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NOTE

DD1.5k, the Gene Preferentially Expressed in Bloodstream Isolates of Vancomycin-Resistant *Enterococcus faecium*

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Vancomycin-resistant *Enterococcus faecium* (VREFM) is becoming a threatening pathogen. We identified a gene called DD1.5K by differential display-PCR, which was preferentially expressed in the bloodstream isolates of VREFM. Due to its amino acid similarity to transfer complex protein, *trsE*, and tissue-specific expression, this gene may be involved in virulence of VREFM.

Key words: *Enterococcus*, Vancomycin Resistance, Virulence Factors

Vancomycin-resistant enterococci (VRE) were first reported in the United Kingdom (Uttley *et al.*, 1988) and in France (Leclercq *et al.*, 1988) in 1988. Shortly after the first reports were made, VRE were detected in hospitals in the United States (Sahm *et al.*, 1989). Since then, VRE have emerged with unanticipated rapidity and, especially in the United States, are now encountered in most hospitals (Martone, 1998). From the earliest reports of VRE clinical isolates, most were VanA phenotype strains of *E. faecium* and were associated with outbreaks in special units with immunocompromised patients on prolonged antimicrobial regimens, with extended lengths of stay and higher severity-of-illness scores (Sahm *et al.*, 1989; Bonten *et al.*, 1998).

Historically, the ratio of *E. faecalis* infections to those due to all other *Enterococcus* species was approximately 10:1. In recent years, there has been a progressive decline in this ratio of enterococcal bacteremia (Shay *et al.*, 1995). This shift might be explained, in part, by the emergence of *E. faecium* as a predominant species among enterococcal isolates. Data from the National Nosocomial Infection Surveillance (NNIS) survey evidences a rising percentage of VRE since 1989, with rates now approaching 20% of all enterococcal isolates (including all species); an equal proportion of VRE isolates occurring in and out of intensive care units; and *E. faecium* as the dominant species identified among VRE (although many enterococci are not identified) (CDC, 1993). In the countries of the European Union (EU), VRE are relatively frequently found in healthy humans in the community and in farm animals, and vancomycin resistance is mostly *vanA* mediated. This considerable pool of possibly transmissible isolates with *vanA*-mediated glycopeptide resistance in the EU is very likely caused by the use of avoparcin (an analogue of vancomycin) as a growth promoter in animal husbandry until April 1997 (van den Bogaard *et al.*, 1999; van den Bogaard *et al.*, 2000). Thus far, comprehensive studies have attempted to identify the relationships between virulence and resistance which enhance the organism's ability to cause disease beyond those abilities commonly associated with enterococci (Mundy *et al.*, 2000).

In order to identify and isolate relevant genes of vancomycin-resistant *Enterococcus faecium* (VREFM) whose expressions are differentially induced during growth or infection, we performed a comparative analysis of gene expression pattern of the VREs isolated from different origins such as blood or urine by using differential display - PCR (DD-PCR).

The 75 vancomycin-resistant *Enterococcus* clinical isolates were obtained from blood (n=27), rectal swab (n=19), stool (n=9), urine (n=10), and from 10 chickens (rectal swab). Single colony grown on *Enterococcus* selective media (Enterococcosel™ agar, Becton Dickinson and Company, USA) was inoculated into 1ml of BHI broth and cultured with shaking for 24 h at 37°C. The agar dilution

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method was performed as recommended by the NCCLS (2002). Antimicrobial agents for minimal inhibitory concentration (MIC) were ampicillin, erythromycin, tetracycline, levofloxacin, vancomycin, teicoplanin, gentamicin, and streptomycin. Results were analyzed using the database, APILIB+ (Bio-Merieux, France). Auxiliary experiments (motility and pigment formation) for *Enterococcus* species other than *Enterococcus faecium* and *Enterococcus faecalis* were also performed.

DNA purification from standard strains and clinical isolates was performed with the QIAamp DNA mini kit (QIAGEN, Germany) in accordance with manufacturer's instructions. RNA extraction was performed using the RNeasy mini kit. A total of 1 to 5 μg of each sample was subjected to RT-PCR or Northern (RNA) blot analysis.

Differential display PCR was performed with the Delta™ Differential Display kit, in accordance with manufacturer's instructions (Clontech, USA). The combination of forward primers (F1 gacttctaatggggaaatttctaaa, F2 gacttctaatggaaaaatttctaaa) and reverse primers (R1 cgtatgatgaagcttccagttta, R2 cgtatgatgaagcttccagggga) was determined based on the alignment analysis of mRNAs from different *Enterococcus* and other bacterial species. Briefly, Two microliter samples containing 10 μg of total RNA with cDNA synthesis primers were incubated at 70°C for 3 min and then ice-cooled for 2 min. Two microliters of cDNA synthesis buffer, containing 100 mM Tris-HCl (pH 8.0), 100 mM KCl, 8 mM $MgCl_2$, 20 mM dithiothreitol, 5 mM deoxynucleoside triphosphates, and 200 units of Moloney murine reverse transcriptase (Stratagene, USA), was added. The cDNA synthesis was carried out at 42°C for 1 h and reactions were terminated by incubating at 75°C for 10 min. After cDNA synthesis, 2 μl of sample was subjected to DD-PCR with the primer or in combination with other primers. The PCR products were labeled with [α-^{33}P]dATP (1,000-3,000 Ci/mmol). The thermal cycling parameters were as follows: 94°C (5 min), 40°C (5 min), 68°C (5 min) for 1st cycle, 94°C (2 min), 40°C (5 min), 68°C (5 min) for 2 cycles, 94°C (1min), 60°C (1 min), 68°C (2 min) for 25 cycles and finally, 68°C (5 min) for the last. The RT-PCR samples were analyzed by standard sequencing gels, and DD-PCR fingerprints were autoradiographically visualized using Kodak XAR film. Different RNA samples prepared from VRE treated under co-culture with PBMC were fingerprinted, and duplicated signals from at least two DD-PCRs were isolated and analyzed. In addition, in each reaction, parallel experiments were carried out with negative and positive control and, therefore, confirmed that the amplified products were not due to DNA contamination. Differentially expressed bands of DD-PCR products were cut from the gel, and the DNA was eluted into 50 μl of TE by heating at 65°C for at least 1 h. Seven microliters of the eluent

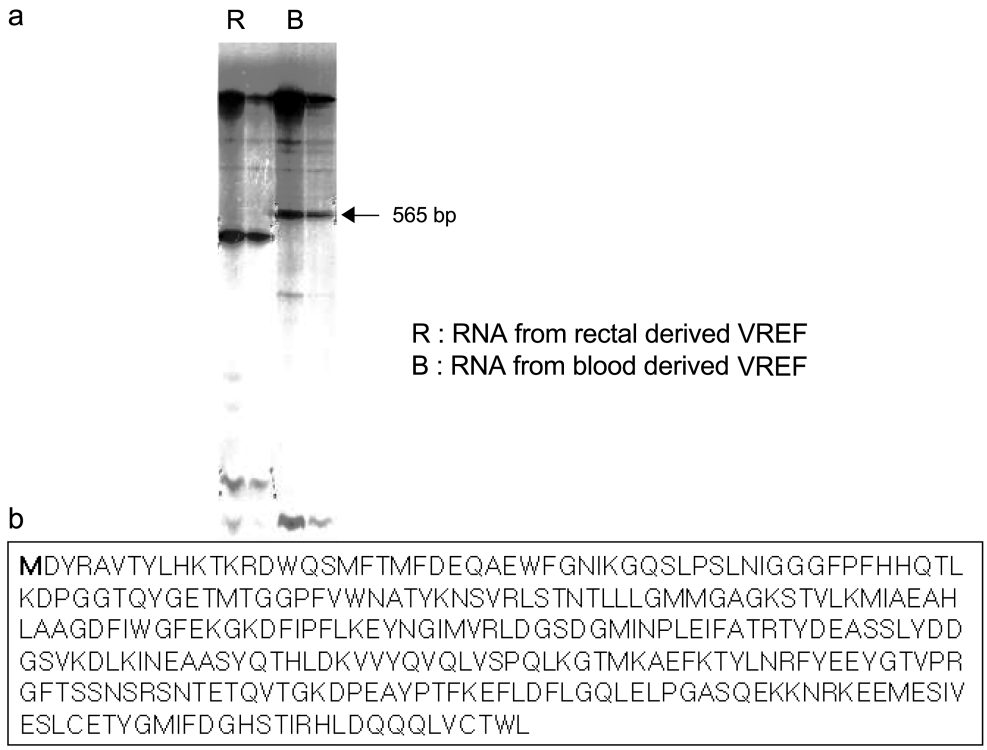

Fig. 1. Identification of DD1.5K by DD-PCR and assignment of conceptual open reading frame (ORF) (GenBank accession no. AY273800)
a) Total RNA was purified from VREFM originating from rectal or blood samples and subjected to DD-PCR. The amplified products were resolved on a denaturating polyacrylamide gel. The band representing DD1.5 K, which is preferentially expressed in the bloodstream isolate of VREFM, is shown by an arrow. b) Using the partial sequence, full-length sequences were retrieved from the TIGR database and Genbank™ via bioinformatics. The conceptual ORF was created by DNA Star software.

Table 1. Sequence homology analysis of translated amino acid of DD1.5K (GenBank accession no. AY273800)

Accession no.	Total Predicted protein	Similar portion of Predicted protein	No. of residues compared (% identity)	Score (bits)
AF188935.1	*Bacillus anthracis* plasmid pXO2	similar to transfer complex protein trsE	108/310 (34%)	202
AE011191.1	*Bacillus anthracis* str. A2012 plasmid pXO2	conserved hypothetical protein	108/310 (34%)	202
AP003515.1	*Clostridium perfringens* plasmid Pcp13	conserved hypothetical protein	98/312 (31%)	157

was PCR amplified with the same sets of primers, cloned into the pGEM-T Easy vector (Promega, USA), and each clone was sequenced with a model ABI Prism 310 genetic analyzer (Perkin-Elmer, Applied Biosystems, USA). We then analyzed the sequence data and performed homology searches utilizing BLAST software and the GenBank database. To confirm that the clones obtained, differentially expressed genes derived from blood origin VRE, Northern blot tests were performed. Probes obtained from RT-PCR were labeled with [α-^{32}P]dATP (Amersham Pharmacia Biotech, USA) utilizing the PCR labeling method. The PCR primer sequence was as follows: DD1.5K (291 bp), sense, 5'-GTTTCACTTCCTCAAAC-TCG-3'; antisense, 5'-TTGTACCCCACGATCTAAAC-3'. Filters were hybridized overnight at 42°C with labeled probes. Washing of the filters was performed at a final stringency of 0.1×SSC (1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS) at 65°C. The blots were then sealed in a plastic bag and subjected to autoradiography.

Clones containing cDNA from temperature-regulated genes were sequenced using a T7-polymerase sequencing kit (Pharmacia, UK). The nucleotide sequences obtained were used to screen the GenBank and EMBL databases in an attempt to identify the transcripts by using the BLASTN and BLASTX programs, available at the National Center for Biotechnology Information website.

All 27 blood stream isolates were resistant (MICs, 32 to >128 μg/ml) to vancomycin as well as teicoplanin (MICs, 16 to >128 μg/ml). Of the 28 rectal or stool isolates (for all of which the vancomycin MICs were 64 to >128 μg/ml), only two were teicoplanin susceptible (MICs, 0.5 to 1μg/ml). All urine and chicken rectal isolates proved to be highly resistant (MICs, 128 to >128 μg/ml) to vancomycin while the range of teicoplanin resistance was high overall in the chicken rectal isolates (MICs, 32 to >128 μg/ml). Ampicillin resistance was seen in *E. faecium* (MICs, 64 to >128 μg/ml), obtained from blood and rectal swab, while susceptibility was demonstrated in *E. faecalis* (MICs, 0.5 to 4 μg/ml). All the chicken rectal isolates were susceptible to levofloxacin and the rest of the clinical isolates were levofloxacin resistant (MICs, 8 to 128 μg/ml), ex-cepting 2 blood isolates (MICs, 2 μg/ml), which were levofloxacin susceptible.

As seen in Fig. 1a, a specific gene fragment was identified by DD-PCR from total RNA from the bloodstream VREFM, which was named DD1.5 k (GenBank accession no. AY273800). From data obtained from the dot blot

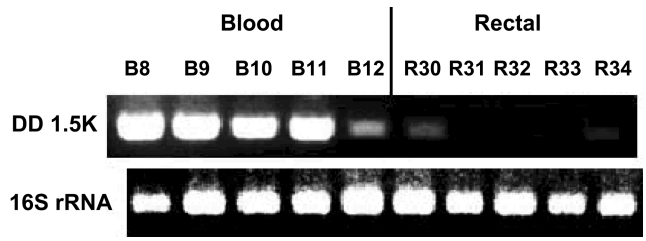

Fig. 2. Preferential expression of DD1.5K in the bloodstream isolate of VREFM: Total RNA was isolated from VREFM from blood or rectal samples. DD1.5 K was detected by RT-PCR. 16S RNA was amplified also, as a control. The resulting products were separated by agarose gel electrophoresis. The marks mentioned were serial numbers of clinical isolates.

analysis, we did not see any gene expressions in other major *Enterococcus* species, such as vancomycin-resistant *E. faecalis* and major gram-negative organisms. Utilizing the TIGR bacterial genome database, we retrieved a full length DNA and a conceptual open reading frame (ORF) was constructed by DNA Star software. Genbank searches revealed no significant homology to any known sequence. However, the translated amino acid sequences (as shown in Fig. 1b) of this gene showed a limited homology to transfer complex protein trsE, a hypothetical ORF of *Bacillus anthracis* plasmid pX02 (34%) and an ORF of *Clostridium perfringens* plasmid pCP13 DNA (31%) (Table 1).

Fig. 2 shows a representative RT-PCR data reflecting preferential expressions of DD1.5k in the bloodstream isolates of VREFM. DD1.5k RNA are fairly abundant (65%, 18 out of 27) in the VREFM derived from blood compared to that from rectal, stool (15%, 4 out of 28), or other isolates from urine (10%, 1 out of 10) and chicken rectal isolates (0%, 0 out of 10).

This data strongly suggests that the expression of DD1.5 k is associated with onset of bacteremia caused by VREFM. It also indicates that the horizontal transfer of this gene, between species such as chicken and human, has not occurred. According to Rice *et al.* (2003), The presence of esp_{efm} was roughly twice that of the newly cloned gene, hyl_{efm}, but both were found primarily in VREFM isolates in non-stool cultures obtained from patients hospitalized in the United States. His data showed that even though variations exist, specific *E. faecium* strains exhibit these virulent genotypes and these genes might cause more serious clinical infections. In our study, similar results have been observed. We believe that the

expression of DD1.5K genes cloned from blood-derived *E. faecium* might be closely related to virulence. The reason is that bacteremic patients infected by blood-derived VREFM eventually died, whereas those infected by stool, rectal, and urine-derived VREFM did not. Furthermore, VREFM, other than blood-derived strains, did not cause any significant symptoms or signs of infection. In summary, for mass surveillance purposes, we postulate that the detection of DD1.5K - *E. faecium*, not only in blood but also from various origins - might play an important role in predicting bacteremic virulence. Furthermore, it is possible that DD1.5k could be used as a marker for diagnostic or prognostic purpose, for bacteremia caused by VREFM.

To our knowledge, this is the first experimental evidence that a specific gene is differentially regulated by bloodstream isolates of VREFM.

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