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ORIGINAL ARTICLE

Comparison of *vanA* gene mRNA levels between vancomycin-resistant *Enterococci* presenting the VanA or VanB phenotype with identical Tn*1546*-like elements

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Tn*1546*-like elements;
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Background/Purpose: During the routine screening of vancomycin-resistant *Enterococci faecium* (VREfm) we found that VanA phenotype–*vanA* genotype and VanB phenotype–*vanA* genotype isolates had an identical Tn*1546*-like element structure. This study aimed to evaluate the genetic background and *vanA* gene expression to identify the mechanisms of the development of the VanB phenotype–*vanA* genotype VREfm.

Methods: Twelve VREfm isolates were collected from a 1500-bed tertiary-care teaching hospital in Beijing. Genetic variations of the Tn*1546*-like element were determined by an overlapping polymerase chain reaction assay and sequencing. The genetic background was determined by pulsed-field gel electrophoresis and mutilocus sequence typing. *vanA* gene expression was evaluated using a TaqMan quantitative real-time polymerase chain reaction.

Results: For the 12 isolates, six isolates with the VanA phenotype–*vanA* genotype and six with the VanB phenotype–*vanA* genotype were identified. According to the structure analysis of the Tn*1546*-like elements, our isolates were divided into two types. In the four isolates of type A, IS*1542* and IS*1216V* were inserted into the *orf2-vanR* and *vanX-vanY* regions, respectively. In the eight isolates of type D, a similar insertion as type A occurred, except for an ISE*fa4* insertion into IS*1542*. A significant difference in *vanA* gene expression was observed between the VanA and VanB phenotype isolates in type A, but not in type D. Mutilocus sequence typing and pulsed-field gel electrophoresis analysis showed that these isolates have a different genetic background.

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Conclusion: Our results indicated that the occurrence of the VanB phenotype—*vanA* genotype might not completely depend on the structure of Tn*1546*-like elements and *vanA* gene expression.

Introduction

Vancomycin-resistant *Enterococci* (VRE) are a major nosocomial pathogen and have become a global public health problem.[1] The treatment of VRE infections is hampered by the emergence of resistance of the bacteria to new classes of antibiotics.[2,3]

Two principal phenotypes of VRE have been described (VanA and VanB), which are encoded by two distinct gene clusters (the *vanA* and *vanB* gene clusters) found on the transposons Tn*1546* and Tn*1547*, respectively.[4] In general, *vanA* genotype VRE show the VanA phenotype, which is characterized by an acquired high-level of resistance to both vancomycin and teicoplanin. The *vanB* genotype shows the VanB phenotype, which is characterized by variable acquired levels of resistance to vancomycin, but not to teicoplanin.[5] The *vanA* gene cluster carried by the Tn*1546*-like element consists of seven genes, including *vanH*, *vanA*, *vanX*, *vanY*, *vanR*, *vanS*, and *vanZ*. It is generally known that the *vanH*, *vanA*, and *vanX* genes, encoding dehydrogenase, ligase, and D,D-dipeptidase, respectively, are essential for vancomycin resistance.[6] The *vanR* and *vanS* genes encode a vanS/vanR two-component signal transduction system and are necessary to promote the transcription of the vancomycin-resistant gene cluster.[7] There are two nonessential genes, *vanY* and *vanZ*. It is reported that the accessory gene *vanY* contributes to vancomycin resistance, whereas *vanZ* confers low-level resistance to teicoplanin.[8,9]

VanB phenotype—*vanA* genotype vancomycin-resistant *Enterococci faecium* (VREfm) has been reported in several Asian countries, mainly South Korea, Japan, and China.[10–18] The mechanisms underlying the VanB phenotype—*vanA* genotype VRE are still unclear. There has been much research on the description of the Tn*1546*-like elements and it has been suggested that variations among the Tn*1546*-like elements could be responsible for the VanB phenotype—*vanA* genotype. However, few studies have been carried out on the relationship between resistant gene expression and VanB phenotype—*vanA* genotype VREfm.[13,14,17]

Recent studies have found that two VREfm isolates have identical Tn*1546*-like element structures, however, one VREfm isolate was susceptible to teicoplanin and the other resistant, thus vancomycin resistance may not be dependent on the Tn*1546*-like element.[10,18] These findings prompted us to suspect that the occurrence of the VanB phenotype—*vanA* genotype VREfm might not be due to a single genetic variation, but due to *vanA* gene expression.

During the course of routine screening of VREfm, we detected 12 *vanA*-positive VREfm isolates with different phenotypes and identical Tn*1546*-like element structures. We evaluated the genetic background and the structure of the Tn*1546*-element and assessed the relationship between the level of *vanA* gene expression and the occurrence of VanB phenotype-*vanA* genotype VREfm.

Materials and methods

Bacterial strains

We studied a total of 12 *vanA* genotype *E. faecium* clinical isolates, collected between 2004 and 2008 from individual patients at the teaching hospital of Capital Medical University in Beijing. The organisms were identified as *E. faecium* using conventional microbiological methods and were further confirmed by 16S rRNA sequencing.[19] The vancomycin-resistant genotype was confirmed by multiplex polymerase chain reaction (PCR) methods as described previously.[20]

Antimicrobial susceptibility testing

Minimal inhibitory concentrations of vancomycin and teicoplanin were measured for the bacterial strains using E test methods according to the manufacture's instruction (AB Biodisk, Solna, Sweden) and interpreted according to the guidelines established by the Clinical and Laboratory Standards Institute.[21]

Structure of Tn*1546*-like elements

For analysis of the DNA sequence of the Tn*1546*-like elements, an overlapping PCR amplification of the internal region of the Tn*1546*-like element was performed as described previously.[20] All the purified PCR fragments were sequenced. The nucleotide sequence was compared with the reference sequence of Tn*1546* published in the GenBank database (Accession no. M97297).

Pulsed-field gel electrophoresis and mutilocus sequence typing

Pulsed-field gel electrophoresis (PFGE) was performed using *SmaI*-digested genomic DNA with separation by electrophoresis through a 1% agarose gel (Bio-Rad, Richmond, CA, USA) using the CHEF mapper system (Bio-Rad) as described previously.[22] The criteria for interpreting the PFGE patterns was based on Tenover *et al.*[23]

Mutilocus sequence typing (MLST) of the isolates was performed as reported previously.[24] The alleles and the sequence type (ST) for each isolate were determined through the MLST database (www.mlst.net).

VanA expression by quantitative real-time PCR

VREfm strains were grown in LB medium at 37°C to the exponential growth phase. The total RNA was isolated using an Eastep Universal RNA Extraction Kit (Progema, Fitchburg, MA, USA). The RNA sample was diluted in RNase-free water. cDNA was synthesized using M-MLV reverse transcriptase and random primer (Progema) according to the manufacturer's instructions. Transcripts were quantified by TaqMan quantitative real-time PCR (qRT-PCR) on an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The transcription level was calculated from the standard curve generated with a serially diluted plasmid obtained by cloning a target gene in the PMD 19 T-vector (Takara, Dalian, China). The mRNA levels of the target genes were normalized to the 16SrRNA level. Amplification was carried out in a final volume of 25 μL containing 500 nM of each primer, 250 nM of the probe, 5 × PCR buffer 2.5 μL, and 1 μL of standard template plasmid or cDNA. The reactions were performed under the following conditions: 5 minutes at 95°C, 30 seconds at 94°C, 32 seconds at 52°C, 10 seconds at 72°C for 35 cycles, and 3 minutes at 72°C. The primers and probes were selected for use in a standard TaqMan amplification protocol. The primers and probes specific for *vanA* and 16SrRNA were designed based on the nucleotide sequences published in the GenBank database (Accession nos. M97297 and AJ301830) using the Primer 5 software (Premier, Port Coquitlam, BC, CA). Table 1 lists the primer sequences and probes.

Statistical analysis

The results for the level of vanA expression are given as mean ± standard error of the mean values. Comparison of the level of expression of the *vanA* gene between the VanA and VanB phenotypes with identical Tn*1546*-like elements was performed using the Student *t* test. A p *value* ≤ 0.05 was considered statistically significant. All analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA).

Results

Antimicrobial resistance

We identified 12 VREfm isolates that were positive for the *vanA* gene by multiplex PCR. All isolates were negative for the *vanB*, *vanC*, *vanD*, *vanE*, and *vanG* genes. Table 2 lists the characteristics of the 12 VREfm isolates. All the isolates displayed moderate to high levels of resistance to vancomycin [minimal inhibitory concentration (MIC) > 128 μg/μL]. Of these, six isolates (EA1, EA2, ED1, ED2, ED3, and ED4) were of the VanB phenotype, which is susceptible to teicoplanin; the other six isolates (EA3, EA4, ED5, ED6, ED7, and ED8) were of the VanA phenotype, which is resistant to teicoplanin.

Table 1 Primers and probes used for TaqMan quantitative real-time polymerase chain reaction analyses

Gene	Primer/probe	Sequence
vanA	Van3F	5′-CTGTGAGGTCGGTTGTGCG-3′
	Van3R	5′-TTTGGTCCACCTCGCCA-3′
	Van3-probe	FAM-5′-CAACTAACGCGGCACTG TTTCCCAAT-3′-TAMRA
16SrRNA	16SF	5′-CGCGGTGCATTAGCTAGTTG-3′
	16SR	5′-CCCTCTCAGGTGCGGCTAT-3′
	16S-probe	FAM-5′-AGGTAACGGCTCACCAAG GCCACG-3′-TAMRA

Structure of Tn*1546*-like elements

Overlapping PCR and DNA sequences of the central region of the Tn*1546*-like elements showed no size variation. The *vanRSHAX* genes were conserved in all 12 VREfm isolates. However, in the left and right side of Tn*1546*, the integration of the insertion sequence accompanied by a deletion adjacent to the insertion sites was found. These 12 isolates were classified into two transposon types by comparison with the Tn*1546* reference sequence of a previous study.[1] Type A was characterized by a copy IS*1542* insertion in the *orf2-vanR* intragenic region and a copy IS*1216V* insertion in *vanX-vanY* intragenic region, accompanied by partial deletion of the downstream insertion site of IS*1216V*. Both the *vanY* and *vanZ* genes were complete. Type D had a similar insertion as type A, except for an IS*Efa4* insertion 66 bp downstream of IS*1542* (Fig. 1).

MLST and PFGE genotyping

We used a combination of MLST and PFGE genotyping to provide the molecular characteristics of the 12 VRE isolates. MLST analysis identified five STs (ST-203, ST-17, ST-192, ST-78, and ST-414; Table 2). Four isolates (EA1, EA2, ED2, and ED6) were genotyped as ST-203, followed by ST-17(2), ST-192(2), ST-78(2), and ST-414(2). Analysis of the 12 VREfm isolates by PFGE found nine different banding patterns (designated as numbers 1, 2, 3, 4, 5, 6, 7, 8, and 9; Table 2). Isolate EA1 was closely related to EA2. ED2 and ED3 were closely related to ED6 and ED7, respectively. The remaining VRE isolates presented as only a PFGE pattern (EA3, EA4, ED1, ED4, ED5, and ED8). We found that the same ST isolates showed different PFGE patterns (Fig. 2). For example, of the ST-17 isolates, we found two PFGE types (2 and 3). This suggested genetic diversity among these VREfm isolates.

VanA gene expression

We compared the *vanA* mRNA levels between the VanA phenotype—*vanA* genotype and the VanB phenotype—*vanA* genotype isolates with the same Tn*1546* type (Fig. 3). The results of the TaqMan qRT-PCR showed that *vanA* gene transcription could occur in all isolates. For the isolates of Tn*1546* type A, VREfm isolates EA3 and EA4 produced approximately three-fold higher levels of the *vanA* gene than EA1 and EA2, indicating that the VanB phenotype—*vanA* genotype isolates might be due to the lower expression of *vanA* transcription. For the isolates of the Tn*1546* type D, VREfm isolate ED8 showed the highest level of *vanA* expression among all the isolates, but a significant

Table 2 Characteristics of VREfm isolates

Tn1546 type	Isolate	Source	Genotype	Phenotype	MLST type	PFGE	MIC (μg/mL)	
							Vancomycin	Teicoplanin
A	EA1	Sputum	A	B	ST-203	1	128 (R)	8 (S)
	EA2	Sputum	A	B	ST-203	1a	128 (R)	8 (S)
	EA3	Ascites	A	A	ST-17	2	>256 (R)	64 (R)
	EA4	Wound pus	A	A	ST-17	3	>256 (R)	64 (R)
D	ED1	Bile	A	B	ST-192	4	128 (R)	8 (S)
	ED2	Urine	A	B	ST-203	5	256 (R)	8 (S)
	ED3	Urine	A	B	ST-78	6	256 (R)	8 (S)
	ED4	Urine	A	B	ST-414	7	512 (R)	8 (S)
	ED5	Ascites	A	A	ST-192	8	256 (R)	32 (R)
	ED6	Ascites	A	A	ST-203	5a	256 (R)	512 (R)
	ED7	Sputum	A	A	ST-78	6a	512 (R)	32 (R)
	ED8	Urine	A	A	ST-414	9	256 (R)	32 (R)

MIC = minimal inhibitory concentration; MLST = mutilocus sequence typing; PFGE = pulsed-field gel electrophoresis; R = resistant; S = susceptible; ST = sequence type; VREfm = vancomycin-resistant *Enterococci faecium*.

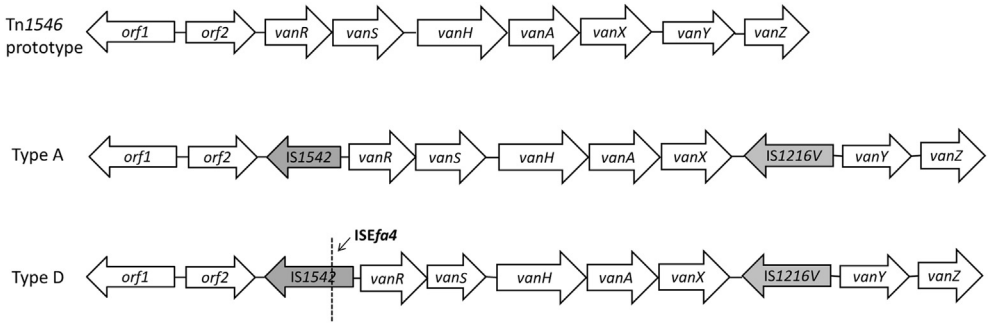

Figure 1. Structures of the Tn*1546*-like elements. The prototype of the Tn*1546*-like element is shown at the top. The arrows indicate the direction of transcription.

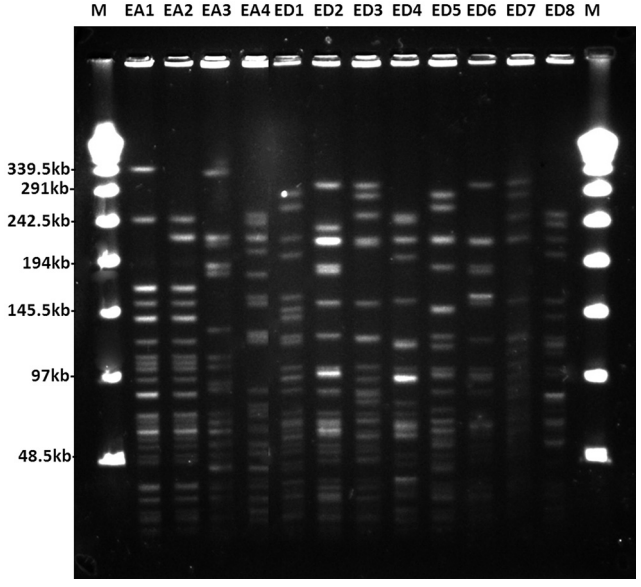

Figure 2. Pulsed-field gel electrophoresis results of 12 vancomycin-resistant *Enterococci faecium*. Lanes 1 and 14, molecular marker; lanes 2—13, EA1 to ED8.

difference was not observed between VanA phenotype and VanB phenotype VREfm isolates.

Discussion

Genotypic and phenotypic varieties of VRE have been investigated in a number of studies. These studies focused on describing VRE with different Tn*1546*-like elements and elucidated the relationship between the Tn*1546*-like elements and vancomycin resistance. In this study, we compared VRE with identical Tn*1546*-like elements and evaluated the relationship between *vanA* gene expression and the VanB phenotype—*vanA* genotype. Twelve VREfm isolates were investigated and all isolates contained the *vanA* gene. These isolates were classified into Tn*1546* type A and type D based on the structure of the Tn*1546*-like element. The isolates of the Tn*1546* type A had two VanA phenotype—*vanA* genotypes and two VanB phenotype—*vanA* genotypes, while the Tn*1546* type D had four VanA phenotype—*vanA* genotypes and four VanB phenotype—*vanA* genotypes.

MLST is used to evaluate the long-term evolution of isolates and PFGE genotyping is used for short-term evolution. The MLST analysis showed five ST types. Our PFGE files showed nine different banding patterns. Some of the same

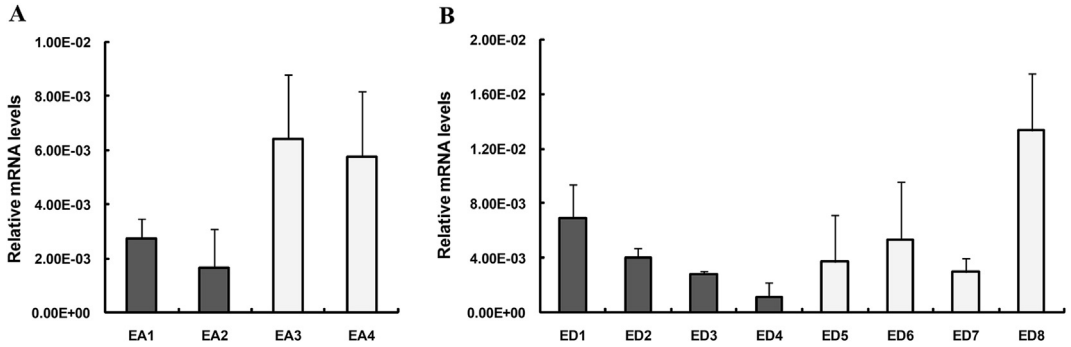

Figure 3. Transcription of mRNA levels of the *vanA* gene in the bacterial strains. (A) Strains with Tn*1546* type A. (B) Strains with Tn*1546* type D. The mRNA level was determined using a TaqMan quantitative real-time polymerase chain reaction. Data are presented as mean ± standard deviation values from three independent experiments. Statistically significant differences in *vanA* gene expression were observed between phenotype A and B isolates with Tn*1546* type A ($p = 0.025$), but were not observed in the isolates with Tn*1546* type D ($p = 0.366$).

STs (ST-17, ST-192, and ST-414) showed different PFGE patterns. Based on this, these isolates had different genetic backgrounds. We found no correlation between the Tn*1546* type, phenotype, and genetic background.

Many studies have reported the emergence of VanB phenotype—*vanA* genotype VRE, but the mechanism has not previously been clear. It has been reported that point mutation in the sensor domain of the *vanS* gene, the absence or impairment of the vanR/vanS two-component system, the impairment of the accessory genes *vanY* and *vanZ*, and the deletion of the *vanY* and *vanZ* genes can lead to a susceptibility to teicoplanin.[13,14,16,17] However, PCR and direct sequencing of the *vanS* gene did not demonstrate point mutation in our 12 isolates; in addition, the deletion of the *vanY* and *vanZ* genes were not found in our isolates.

An interesting finding in this study was that the same genotype, but different phenotype, VREfm isolates had identical Tn*1546*-like elements. Therefore the Tn*1546*-like element might not be sufficient to explain the loss of teicoplanin resistance in the VanB phenotype—*vanA* genotype isolates. This finding was similar to that in a previous study, in which two *E.faecium* strains collected from one patient carried the intact *vanRHAX* gene and an 88-bp deletion of the *vanS* gene. One strain was susceptible to vancomycin and teicoplanin, whereas the other was resistant to vancomycin and teicoplanin, despite possessing the same structure of the Tn*1546*-like element.[18] Another study also reported a vancomycin-susceptible isolate missing the *orf1, orf2*, *vanS*, and *vanR* genes of the Tn*1546*-like element, similar to the vancomycin-resistant isolates reported in another study.[25,26] These results demonstrated that the occurrence of the VanB phenotype—*vanA* genotype might not completely depend on the genetic variation in the Tn*1546*-like elements.

To further understand the mechanism by which isolates can possess the same Tn*1546*-like elements with different phenotypes, we tested *vanA* gene expression by TaqMan qRT-PCR. In the isolates with Tn*1546* type A elements, two VanB phenotype isolates showed lower levels of *vanA* expression than the other two VanA phenotype isolates. We therefore propose that this phenotypic difference is a result of decreased *vanA* gene expression. This is similar to a previous study.[18] A limitation of this study was that it was difficult for us to find more isolates. However, in the isolates with the Tn*1546* type D elements, no distinction could be found between the two phenotypes. Thus we propose that the occurrence of the VanB phenotype might not be a result of *vanA* gene expression. It is possible that the ISE*fa4* insertion could cause the difference in results between the Tn*1546* type A and type D isolates.

The occurrence of the VanB phenotype—*vanA* genotype VREfm might not be a result of genetic variation in the Tn*1546*-like elements and *vanA* gene transcription. Experiments similar to those reported here should be conducted using more isolates. Further study is required to study the effect of the expression of other resistant genes on the occurrence of VanB phenotype—*vanA* genotype VREfm. This may provide an insight into developing new strategies to infection with VRE.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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