ANTIMICROBIAL AGENTS AND RESISTANCE

Characterisation of VanA and VanB elements from glycopeptide-resistant *Enterococcus faecium* from Greece

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Ten glycopeptide-resistant *Enterococcus faecium* isolates from separate patients in Laikon General Hospital, Athens were studied. Eight isolates had the VanA phenotype and represented variants of three strains based on *Sma*I macrorestriction banding patterns. Their VanA elements were compared with the prototype element, Tn1546, by an overlapping PCR method. Three related isolates contained resistance elements indistinguishable from Tn1546 (designated Greek type I). The other five isolates all contained identical elements that differed from Tn1546 by the presence of IS1251 between *vanS* and *vanH*, by a point mutation (G → T) at nucleotide position 8234 within *vanX* and by a partial loss of transposition gene *orf1* (designated Greek type II). Two distinct strains of *E. faecium* with the VanB phenotype were obtained. *Hha*I digestion of an amplified fragment of the *vanB* gene indicated that both strains contained the *vanB2* allele, and further PCR assays confirmed that the *vanB2* gene cluster was located within a Tn5382-like element.

Introduction

The spread of glycopeptide-resistant enterococci (GRE) has caused increasing therapeutic problems in the USA and some parts of Europe, including the UK [1]. Enterococci may have intrinsic or acquired resistance to glycopeptides. Intrinsic resistance is represented by the VanC phenotype of *Enterococcus gallinarum* and *E. casseliflavus*/*E. flavescens*, and is due to the presence of a modified terminal dipeptide (D-alanyl-D-serine, instead of D-alanyl-D-alanine) in the peptidoglycan precursors. To date, four phenotypes of acquired glycopeptide resistance have been reported. Enterococci with the VanA, VanB and VanD phenotypes have peptidoglycan precursors that terminate in the depsipeptide D-alanyl-D-lactate [1], whereas VanE strains have D-alanyl-D-serine [2]. The vast majority of clinical problems caused by GRE are associated with isolates of *E. faecium* with the VanA or VanB phenotypes.

The prototype VanA element is the 10.8-kb transposon Tn1546, which carries the *vanRSHXYZ* genes and transposition genes *orf1* and *orf2* [3]. VanA elements among UK isolates have been divided into 24 distinct groups [4], and further elements have been described in isolates from other countries. Each of these diverse elements shares the *vanRSHAX* cluster [5] and is believed to have evolved from a Tn1546 progenitor [6]. Sequences of the resistance genes are remarkably conserved, with only a few point mutations identified [6, 7]. Rather, VanA elements vary by the presence of deletions and insertion sequences (IS) in the non-essential genes (*orf1*, *orf2*, *vanY* and *vanZ*) and the intergenic regions [5–9]. Thus, pinpointing IS elements may be useful for monitoring the dissemination and evolution of particular VanA elements [10].

In contrast, the *vanB* gene clusters that are responsible for the VanB phenotype show greater sequence divergence. Three *vanB* alleles, designated *vanB1*, *vanB2* and *vanB3*, have been described [11–13]. The *vanB2* gene cluster appears to be the most widespread form among *E. faecium* in both the USA [14] and Europe [11, 15]. The *vanB2* gene cluster has been associated with two closely related transposons, Tn5382 [16, 17] and Tn1549 [18].

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This paper reports the characterisation of eight VanA and two VanB isolates of *E. faecium* and their glycopeptide resistance elements from Laikon General Hospital in Athens.

Materials and methods

Isolates

Between April 1998 and Sept. 1999, 10 isolates of *E. faecium* with VanA or VanB glycopeptide resistance were isolated at Laikon General Hospital in Athens. Eight VanA isolates included three from clinical samples of separate patients and five from rectal swabs of patients in the ICU and haematology units. Two VanB isolates were from clinical samples of patients in the ICU. These isolates were included in the present study for the molecular characterisation of their VanA and VanB elements. Strains used as controls were: *E. faecium* BM4147 containing Tn1546 [3]; *E. faecium* Tn#3 with a VanA element assigned to group D (from a study that defined 24 distinct VanA elements present in GRE from the UK) [5]; *E. faecalis* ATCC 51299 with the *vanB1* allele [15]; and *E. faecium* strains D-002 and 2781 with *vanB2* alleles [15]. No control strain with the *vanB3* allele was available for inclusion in this study.

Species identification and genotype determination

Species identification and glycopeptide resistance genotypes were confirmed by the multiplex PCR methods described by Dutka-Malen *et al.* [19] and Woodford *et al.* [20], respectively. The identification assay was modified by the addition of the primers specific for *ddl_{E. avium}* (*E. avium*-A 5'-GTT CCA GTC CTA AAA AAC C-3' and *E. avium*-B 5'-GCT TCT TCC TTT ACG ACC-3'), designed to amplify a 290-bp fragment corresponding to nucleotides 121–411 of GenBank U39774.

Susceptibility testing

The susceptibilities of isolates to vancomycin and teicoplanin were determined on Diagnostic Sensitivity Test (DST) Agar (Oxoid) supplemented with lysed horse blood 5%. The inoculum was 10^4 – 10^5 cfu/spot. MICs were read after incubation for 18 h at 37°C.

DNA banding pattern polymorphism

Pulsed-field gel electrophoresis (PFGE) of *Sma*I-digested genomic DNA was performed by a modification of a method described previously [21]. Electrophoresis was performed by the contour-clamped homogeneous electric field method with a CHEF-DR11 drive module (BioRad, Hemel Hempstead, Hertfordshire) at 200 V for 30 h, with a ramp rate of 1–35 s through agarose 1.2% at 12°C. Banding patterns were analysed with GelCompar software (Applied Maths,

Kortrijk, Belgium). The patterns were interpreted initially in accordance with the general criteria proposed by Tenover *et al.* [22]; thus, isolates showing three or fewer band differences were regarded as closely related and those with four-to-six differences as possibly related. Patterns were also considered according to the specific recommendations of Morrison *et al.* for *E. faecium*, which emphasised the large degree of PFGE band polymorphism possible among epidemiologically related isolates considered to belong to the same strain when analysed by multiple methods [23].

Characterisation of VanA resistance elements

VanA resistance elements were initially characterised by PCR with 10 pairs of overlapping primers [3]. The elements were compared with Tn1546 for the presence, absence and relative sizes of amplicons obtained with each primer pair as described previously [4].

A 424-bp fragment of the *vanX* gene (nucleotides 8082–8505 of Tn1546) was amplified by PCR and a G → T point mutation at position 8234 was sought by *Dde*I digestion of the amplicon as described previously [5].

Isolates were screened with primers designed to detect an IS1216V/IS3 complex and the linkage of IS3 with *orf1*, both of which have been found in VanA elements with the mutation at position 8234 [5, 7]. They were also screened with primers specific for IS1251 [7, 9]. An IS1251 amplicon was labelled with digoxigenin (Roche, Lewes) and used in hybridisation studies with Southern blots prepared from selected PCR amplicons under stringent conditions at 68°C. Hybrids were detected colorimetrically.

Characterisation of VanB resistance elements

A 589-bp fragment of the *vanB* gene [24] was amplified and digested with *Hha*I to identify the particular allele present [15]. Based on analysis of sequences in GenBank, amplicons of *vanB1* alleles were predicted to give *Hha*I fragments of 323 bp and 266 bp; those of *vanB2* alleles, fragments of 429 bp and 160 bp; those of *vanB3* alleles, fragments of 357 bp, 160 bp and 72 bp. The amplification conditions were as described previously [24].

The association of *vanB2* alleles with a Tn5382-like element was sought by using two pairs of primers [16, 25]. A 311-bp fragment of the left end of Tn5382 was amplified with primers 5'-ACG CCA TGC TAT TTA CTT CCG GC-3' and 5'-GTT CTT ATT CCG CAG GTG GTG ATT-3'. A 1581-bp fragment containing *vanXB2* and part of the right end of Tn5382 was amplified with primers 5'-TTG CAT GGT GTT CGT TGG-3' and 5'-CGG CAT CAA CGC CTT TAG-3'.

Results

Species identification, genotyping and susceptibility testing

The 10 isolates of GRE included in this study were all confirmed as *E. faecium* in a multiplex PCR assay. The *vanA* gene was detected in eight isolates, with *vanB* detected in the two others. All isolates were resistant to vancomycin (MICs >32 mg/L). The glycopeptide resistance phenotype of each isolate was consistent with its genotype, i.e., *vanA* isolates were resistant to teicoplanin (MICs >32 mg/L), whereas *vanB* isolates were sensitive to teicoplanin (the MIC for one isolate was 2 mg/L and the MIC for the other was 4 mg/L).

Characterisation of GRE by PFGE

The eight VanA isolates showed seven macrorestriction banding patterns (Fig. 1a). Isolates 1 and 2 were closely related, but isolate 8 also showed a similar banding pattern to these and was considered to be related, although a skewed gel prevented its satisfactory normalisation. The remaining five VanA isolates each had different banding patterns and were considered to be distinct by rigid interpretative criteria [22]. However, allowing for the banding polymorphism possible

between isolates of *E. faecium* known to belong to a single strain [23], isolates 3 and 4 and isolates 5, 6 and 7 were considered to represent variants of two strains. The two VanB isolates (9 and 10) gave PFGE banding patterns that were distinct from each other (Fig. 1b) and from the patterns observed among the VanA strains.

Characterisation of VanA resistance elements

Overlapping fragments, amplified with 10 pairs of primers, indicated that isolates 1, 2 and 8 contained VanA elements indistinguishable from Tn1546 (Table 1). Interestingly, these isolates appeared related by PFGE. These elements were designated Greek type I. The remaining five VanA isolates had elements that had only eight of the 10 amplicons in common with Tn1546. None of these isolates gave a product with primer pair p1p2, which corresponds to nucleotides 22–1330 of Tn1546 (including part of *orf1* and of the left inverted repeat region), and all gave a larger amplicon than Tn1546 with primer pair p11p12, which span nucleotides 4675 (in *vanR*) –6353 (in *vanH*) of Tn1546 (Table 1). The elements from these five isolates were designated Greek type II.

Fig. 1. PFGE of *Sma*I-digested DNA of (a) VanA and (b) VanB enterococci from Laikon General Hospital. The mol. wt marker is a concatamer of phage λ DNA and the 48.5-kb band is marked with an arrow.

Table 1. Characteristics of VanA elements from clinical isolates of *E. faecium* from Greece, with control elements Tn1546 and group D

Isolate	Hospital unit	Amplification with specified primer pair*										Hybridisation of p11p12 product with IS/251-specific probe	Presence of G → T mutation at position 8234 (in <i>vanX</i>)	
		p1p2	p3p4	p5p6	p7p8	p9p10	p11p12	p13p14	p15p16	p17p18	p19p1			IS1216V-IS3
Tn1546 Group D														
1	ICU	+	+	+	+	+	+	+	+	+	+	+	+	+
2	ICU	+	+	+	+	+	+	+	+	+	+	+	+	+
3	ICU	+	+	+	+	+	+	+	+	+	+	+	+	+
4	Haematology	+	+	+	+	+	+	+	+	+	+	+	+	+
5	Haematology	+	+	+	+	+	+	+	+	+	+	+	+	+
6	ICU	+	+	+	+	+	+	+	+	+	+	+	+	+
7	ICU	+	+	+	+	+	+	+	+	+	+	+	+	+
8	Haematology	+	+	+	+	+	+	+	+	+	+	+	+	+

ND, not done.

*+, amplicon present; -, amplicon absent; ++ amplicon larger than that from control.

DdeI digestion of a 424-bp internal fragment of the *vanX* gene that had been amplified from the three isolates with type I VanA elements yielded three fragments of 185 bp, 149 bp and 90 bp, which were indistinguishable in size from those obtained from a Tn1546 control. However, *vanX* amplicons from the five isolates with type II VanA elements yielded only two *DdeI* fragments of 239 bp and 185 bp, as observed in control Tn#3 with a group D element. These two fragments were consistent with the presence of the G → T point mutation at position 8234 (Table 1) [5]. We have shown previously that VanA elements belonging to group D lack a p1p2 amplicon and have the mutation at position 8234. In group D elements the first 120 bases of Tn1546 are replaced by an IS1216V/IS3 complex linked to the remaining part of *orf1* (Table 1) [5]. Therefore, the Greek type II elements were compared further with a group D control. However, these elements did not yield amplicons with primers specific for the IS1216V/IS3 complex or IS3-*orf1* linkage (Table 1).

The large p11p12 amplicon observed in Greek type II VanA elements suggested a possible insertion in the *vanS-vanH* intergenic region. IS1251 has been reported at this position in some VanA elements from the USA [9]. A 427-bp IS1251-specific fragment was amplified from all five isolates harbouring type II VanA elements. Furthermore, an IS1251-specific probe hybridised with Southern blots prepared from the p11p12 amplicons of the five type II elements, confirming the presence of this IS element on this fragment of the VanA elements. The IS1251 probe did not hybridise with the p11p12 amplicons of Tn1546 or Greek type I elements (Table 1).

Characterisation of *VanB* resistance elements

Amplification with *vanB*-specific primers yielded a 589-bp fragment from two isolates. Restriction fragment-length polymorphism (RFLP) analysis with *HhaI* indicated that the amplicons from both isolates were derived from *vanB2* alleles (Fig. 2). Both of the VanB isolates also gave products of 311 bp and 1581 bp when amplified with primers specific for the left and right ends of Tn5382, which confirmed that their *vanB2* gene clusters were contained within a Tn5382-like element.

Discussion

We believe that this report represents the first characterisation of elements responsible for acquired glycopeptide resistance in enterococci from Greece. Eight isolates expressing the VanA phenotype had seven different macrorestriction banding patterns, as interpreted by published general criteria [22], but when the patterns were interpreted according to guidelines specifically designed for *E. faecium* [23], the isolates

Fig. 2. Representative results for PCR-RFLP analysis of *vanB* amplicons with *HhaI*. Lane 1, *vanB1* allele; 2, *vanB2* allele; 3, 123-bp ladder (Life Technologies, Paisley).

were considered to represent variants of only three strains. Two VanB isolates were also studied; their macrorestriction banding patterns were distinct from each other and from those of the VanA strains.

Despite diversity among the resistant strains, the VanA resistance elements were divided into only two types. Elements designated Greek type I were identified in three related isolates and were indistinguishable from Tn1546 [3] and group A elements from the UK [4]. Greek type II elements were identified in five isolates that represented variants of two strains. These elements contained IS1251 in the *vanS*–*vanH* intergenic region, had a mutation within *vanX* and a deletion in *orf1*. Type II elements were distinct from all of the 24 groups of VanA elements identified among GRE from the UK [4]; they shared the mutation in *vanX* and absence of a *p1p2* amplicon with group D elements, but differed by the presence of IS1251 and by the absence of an IS1216V/IS3-*orf1* complex at the 5' end of the element [5]. They resemble elements designated types F1 and F2 that were characterised from enterococci from the USA [6]. Indeed, VanA elements containing IS1251 were originally identified in GRE from the USA, where they are locally prevalent [9]. Such elements have recently been identified in one GRE from Norway and in two from the Republic of Ireland [26], but apparently remain rare in Europe as a whole [4, 6, 7]. The reason for the appearance of VanA elements containing IS1251 in Laikon General Hospital is unclear. There were no discernible epidemiological links with countries from which these elements have been reported previously.

Two distinct strains of *E. faecium* with VanB gly-

copeptide resistance were obtained. Both contained the *vanB2* allele as part of a gene cluster within a Tn5382-like element. These findings are consistent with other studies of the genetic basis of VanB resistance in enterococci from Europe [11, 15, 25]. Horizontal transmission of *vanB2* as part of Tn5382 has also been documented in diverse strains from across the USA [14].

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