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Vancomycin-Dependent *Enterococcus faecium* Isolated from Stool following Oral Vancomycin Therapy

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The isolation of clinical strains of enterococci requiring vancomycin for growth has only recently been reported. We describe the isolation of *Enterococcus faecium* requiring vancomycin for growth from the stool of a patient who had completed oral vancomycin therapy. Growth of the vancomycin-dependent *E. faecium* was supported by ristocetin and D-alanyl–D-alanine but not by daptomycin, teicoplanin, or D,L-alanine. Spontaneous revertants not requiring vancomycin occurred at a rate of 1 in 10^6. Both the vancomycin-dependent *E. faecium* and the revertant hybridized with a *vanB* gene probe and had identical contour-clamped homogeneous electrophoresis patterns. The majority of revertant colonies were resistant to teicoplanin, suggesting constitutive production of the *vanB* ligase. We believe the vancomycin-dependent *E. faecium* evolved from a vancomycin-resistant, vancomycin-independent *E. faecium* in the presence of high concentrations of vancomycin in the intestine.

The past 5 years have witnessed a dramatic increase in the isolation of vancomycin-resistant enterococci from hospitalized patients (4, 5). Although enterococci have generally been regarded as having limited virulence, their intrinsic resistance to antibiotics and the ease with which they adapt to their environment and acquire resistance to antibiotics provide them with distinct survival advantages over other more susceptible bacterial pathogens. Clinical isolates of enterococci that not only are resistant to high concentrations of vancomycin but actually require vancomycin for growth have recently been described (9, 12, 22). This phenomenon may represent the ultimate in microbial evolution in response to an antimicrobial agent.

We report the isolation and characterization of vancomycin-dependent *Enterococcus faecium* (VDEF) from the stool of a patient who had received oral vancomycin therapy for the treatment of presumed *Clostridium difficile* colitis. The oral use of vancomycin has been associated with the isolation of fecal vancomycin-resistant *E. faecium* (VREF) (20); however, the isolation of *E. faecium* requiring vancomycin for growth from the stool of a patient after oral administration of vancomycin has not been reported.

Case report. An 82-year-old man with fever, cough, and diarrhea was admitted from a nursing home. He had received oral ciprofloxacin and amoxicillin for 10 days prior to admission. A physical examination revealed crackles over the right lower lung fields. A right lower lobe infiltrate was present on a chest radiograph. The stool was positive for occult blood. Empiric treatment with intravenous ceftazidime for pneumonia and oral vancomycin, 125 mg every 6 h, for presumed *C. difficile* colitis was begun. A *C. difficile* toxin assay was requested, but the sample collected was insufficient for testing.

Stool collected from this patient on admission as part of a surveillance study for vancomycin-resistant enterococci (23) grew VREF. After 10 days of oral vancomycin treatment, *E. faecium* (VDEF) that would only grow on media containing vancomycin was recovered from the stool. The *C. difficile* stool toxin assay was negative. After vancomycin was discontinued, three more stool cultures were obtained at 10-day intervals and only VREF not requiring vancomycin for growth was isolated. The patient's pneumonia resolved after 14 days of ceftazidime treatment. He subsequently developed a left upper lobe infiltrate and responded clinically to a 14-day course of intravenous vancomycin and cefotetan. VDEF was not isolated from stool cultures or from cultures of other sites during or after the time he received intravenous vancomycin.

Methods and results. Campylobacter agar (Becton-Dickinson Microbiology Systems, Cockeysville, Md.) containing 10% sheep blood with antibiotic supplements, including 10 μg of vancomycin per ml, was used to selectively recover vancomycin-resistant enterococci from stool or rectal swabs (7). After inoculation, plates were incubated at 42°C in 5% O_2–10% CO_2–85% N_2 (Campy Pouch; Becton-Dickinson). Enterococci were identified to the species level by using a combination of conventional biochemical tests (8) and the API 20S system (Analytab Products, Plainview, N.Y.). The VDEF isolate was alpha-hemolytic and catalase negative and did not grow on subculture to a variety of nutrient broths and solid media, including chocolate and blood agar incubated at 35°C in 5% CO_2, and under anaerobic conditions. The isolate was identified as *E. faecium* with the API 20S system, which does not require additional growth of the organism for identification. There was insufficient growth for identification of the isolate with the Vitek system (bioMerieux Vitek, Inc., Hazelwood, Mo.), even when the inoculum was supplemented with 10 μg of vancomycin per ml.

The growth requirement of the isolate was first suspected when growth was observed around only the 30-μg vancomycin disk on agar diffusion antibiotic susceptibility testing plates (Fig. 1A). The growth of VDEF was more rapid and the colonies were larger when the agar was supplemented with blood. To further identify requirements for growth of VDEF, Mueller-Hinton and blood agars were inoculated with a suspension of organisms adjusted to a 0.5 McFarland turbidity standard. Filter paper disks saturated with a 20-μl volume containing 10

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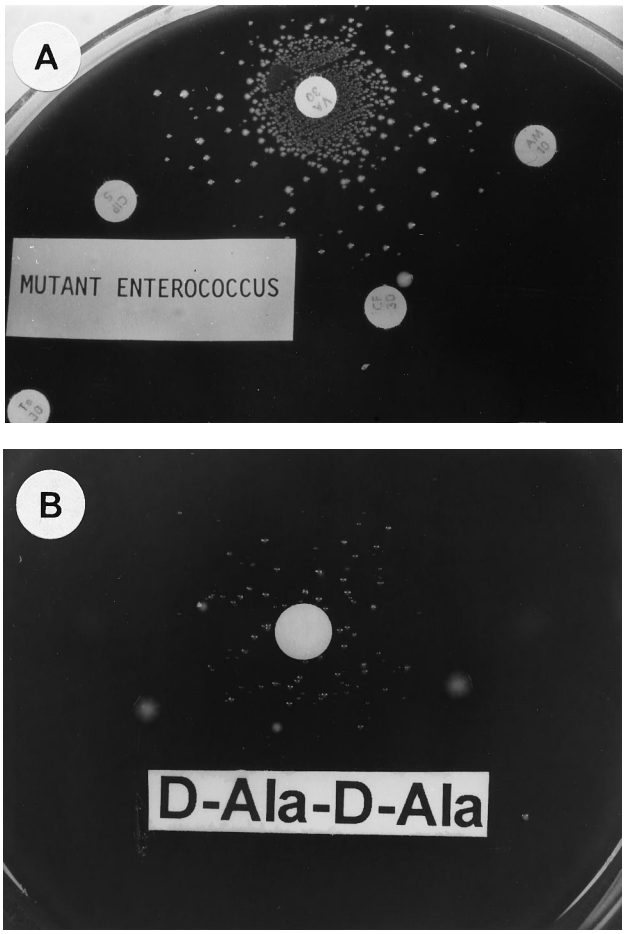

FIG. 1. Growth dependence of *E. faecium* isolate. (A) Growth around a 30-μg vancomycin disk on Mueller-Hinton agar supplemented with blood. A large spontaneous revertant colony not requiring vancomycin for growth is shown adjacent to the cephalothin (CF) disk. (B) Growth around a disk containing 1,000 μg of D-alanyl–D-alanine on blood agar. Growth was less optimal with D-alanyl–D-alanine than with vancomycin. Large spontaneous revertant colonies not requiring D-alanyl–D-alanine for growth can be seen away from the disk.

μg of vancomycin, ristocetin, teicoplanin, or daptomycin and concentrations ranging from 10 to 10,000 μg/ml of D-alanyl–D-alanine and D,L-alanine were applied to inoculated plates. The plates were incubated aerobically at 35°C and observed for growth. To determine concentrations supporting growth, vancomycin, ristocetin, teicoplanin, and daptomycin were diluted in Mueller-Hinton broth and twofold serial dilutions were made in 96-well microtiter trays. Concentrations of vancomycin tested ranged from 0.001 to 50,000 μg/ml, and those of ristocetin, teicoplanin, and daptomycin ranged from 0.125 to 64 μg/ml. The VDEF isolate was suspended in Mueller-Hinton broth, and the trays were inoculated to a final concentration of 5×10^5 CFU/ml. The trays were incubated aerobically at 35°C and observed for visible growth for 72 h. Agar dilution to verify vancomycin growth requirements was also performed with Mueller-Hinton agar with added vancomycin (concentration range, 0.001 to 10 μg/ml).

The VDEF isolate required a minimum of 0.125 μg of vancomycin per ml to support growth and was able to grow in concentrations of vancomycin of up to 50,000 μg/ml. Growth was also supported by the glycopeptide antibiotic ristocetin and by the dipeptide D-alanyl–D-alanine (Fig. 1B). Growth was not supported by the glycopeptide antibiotic teicoplanin, the lipopeptide antibiotic daptomycin, or D,L-alanine.

The rate at which spontaneous revertant colonies not requiring vancomycin for growth appeared was determined by plating serial dilutions of a VDEF suspension adjusted to a McFarland 0.5 standard in triplicate to Mueller-Hinton agar with and without vancomycin (10 μg/ml). The number of revertant colonies appearing on the media lacking vancomycin was compared with the total number of colonies growing on the vancomycin-containing agar. Spontaneous revertant colonies not requiring vancomycin for growth occurred at a rate of 1 in 10^6. The revertant strain was identified as *E. faecium* both by conventional biochemical tests and with the Vitek system (bioMerieux Vitek, Inc.). The Vitek system identification matched that for the initial VREF stool isolate.

Agar disk diffusion (18) and microdilution MIC methods (17) were used for susceptibility testing of VDEF and the spontaneous revertant isolate. For the VDEF isolate, 10 μg of vancomycin per ml was added to all agar and broth media. Brain heart infusion broth was used to screen for high-level gentamicin and streptomycin resistance (17). The susceptibility of the revertant strain was identical to that of the vancomycin-dependent strain regardless of the presence or absence of vancomycin during testing. Both VDEF and the revertant were β-lactamase negative (Cefinase disks; Becton-Dickinson) and were susceptible by agar disk diffusion testing to tetracycline, chloramphenicol, rifampin, nitrofurantoin, and the oral streptogramin pristinamycin (Rhône-Poulenc Rorer, Collegeville, Pa.). Both were resistant to penicillin G, ampicillin, erythromycin, clindamycin, and ciprofloxacin. VDEF and the spontaneous revertant were susceptible to the investigational streptogramin antibiotic combination quinupristin-dalfopristin (RP 59500) (Rhône-Poulenc Rorer), which had an MIC of 1 μg/ml. The MIC of gentamicin was >500 μg/ml and that of streptomycin was >1,000 μg/ml for both VDEF and the revertant. The MIC of vancomycin for the revertant strain was >256 μg/ml. Twenty-five spontaneous revertant colonies were selected for microdilution susceptibility testing of teicoplanin with media not supplemented with vancomycin; 20 of these were resistant to teicoplanin, with MICs of teicoplanin of ≥16 μg/ml, and 5 were susceptible, with MICs of the antibiotic of ≤0.5 μg/ml.

Genomic DNA from both VDEF and the spontaneous revertant was purified by a previously published method (14) after overnight growth in brain heart infusion broth with 10 μg of vancomycin per ml. Extracted DNA was digested overnight with *Eco*RI according to the manufacturer's recommendations (Gibco BRL, Bethesda, Md.). Restriction-digested DNA was separated on 0.8% agarose gels by electrophoresis and transferred to nylon membranes with a vacuum blotting apparatus. DNA hybridization with digoxigenin-labeled *vanA* and *vanB* gene probes was performed as described previously (14). The *vanA* probe used was a 3.2-kb fragment of pHKK100 (14). The *vanB* probe used was a 0.63-kb fragment derived from *Enterococcus faecalis* SF300 (a gift from Robert C. Moellering, Jr.) (11). Genomic DNA was prepared for contour-clamped homogeneous electric field electrophoresis and digested with *Sma*I (Gibco BRL) by methods previously reported (6). Restriction-digested DNA was separated on a 1.0% agarose gel in a contour-clamped homogeneous electric field apparatus (CHEF-DR II; Bio-Rad, Richmond, Calif.). The pulse time was linearly ramped from 5 to 35 s at 200 V for 26 h at 4°C. DNA in the gel was visualized by ethidium bromide staining. *Eco*RI digests of the vancomycin-dependent isolate and the revertant hybridized with a *vanB* probe but not with a *vanA*

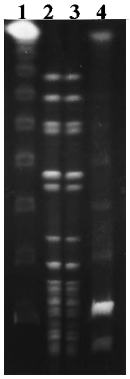

FIG. 2. Contour-clamped homogeneous field electrophoresis of *Sma*I-digested chromosomal DNA of VREF (lane 2) and the spontaneous vancomycin-independent revertant (lane 3). Lanes 1 and 4 contain lambda concatemer molecular size markers in multiples of 48.5 kb.

probe. *Sma*I restriction enzyme patterns by contour-clamped homogeneous electric field electrophoresis of VDEF and the spontaneous revertant digested with *Sma*I were identical, as is shown in Fig. 2.

Discussion. The gastrointestinal tract is a complex environment well suited to enterococci and serves as the main reservoir for these organisms. They may be present there in concentrations of 10^6 to 10^7/g of feces (16, 21). Vancomycin is poorly absorbed from the gastrointestinal tract after oral administration, and concentrations can exceed 30 mg/g of feces at doses of 2 g daily (10). It is not surprising that this environment promotes the selection of organisms resistant to vancomycin. The initial VREF stool isolate from our patient had the same biochemical reactions and antibiotic susceptibility patterns as VDEF and the revertant, and it is reasonable to suspect that the VDEF isolate arose from it in the presence of vancomycin.

Fraimow and colleagues (9) reported the isolation of *E. faecalis* dependent on vancomycin for growth from the urine of a patient receiving intravenous vancomycin. Green et al. (12) described the isolation of VDEF from the blood of a child treated with intravenous vancomycin. Vancomycin-dependent isolates in these reports were of the *vanB* genotype, as was our isolate. Rosato and coworkers (19) also recently described the selection of a vancomycin-dependent mutant from a clinical isolate of a VanA-type *Enterococcus avium*. In each of these cases, investigators proposed that vancomycin-dependent enterococci might lack a functional D-alanine–D-alanine ligase. Vancomycin induction of the *vanA* or *vanB* ligase would compensate for the absence of the native ligase by producing the depsipeptide D-alanyl–D-lactate, allowing for cell wall precursor synthesis (13). Since these ligases are only induced in the presence of vancomycin, the organism cannot grow in the absence of this antibiotic unless it reverts to the vancomycin-independent form. This mechanism might also explain the vancomycin dependency we observed with our *E. faecium* isolate.

Our studies of the growth requirements of the VDEF strain are consistent with those of Fraimow et al. (9) and further support the proposed mechanism for vancomycin dependency. Growth was supported by compounds that either induced the production of the *vanB* ligase or could be substituted for the products of the missing native ligase. For example, ristocetin, a glycopeptide closely related structurally to vancomycin, most likely induces the production of the *vanB* ligase. In contrast, the dipeptide D-alanyl–D-alanine is likely to be incorporated into peptidoglycan precursors substituting for the D-alanyl–D-lactate made by the *vanB* ligase.

Although VanB strains of enterococci were originally described as those with moderate-level resistance to vancomycin and susceptibility to teicoplanin, it is now apparent that the *vanB* gene cluster can confer a wide range of vancomycin and teicoplanin resistance (1–3, 15). Both teicoplanin-resistant and -susceptible colonies were present among the revertants of our *E. faecium* strain. However, the majority of colonies tested were resistant to teicoplanin, suggesting constitutive production of the *vanB* ligase.

The true prevalence of vancomycin-dependent strains of enterococci is unknown. In fact, the recent flurry of reports (9, 12, 22) suggests that clinical isolates of vancomycin-dependent enterococci may be increasingly recognized when appropriate culture techniques are used. Although routine screening for vancomycin-dependent enterococci in patients receiving vancomycin is not indicated, it may be warranted for culture-negative patients who have continuing evidence of infection and have previously had positive cultures for vancomycin-resistant enterococci. In these situations, additional specimens should be obtained and cultured on either media containing vancomycin or media with vancomycin disks added (22).

In summary, we describe a fecal VREF that became dependent on vancomycin for growth during oral therapy. Our observations demonstrate the versatility of the enterococcus under the extremes of antibiotic pressure and provide support for recommendations to limit the use of both oral and intravenous vancomycin.

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