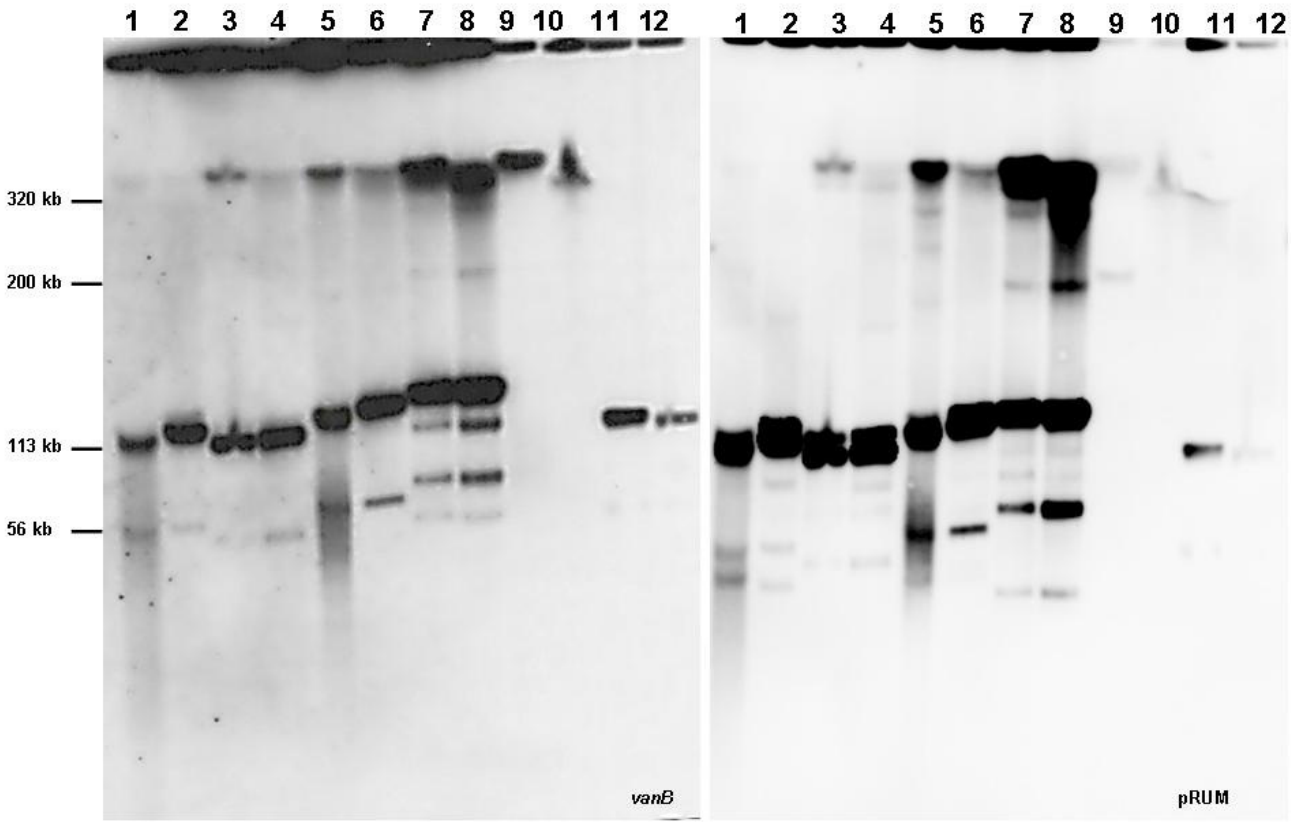

FIG. 2. Southern hybridisation with pRUM *repA* (left) and *vanB* probe (right) on S1 nuclease digested genomic DNA from donors and transconjugants obtained from matings with BM4105-RF as recipient. Lane 1, Donor 6a; Lane 2, Transconjugant 6a x BM4105-RF; Lane 3, Donor 7; Lane 4, Transconjugant 7 x BM4105-RF; Lane 5, Donor 8; Lane 6, Transconjugant 8 x BM4105-RF; Lane 7, donor 9; Lane 8, Transconjugant 9 x BM4105-RF; Lane 9, Donor 1; Lane 10, Transconjugant 1 x BM4105-RF; Lane 11, Donor 2a; Lane 12, Transconjugant 2a x BM4105-RF. *Sma*I digested V583 was used as marker.

TABLE 1. Epidemiological characteristics of vancomycin resistant *E. faecium* isolated at Örebro University Hospital from November 2002 to April 2004.

Case	Isolate designation in this study	Date of isolation	Age Sex	Antimicrobial therapy [b]	MIC (mg/L) [b] VAN	AMP	TEC	CIP	Hospital department [c]	Patient diagnosis [d]	VRE source [e]	Virulence genes [f]	PFGE type [g]	ST type [h]
1	1 (02B814)[a]	2002-11-01	49 M	Unknown	24	32	>32	>32	Infection	Organ transplantation, DM, IS	**blood**, abscess, faeces	*acm*, *ecbA*	II	17
2	2a (02T878)	2002-12-12	65 M	F, M, V	96	48	0.125	>32	Nephrology, HD	RI, DM	**wound**	*acm*, *hyl*, *ecbA*	IIIc	17
2	2b (03T069)	2003-01-27			16	>256	0.25	>32			**faeces**	*acm*, *hyl*, *ecbA*	IIId	17
3	3 (03T039)	2003-01-15	59 M	AG, C, V	24	96	>32	>32	Nephrology, HD	RI, staphylococcal septicaemia	**faeces**	*acm*	V	18
4	4 (03T119)	2003-02-20	56 F	C, F	24	96	>32	1,5	Nephrology, HD	RI, IS	**faeces**	*acm*	V	18
5	5 (03T118)	2003-02-20	37 M	C, M	16	96	>32	>32	Nephrology, HD	RI, DM	**wound**, faeces	*ecbA*	IIIb	17
6	6a (03T004)	2003-01-03	49 M	F,M,V	>256	16	>32	>32	Nephrology, HD	RI, DM	**wound**, faeces	*acm*, *hyl*, *ecbA*	IIIa	460
6	6b (03T213)	2003-03-18			12	>256	>32	>32			**wound**	*hyl*, *ecbA*	Ic	125
7	7 (03T418)	2003-06-27	48 M	AG, C, V	48	48	0.125	>32	Nephrology, ICU, Infection	DM with hyperosmolality, sepsis, endocarditis	**urine catheter**, wound, faeces	*acm*, *hyl*, *ecbA*	IV	17
8	8 (03T468)	2003-07-21	64 F	V, F	>256	64	0.19	>32	Nephrology HD	RI, DM	**urine**	*acm*, *esp*	VI	262
9[i]	9 (03T643)	2003-10-07	55 M	AG, C, M, V	>256	>256	>32	>32	Haematology	Haematologic malignancy, neutropenia, IS	**abscess**	*acm*, *ecbA*	Ib	125
10[i]	10 (03B699)	2003-10-25	54 M	F	16	128	>32	>32	Haematology	Haematologic malignancy, neutropenia, IS	**blood**, faeces	*acm*, *ecbA*	Ib	125
11	11 (03T733)	2003-11-06	89 F	C	24	96	>32	>32	Outpatient (Orthopedics)	Wound infection, recent surgery	**wound**	*acm*, *ecbA*	Ic	125
12	12 (03T734)	2003-11-10	46 F	M	8	>256	>32	>32	Gastro-enterology	Hepatic failure, ascites drainage	**ascites**, faeces	*acm*, *ecbA*	Id	18
13[i]	13 (04B252)	2004-04-01	56 F	F, M	24	64	>32	>32	Surgery, ICU	Rectal cancer, postoperative perianal abscess	**blood**, abscess	*acm*, *ecbA*	Ia	125
14[i]	14 (04T227)	2004-04-08	88 F	C, M	32	128	>32	>32	Surgery, ICU	Ileal bladder, hip replacement operation	abscess, urine, **faeces**	*acm*, *ecbA*	Ia	125
15	15 (04T217)	2004-04-06	89 F	C	32	64	>32	>32	Infection	Infection of unknown origin, DM	**urine**, faeces	*acm*, *hyl*, *ecbA*	Ib	125

[a] Reference number at Örebro University Hospital in parenthesis.

[b] AG= aminoglycosides, C=cephalosporins, F= fluroquinolones, M= metronidazole, VAN=vancomycin (MIC breakpoint R > 4 mg/L), AMP= ampicillin (MIC breakpoint R > 8 mg/L), TEC= Trimethoprim (MIC breakpoint R > 1 mg/L), CIP=ciprofloxacin (MIC breakpoint for high level R >32 mg/L)

[c] HD=haemodialysis unit, ICU=intensive care unit

[d] DM=diabetes mellitus, IS=immunosuppression, RI=renal insufficiency

[e] Isolates selected for further analysis are given in bold.

[f] All isolates were positive for *EfaAfm* and *sgrA* in addition to the virulence gene results showed in this table.

[g] The PFGE types and subtypes have been determined according to Carrico *et al.* (7). 81% threshold similarity value of Dice dendrogam is used to designate type (Capital roman number) and 97% to designate subtype (small letter).

[h] ST460 is a novel single locus variant (SLV) of ST17, while ST125 and ST262 are SLVs of ST18

[i] Case 9 and 10 and Case 13 and 14 shared ward room