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G. Kerbauy, M.R.E. Perugini, L.M. Yamauchi and S.F. Yamada-Ogatta

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Vancomycin-dependent *Enterococcus faecium van*A: characterization of the first case isolated in a university hospital in Brazil

G. Kerbauy[1], M.R.E. Perugini[2], L.M. Yamauchi[1] and S.F. Yamada-Ogatta[1]

[1]Laboratório de Biologia Molecular de Microrganismos, Departamento de Microbiologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, PR, Brasil
[2]Laboratório de Microbiologia Clínica, Departamento de Patologia, Análises Clínicas e Toxicológicas, Centro de Ciências da Saúde, Universidade Estadual de Londrina, Londrina, PR, Brasil

Abstract

In this study, we report the characterization of a strain of *Enterococcus faecium van*A, which grows only in the presence of vancomycin (VDEfm-UEL). The bacterium was isolated from the feces of a female patient who had undergone surgical treatment of Reinke's edema and was receiving intravenous vancomycin therapy for infection with methicillin/oxacillin-resistant *Staphylococcus aureus*, a postoperative complication. Antimicrobial dependence was further confirmed by the vancomycin *E*-test. VDEfm-UEL was also shown to be resistant to ampicillin, ciprofloxacin, chloramphenicol, erythromycin, levofloxacin, penicillin, rifampicin, and teicoplanin. The putative virulence genes *efa*A, *gel*E and *esp* were detected by PCR. The *ddl* gene from VDEfm-UEL was cloned and sequenced. Vancomycin dependence seems to be associated with the insertion of a nucleotide in that sequence, which results in a frame-shift mutation, introducing a premature stop codon. This is the first report of vancomycin-dependent *E. faecium* isolation in a university hospital in Brazil.

Key words: *Enterococcus faecium*; Vancomycin; Dependence; *van*A genotype; Virulence factors

Introduction

Over the last decades, infections due to glycopeptide-resistant enterococci in healthcare-associated settings have been reported worldwide. Among enterococci, glycopeptide resistance is detected most commonly in *Enterococcus faecium*, which is often resistant to other classes of antibiotics (1,2), and this feature has resulted in limited therapeutic options. Besides showing antimicrobial resistance, enterococci that require glycopeptides for growth have been isolated, particularly from patients on previous vancomycin therapy (3,4).

We report here the isolation and characterization of vancomycin-dependent *Enterococcus faecium* (VDEfm) isolated from a rectal swab of a patient who had received prolonged intravenous vancomycin therapy for the treatment of methicillin/oxacillin-resistant *Staphylococcus aureus* (MRSA) infections. This is the first case of the isolation of VDEfm in Brazil, which occurred at the University Hospital of Londrina, Paraná, Brazil.

Material and Methods

Case report

A 65-year-old female patient was admitted to the University Hospital of Londrina, Paraná, Brazil, for surgical treatment of Reinke's edema. She stayed in a nursing home for preoperative analysis for 6 days. After the surgical procedure, she was transferred to the intensive care unit where she stayed for 4 days. Her clinical course was complicated by the development of meningitis and sepsis on the 5th postoperative day. MRSA was isolated from a blood culture and, on the basis of the antimicrobial susceptibility of the etiological agent, intravenous vancomycin (500 mg every 6 h) and meropenen (2000 mg every 8 h) were used for 22 days for the treatment of the infections. On the 16th day of antibiotic therapy, a rectal swab was taken from the patient as part of the hospital surveillance study for vancomycin-resistant *enterococci* (VRE). Colonies

Correspondence: S.F. Yamada-Ogatta, Departamento de Microbiologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR 445, km 380, 86051-980 Londrina, PR, Brasil. Fax: +55-43-3371-4788. E-mail: ogatta@uel.br

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growing on VRE agar (Oxoid, UK) supplemented with 6 µg/mL vancomycin were identified as *E. faecium,* which was found to be growing only around the glycopeptide-impregnated discs in the agar diffusion assay (Figure 1A). The *E-test* further showed bacterial growth contiguous with the end of the strips containing the highest concentration of vancomycin (Figure 1B). The administration of antibiotics was discontinued because therapy was complete and a negative blood culture was confirmed. The patient was cured of infectious complications and left the hospital after 40 days of hospitalization. After 6 months, a rectal swab culture for VRE was negative, indicating that cessation of vancomycin had led to the clearance of VRE.

Hospital, surveillance and microorganism isolation

The University Hospital of Londrina is a 353-bed tertiary care center that serves the city of Londrina, in addition to several localities in the States of Paraná, São Paulo, and Mato Grosso do Sul. The intensive care center houses 35 patients distributed among 17 beds for general cases, 6 beds for burn cases, 7 beds for neonatal cases, and 5 beds for pediatric cases. Surveillance cultures of stools were examined weekly for all patients housed in intensive care units and for all patients found to be colonized by, or infected with VRE. A rectal swab was obtained from the patient and the sample was transferred to VRE broth (Oxoid) supplemented with 6 µg/mL vancomycin, 6 µg/mL ciprofloxacin and 8 µg/mL colistin. After a 18-h incubation at 37°C, a 100-µL sample was spread on VRE agar supplemented with 6 µg/mL vancomycin. The culture was further incubated at 37°C for 24 h under aerobic conditions. Bacteria were stored at -80°C in 20% glycerol-brain heart infusion broth (Himedia, India) supplemented with 10 µg/mL vancomycin. The study protocol was approved by the Ethics Committee of the Universidade Estadual de Londrina (Protocol #186-09/CEP-UEL). The patient gave written informed consent to participate in the study.

Phenotypic characterization

Species identification was based on colony morphology, Gram stain, catalase assay, and the profile determined by the automated MicroScan WalkAway 96 Instrument (Dade MicroScan, USA). The disk diffusion method on Muller Hinton agar medium (Himedia) was carried out according to Clinical Laboratory Standard Institute (CLSI) (5) guidelines in order to determine the profile of antimicrobial susceptibility to linezolide (30 µg), teicoplanin (30 µg), tigecycline (15 µg), and vancomycin (30 µg). As bacterial growth was detected only around the glycopeptide-impregnated discs, the isolate was tested for susceptibility to 12 other antimicrobials (ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, levofloxacin, linezolide, penicillin, rifampicin, streptomycin, tetracycline, and tigecycline), using the automated broth microdilution panel of MicroScan WalkAway 96 according to manufacturer recommendations. The results reported here were those obtained after 24 h of incubation. The susceptibility breakpoints used were those recommended by the CLSI (5). *E. faecalis* ATCC 29212 and ATCC 51299 were used for quality control. The isolated bacterium was further tested by the vancomycin

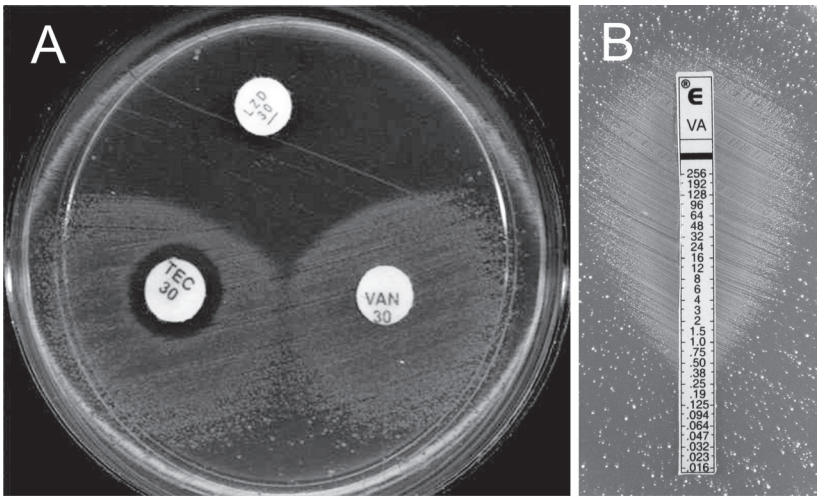

Figure 1. Growth dependence of *van*A *Enterococcus faecium* UEL (VDEfm-UEL). *A*, Discs impregnated with 30 µg linezolide (LZD), teicoplanin (TEC), and vancomycin (VAN) were placed on the surface of Muller Hinton agar plates previously inoculated with a suspension of VDEfm-UEL. After a 24-h incubation at 37°C, bacterial growth was observed only around the TEC and VAN discs. *B*, Vancomycin *E*-test showing the greater bacterial growth around the strips containing the highest concentration of the antimicrobial agent.

E-test (AB BIODISK, Sweden).

Genotypic characterization

The vancomycin-resistance genotype and the putative virulence genes *cyl*A (activator of cytolysin, a secreted protein with hemolysin/bacteriocin activities), *efa*A (cell wall *E. faecalis* antigen A, an endocarditis-associated virulence factor), *esp* (enterococcal surface protein), and *gel*E (gelatinase) were determined by PCR. The vancomycin resistance gene was determined using multiplex PCR as described by Petrich et al. (6). Genomic DNA of the enterococcal strain was extracted by the boiling method, and the virulence genes were determined as described by Ruzon et al. (7).

Cloning and sequencing of the *ddl* gene

The DNA fragments to be sequenced were amplified with the following primers based on the *ddl* gene of *E. faecium* BM 4339 (8): forward 5' GAGTAAATCACTGAACGATT 3' and reverse 5' GGTTACGCAATCACTCCAGCCT 3'. PCR was performed in a final volume of 20 µL containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer, 2.5 U *Pfx* DNA polymerase (Invitrogen, Brazil), and 10 µL genomic DNA. The PCR product was purified from agarose gel with the QIAquick Gel Extraction kit (Qiagen, USA) and was inserted into the pCR®2.1 vector using the Original TA Cloning kit (Invitrogen) according to manufacturer recommendations. The insert was sequenced with a 3730xl DNA analyzer (Applied Biosystems, USA) using the Big Dye® Terminator v.3.1 Cycle Sequencing kit. A search for homologies in the GenBank/EMBL databases was carried out with the Blast algorithm (http://www.ncbi.nlm.nih.gov). The deduced amino acid sequence was analyzed with the ExPASy-Prosite program of the Swiss Institute of Bioinformatics, and the alignment of the sequences was carried out with ClustalW2 (http://www.cmbi.kun.nl/bioinf/tools/clustalw.shtml) of the EMBL-EBI software package.

Results and Discussion

The first VDE was isolated in 1994 from the urine of a female patient receiving long-term vancomycin therapy (3). Since then, other cases of VDE isolation from different clinical specimens of patients receiving previous vancomycin therapy have been reported (4). A *van*A-type *E. faecalis* vancomycin-dependent strain from a non-human source was also reported by Tanimoto et al. (9). In that case, VDE was isolated from chicken meat imported from China. For the cases of VDE-infected/colonized patients, 7 were on vancomycin to treat non-enterococcal infections: 1 for diarrhea caused by *Clostridium difficile*, 5 for invasive staphylococcal infections, and 1 for bacteremia by *Corynebacterium* spp. resistant to ß-lactam antibiotics (4). In the remaining cases, VDE was considered to be the primary cause of infection.

In the present study, we report the first case of VDE isolation at a university hospital in Brazil (VDEfm-UEL). As reported in the other cases, the patient was receiving intravenous vancomycin therapy for non-enterococcal infection. This isolation was possible because of the protocol for detecting VRE in a selective culture medium supplemented with vancomycin, which is utilized in the infection prevention and control laboratory of the hospital. Besides requiring glycopeptides, the isolated bacterium was also resistant to ampicillin, ciprofloxacin, chloramphenicol, erythromycin, levofloxacin, penicillin, and rifampicin.

Although the isolated bacterium was considered to be colonizing *Enterococcus* according to CDC definitions of healthcare-associated infections (10), it harbored the putative virulence genes *efa*A, *gel*E and *esp*, indicating a potential risk of infection. Low prevalence of the virulence markers has been shown for *E. faecium* isolates from different sources (11). In contrast to those results, we previously demonstrated a high prevalence of vancomycin-resistant *E. faecium* (VREfm) harboring virulence genes isolated from different sources at the University Hospital of Londrina, including the *efa*A, *gel*E and *esp* genes (7). Camargo et al. (12) showed that the *esp* gene was restricted to VREfm isolates from the southern region of Brazil, which also harbored the hyaluronidase gene (*hyl*). Studies with the *E. faecalis efa*A$^-$ mutant showed attenuation of virulence in a mouse peritonitis model when compared to the wild-type strain, suggesting that EfaA is a virulence factor (13). The chromosomal *gel*E gene encodes an extracellular zinc metalloprotease that can participate in the translocation of bacteria across intestinal cell layers (14) and can contribute to bacterial virulence in a mouse peritonitis model (15). The presence of the *esp* gene, which encodes an enterococcal surface protein, has been associated with colonization and persistence of enterococci in ascending urinary tract infection in mice (16) and with biofilm formation on an abiotic surface (17).

Acquired vancomycin resistance in enterococci depends on the transcription of the *van* operon, leading to the production of the alternative structure D-alanine-D-lactate (D-Ala-D-Lac), instead of the dipeptide D-alanine-D-alanine (D-Ala-D-Ala) found in susceptible bacteria, where D-Ala-D-Lac can be used as a cell wall constituent (18). On the other hand, vancomycin dependence has been associated with mutations in the *ddl* gene, which codes for D-Ala-D-Ala ligase, an enzyme that plays a key role in cell wall biosynthesis (4,8,9,19). In the absence of vancomycin, VDE is unable to synthesize either D-Ala-D-Ala or D-Ala-D-Lac, which could explain the glycopeptide dependence (19). A 1.15-kb fragment corresponding to the *ddl* sequence of *E. faecium van*A, which grows only in the presence of vancomycin (VDEfm-UEL) was cloned and sequenced. The nucleotide sequence of this fragment was deposited in GenBank with the accession No. HQ015715 and showed 98% identity to

Table 1. Point mutations in the *ddl* gene of vancomycin-dependent *Enterococcus faecium*.

Mutation (BM4147 → VDEfm-UEL)	Position	Mechanism	Amino acid change
GGC → AGC	615	Transition	Gly → Ser
- → A	710	Insertion	Frame-shift
TGT → CGT	739	Transition	Cys → Arg
C → -	799	Deletion	Frame-shift
GGA → GTA	910	Transversion	Gly → Val
ACG → CCG	988	Transversion	Thr → Pro

(-) = absent.

the *ddl* gene of *E. faecium* BM4147 (GenBank accession No. U39790). The alignment of the two nucleotide sequences showed several mutations in the *ddl* gene of VDEfm-UEL compared to that of the VRE BM4147 strain, most of them being silent point mutations. The insertion of an adenine at nucleotide position 710 of the *ddl* gene of VDEfm-UEL caused a frame-shift mutation, introducing a premature stop codon at position 733 of the sequence (Table 1) and thereby resulting in an inactive enzyme.

The isolation of an antibiotic-dependent strain alerts us to the importance of the control of healthcare-associated infections. This should be structured as an effective policy controlling the use of antibiotics in association with a laboratory capable of identifying microorganisms that show resistance to and also dependence on antibiotics. This can be reflected in the appropriate management of therapy and early adoption of precautionary measures to prevent the spread of infections by antibiotic resistant/dependent microorganisms.

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