Vancomycin-Resistant *Enterococcus faecium*: Catheter Colonization, *esp* Gene, and Decreased Susceptibility to Antibiotics in Biofilm

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To evaluate the molecular characteristics and antibiotic susceptibility in biofilm of vancomycin-resistant *Enterococcus faecium* (VREF) organisms that had caused catheter-related VREF bacteremia (VREF-CRB), we compared 22 isolates causing bacteremia obtained from patients with VREF-CRB with 30 isolates from control patients with gastrointestinal colonization by VREF. Using pulsed-field gel electrophoresis, we identified 17 unique strains among the 22 VREF-CRB isolates and 23 strains among the gastrointestinal isolates. The *esp* gene was detected in 53% (9 of 17) of the VREF-CRB and 61% (14 of 23) of the control strains ($P = 0.6$). VREF-CRB produced heavier biofilm colonization of silicone disks than did control organisms ($P < 0.001$). Daptomycin, minocycline, and quinupristin-dalfopristin were each independently more active than linezolid in reducing biofilm colonization by VREF-CRB ($P < 0.01$), with daptomycin being the most active, followed by minocycline. In conclusion, the *esp* gene in VREF is not associated with heavy biofilm colonization or catheter-related bacteremia. In biofilm, daptomycin and minocycline were the most active antibiotics against VREF, and linezolid was the least active.

Infections caused by vancomycin-resistant *Enterococcus faecium* (VREF) have emerged as a significant problem among hospitalized patients, particularly in those who are critically ill or immunocompromised (8, 10, 22, 26). Furthermore, during the last two decades, the rate of enterococcal bacteremia associated with central venous catheters (CVCs) has progressively increased (12, 18, 27, 38). Several reports have described the management of VREF bacteremia, including cases with catheter-related VREF bacteremias (VREF-CRB) (17, 20).

Microbial organisms causing catheter-related bacteremias colonize the indwelling catheter by producing biofilm (7). By embedding themselves in the biofilm layer, they become less susceptible to the antimicrobial activity of antibiotics (9, 14, 29, 40). The *esp* gene has been associated with the ability of *Enterococcus faecalis* to form biofilm and adhere to plastic surfaces (42, 44). In this study, we investigated the molecular profile of VREF organisms that had caused catheter-related bloodstream infections, the presence of the *esp* gene in the organisms, the organisms' abilities to produce and colonize biofilm, and the organisms' susceptibilities to antibiotics in the biofilm environment.

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MATERIALS AND METHODS

Molecular profile and analysis of *esp* gene. Molecular typing was performed by pulsed-field gel electrophoresis (PFGE) on all 22 isolates that caused VREF-CRB as well as the 30 control isolates associated with gastrointestinal colonization as previously described (24). Isolates were considered to represent the same strain and were designated as genetically indistinguishable if their restriction patterns had the same number of bands and the corresponding bands were of the same apparent size, whereas isolates were considered closely related if they differed by up to three bands (43). To confirm the identification of VREF, all isolates, obtained from the clinical microbiology laboratory at the University of Texas M. D. Anderson Cancer Center, were inoculated onto Enterococcosel agar (Becton Dickinson, Sparks, Maryland) supplemented with vancomycin (6 μg/ml). Plates were examined after 48 h of incubation at 37°C. All isolates of VRE were identified to the species level as *E. faecium*. Confirmations, as well as determinations of the presence of the *esp* gene, were obtained through colony hybridization (40).

Colony lysates containing denatured VREF genomic DNA were prepared, and colony hybridization was carried out under highly stringent conditions, as previously described (39). VREF isolates on filters (that had been previously identified by other schemes) were reconfirmed by hybridizing with the *E. faecium*-specific probe aac(6′)-Ii (6). To determine the presence of the *esp* gene in VREF isolates, an intragenic fragment of *esp* was amplified using EspF (5′-TTG CTA ATG CTA GTC CAC GAC C-3′) and EspR (5′-GCG TCA ACA CTT GCA TTG CCG A-3′) primers by PCR and verified by sequencing and used as probes for colony hybridization.

Definition. VREF-CRB was defined, as per the guidelines for management of intravascular catheter-related infections by the Infectious Diseases Society of America, as the isolation of at least one blood culture positive for VREF obtained from a peripheral vein of a patient with clinical manifestations of infection (such as fever, chills, or hypotension) and no apparent source for the bacteremia except the catheter (19). In addition, the Infectious Diseases Society of America guidelines required one of the following: a positive catheter tip culture by a semiquantitative catheter culture technique with ≥15 CFU per catheter segment or a positive quantitative culture ($\geq 10^2$ CFU per catheter segment) whereby the same organism is isolated from the catheter segment and peripheral blood sample or simultaneous quantitative blood cultures whereby the number of colonies isolated from the blood culture drawn through the CVC is at least fivefold higher than the one drawn from the peripheral vein. Control organisms consisted of VREF isolates that caused only gastrointestinal coloni-

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TABLE 1. Presence of *esp* gene on VREF strains associated with catheter-related bloodstream infections or gastrointestinal colonization

Source	No. of strains	Ratio (%) of strains with Esp	*P* value
Bloodstream (catheter-related)	17	9/17 (53)	0.6
Gastrointestinal colonization	23	14/23 (61)	

zation and were isolated by rectal swab within a month of the isolation of the organisms that had caused VRE-CRB.

Bioprosthetic colonization. The ability of VREF organisms to cause VREF-CRB and gastrointestinal colonization and to bioprosthetically colonize a catheter in biofilm was determined by a modification of a method previously described by Kuhn et al. (15). A sterile silicone disk was placed in each well of a flat-bottomed 24-well culture plate. One milliliter of pooled human plasma was added to each well, and the plates were incubated in a shaker for 24 h at 37°C. The plasma was then removed from the wells, leaving the silicone disks. Four or five uniform colonies of each tested isolate were used to inoculate 50 ml of Mueller-Hinton broth. This was done for each of the 22 VREF-CRB and 30 gastrointestinal colonizing organisms. One milliliter of inoculum was added to the wells containing the silicone disks, and the wells were incubated in a shaker at 37°C for 24 h. Each organism was tested in at least three disks. The inoculated Mueller-Hinton broth was then removed and replaced with 1 ml of 0.9% saline, and the wells were shaken in an incubator for 30 min. The saline was discarded to remove any planktonic organisms. Gross biofilm was visually observed as a uniform layer of bacteria on the silicone disks. The disks were then placed in 15-ml polystyrene tubes containing 5 ml of 0.9% saline. The tubes were sonicated for 15 min and vortexed for 30 seconds. The spiral plate method was used to plate the cultures, followed by a 24-hour incubation at 37°C. The number of colonies per milliliter was read using a plate scanner.

Antibiotic activity in biofilm. Silicone disks were colonized by the 22 VREF-CRB organisms as described above. After a 30-min rinse in saline to remove planktonic organisms, the silicone disks were placed into new wells containing 1 ml of water (control) or one of the following drugs diluted to a 2-mg/ml concentration in water: quinupristin-dalfopristin (Q-D), minocycline, linezolid, or daptomycin (supplemented to a physiologic level of 50 mg/liter Ca^{2+}). The disks were then incubated in a shaker incubator for 24 h at 37°C. A total of 660 disks were used in evaluating the activity of antibiotics in biofilm, while six disks were used in testing the susceptibility of one VREF-CRB isolate in biofilm to a particular antibiotic or water, resulting in a total of 132 disks used per antibiotic or control (water). Because linezolid and Q-D are given intravenously through the catheter at a concentration of 2 mg/ml (2,000 mg/liter), we used all four antibiotics at this concentration to determine the ability of these drugs to suppress organisms in biofilm when given through the lumen of the catheter. The disks were then placed in 15-ml polystyrene tubes containing 5 ml of 0.9% saline. The tubes were sonicated for 15 min and vortexed for 30 seconds. Of these 5 ml, 100 μl was plated onto Trypticase soy agar blood plates. This was followed by a 24-hour incubation period at 37°C. Colonies were then counted and multiplied by 50 to account for the dilution factor. Colonies were counted up to 100, with the maximum count for any plate being 5,000, and reported in terms of the mean plus or minus standard error of the mean.

Susceptibility testing method. Susceptibility testing was performed in duplicate using microtiter broth dilution methods in accordance with the guidelines established by the National Committee for Clinical Laboratory Standards (25). Briefly, organisms were inoculated into broth and were incubated overnight at

37°C. Appropriate dilutions were made so that the final inoculum tested was 5×10^5 CFU/ml. The test medium used was cation-adjusted Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) for all organisms tested. Antibiotic concentrations were prepared manually, with serial twofold dilutions ranging from 128.0 to 0.06 μg/ml and dispensed automatically with an MIC-2000 apparatus (Dynatech Laboratories, Inc., Alexandria, Va.). *Staphylococcus aureus* ATCC 25933, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains to ensure the validity of the results. The MIC was defined as the lowest concentration of each antimicrobial agent that inhibited visible growth after 16 to 20 h of incubation at 35°C.

Statistical analysis. The significance of differences between the number of the colonies in biofilm of VREF-CRB and that of gastrointestinal-colonizing VREF was determined by the use of Student's *t* test or the Wilcoxon rank sum test for continuous variables. The two groups were compared in terms of the presence of the *esp* gene by Fisher's exact test. All *P* values were based on two-tailed tests of significance. A *P* value of ≤0.05 was considered significant. All computations were performed with SPSS software (version 11.00; SPSS, Inc., Chicago, IL).

RESULTS

Molecular profile and Esp expression. Five (23%) of the 22 VREF isolates that had caused catheter-related bacteremia had indistinguishable DNA profiles by PFGE, with two additional CRB-VREF isolates being closely related (a difference in 1 to 3 bands) to these five. Hence, there were 17 unique strains in the VREF-CRB group because five were indistinguishable. The 30 gastrointestinal-colonizing control isolates consisted of 23 unique strains. Of the 17 VREF-CRB strains, only 9 had the *esp* gene relative to 14 of the 23 control gastrointestinal-colonizing strains ($P = 0.6$) (Table 1).

Bioprosthetic catheter colonization. VREF-CRB isolates demonstrated biofilm colonization growth in broth on bioprosthetic surfaces of silicone catheters or disks with a mean concentration (plus or minus standard error of the mean) of 8.8×10^4 ($\pm 1.5 \times 10^4$) CFU per silicone disk (Table 2). The biofilm colonization of silicone disks produced by the gastrointestinal-colonizing organisms was significantly lower than that produced by VREF-CRB organisms; mean plus or minus standard error of the mean was $6.5 \times 10^4 \pm 1.9 \times 10^4$ CFU/silicone disk ($P < 0.001$) (Table 2). The VREF strains that lacked the *esp* gene, regardless of whether they had caused CRB or gastrointestinal colonization, tended to be associated with heavier biofilm colonization than the *esp* gene-positive strains (mean plus or minus standard error of the mean was $9.0 \times 10^4 \pm 2.9 \times 10^4$ versus $2.8 \times 10^4 \pm 3.3 \times 10^3$, $P = 0.07$).

Antibiotic activity in biofilm. The 22 VREF-CRB isolates were highly susceptible in suspension to Q-D, linezolid, daptomycin, and minocycline, with respective MIC_{90}s of 2 mg/liter, 2 mg/liter, 8 mg/liter, and 4 mg/liter (Table 3). Neither linezolid, Q-D, daptomycin, nor minocycline completely inhibited the growth of the VREF-CRB isolates in biofilm (Table 3).

TABLE 2. Distribution of VREF isolates according to clinical source and respective biofilm colonization of silicone disks

Parameter	Value of parameter for VREF isolate from source		*P* value
	Catheter-related bloodstream	Gastrointestinal colonization	
No. of isolates	22	30	
No. of silicone disks tested	94	90	
Biofilm colonization per disk in broth (means ± standard errors of the means)	$8.8 \times 10^4 \pm 1.5 \times 10^4$	$6.5 \times 10^4 \pm 1.9 \times 10^4$	<0.001
No. of disks with ≥5.0×10^4 CFU	64 (68.1%)	21 (23.3%)	<0.001

TABLE 3. Activity of antibiotics or control against 22 VREF isolates causing catheter-related bacteremia in suspension (MIC) and in biofilm

Antibiotic or control	MIC range (mg/liter)	Biofilm (mean CFU per disk ± SEM)[a]
Daptomycin	2.0–8.0	$1.3 \times 10^2 \pm 2.7 \times 10^1$
Minocycline	≥0.06–8.0	$5.6 \times 10^2 \pm 1.2 \times 10^2$
Quinupristin-dalfopristin	≥0.06–2.0	$3.0 \times 10^3 \pm 1.8 \times 10^2$
Linezolid	0.5–2.0	$4.3 \times 10^3 \pm 1.4 \times 10^2$
Control (water)		$5.0 \times 10^3 \pm 0$

[a] Colonization data are after 24 h of exposure to 2,000 mg/liter of antibiotic. All antibiotics significantly reduced biofilm colonization compared with control (P, ≤0.01). Daptomycin was more effective than minocycline (P, <0.001). Minocycline was significantly more effective than quinupristin-dalfopristin (P, <0.01), and quinupristin-dalfopristin was significantly more effective than linezolid (P, <0.01). A total of 660 disks were tested using 6 disks per isolate and a particular antibiotic or water.

The density of biofilm colonization of VREF-CRB organisms exposed to linezolid (2 mg/ml) for 24 h was $4.3 \times 10^3 \pm 1.4 \times 10^2$ CFU per silicone disk and was comparable to that produced by isolates exposed to water ($4.9 \times 10^3 \pm 4.8 \times 10^1$ CFU per silicone disk) (Table 3). On the other hand, Q-D further reduced the growth of VREF-CRB isolates in biofilm to a mean level of $3.0 \times 10^3 \pm 1.8 \times 10^2$ CFU per silicone disk, which was significantly superior to the activity of linezolid ($P <$ 0.001). Daptomycin and minocycline were the most active antibiotics against VREF in biofilm, leading to a significant reduction in the growth of VREF-CRB organisms in biofilm ($1.31 \times 10^2 \pm 2.7 \times 10^1$ and $5.6 \times 10^3 \pm 1.2 \times 10^2$, respectively) (Table 3), and they were significantly superior to Q-D ($P < 0.001$) and linezolid or control/water ($P < 0.001$). However, the activity of daptomycin was superior to that of minocycline ($P < 0.001$).

DISCUSSION

In a recent report, Gray et al. found that 35% of catheter-related bloodstream infections were caused by enterococci (12). In addition, several studies have reported on episodes of VREF-CRB (17, 20, 35). Lai reported six cases of VRE bacteremia, four of which were CVC related, that were resolved upon the removal of the intravascular catheter (16). However, the molecular bioprosthetic characteristics (such as the *esp* gene, biofilm colonization, and susceptibility to active antibiotics in biofilm) that are associated with VREF-CRB have not well been defined.

Gristina (13) showed that adherence of bacteria to catheter surfaces involves cell-to-cell adherence and bacterial aggregation in biofilm. VREF organisms that caused catheter-related bloodstream infections in our study produced heavier biofilm bioprosthetic colonization compared with gastrointestinal-colonizing organisms. It is possible that when organisms with biofilm-forming capabilities exist in the appropriate clinical setting, colonization of the CVC and subsequent VREF bacteremia occur.

Esp is a large enterococcal surface protein consisting of 1,873 amino acids with an N-terminal domain (amino acids 50 to 743). Although the *esp* gene has been suggested as a virulence factor in *E. faecalis* (37), whereby in one study it was detected in infection-derived *E. faecalis* strains but not in less

pathogenic enterococcal species, this issue remains controversial. In contrast, in another study by Shankar et al. (36), the presence of the *esp* gene in *E. faecalis* strains did not influence histopathologic changes associated with acute urinary tract infections but contributed to urinary tract colonization. On the other hand, the *esp* gene was found to be highly conserved in VREF isolates that were associated with hospital outbreaks across three continents and was absent in nonepidemic and animal VREF isolates, suggesting a role in VREF infections (47). More recently, Rice et al. (32) showed that among patients hospitalized in the United States, the *esp* gene was detected in 65% of nonstool VREF isolates, which was comparable to the 78% isolated from stools.

The *esp* gene was associated with the capacity of *E. faecalis* to form biofilm and adhere to polystyrene surfaces (44). More recently, Tendolkar et al. (42) found similar results when they used isogenic mutants, with and without the *esp* gene. Also Mohamed et al. similarly found that *esp* gene presence enhanced the biofilm amount of *E. faecalis* (21). However, unlike what has been reported regarding *E. faecalis*, our data showed that the *esp* gene in VREF was not associated with catheter-related bacteremia or heavy biofilm colonization. In fact, the VREF isolates that lacked the *esp* gene tended to be associated with heavier biofilm colonization than *esp* gene-positive isolates ($P = 0.07$). Further studies, particularly those conducted with isogenic mutants of VREF, are required to further define the role of the *esp* gene as it relates to catheters and biofilm formation.

The biofilm matrix has been defined as a resistance factor (7, 9, 40). In the biofilm environment, organisms that are highly susceptible to antibiotics in suspension become resistant to these same antibiotics. Khardori et al. (14) reported that *Staphylococcus epidermidis* isolated from catheter tips was highly susceptible to vancomycin in homogeneous suspension but became highly tolerant to this same antibiotic in the biofilm environment. Amorena et al. (1) demonstrated that in the biofilm environment, *S. aureus* required antibiotic concentrations of ≥100 mg/ml, which are much higher than the minimal bactericidal concentration. Wilcox et al. showed that vancomycin and linezolid failed to eradicate staphylococci and enterococci in catheter biofilm (46). Similar observations were noted in our study: in homogeneous suspensions, linezolid, Q-D, daptomycin, and minocycline, which have been shown to be active against VREF bacteremia (4, 17, 20, 30), had MICs of ≤8.0 mg/ml against VREF-CRB. However, in biofilm, a 2-mg/ml concentration of these antibiotics failed to completely inhibit the growth of the VREF-CRB organisms. The concentration of 2 mg/ml was used because this is the concentration that is used clinically in infusing linezolid and Q-D intravenously through a vascular catheter. Although the activity of antibiotics tested against VREF-CRB in the biofilm was reduced, some antibiotics were significantly more active than others. Daptomycin was the most active antibiotic against VREF-CRB organisms embedded in biofilm, significantly reducing the density of VREF colonization on silicone disk surfaces by more than 39-fold relative to the control, and was significantly more active than minocycline, Q-D, or linezolid ($P < 0.001$).

Minocycline was significantly more efficient in reducing the density of VREF colonization in biofilm relative to Q-D or

linezolid. Linezolid was the least active. Recent guidelines for the management of intravascular catheter-related infections from the Infectious Diseases Society of America have drawn attention to the role of antibiotic catheter lock solution containing antibiotics active against organisms embedded in biofilm (19). Hence, flush solutions that include daptomycin should be considered in patients with VREF catheter-related bacteremias whose catheters cannot be removed if the organisms are susceptible to this agent. Minocycline may also be effective in eradicating staphylococci in biofilm (29).

In addition, antibiotic lock solution consisting of minocline and EDTA was recently found to be highly effective in preventing ongoing catheter-related bloodstream infections in an animal model as well as in clinical studies (3, 5, 28, 31). Daptomycin, on the other hand, was found to be highly active in vitro against *S. epidermidis*, *S. aureus*, and VREF (2, 4, 33) in an experimental model of chronic foreign body infection due to methicillin-resistant *S. aureus* (45), and in treating two bone marrow transplant patients with catheter-associated *Leuconostoc* bacteremia (11). However, daptomycin is dependent on the presence of high concentrations of calcium and should not be used in combination with a calcium-chelating anticoagulant such as disodium EDTA. Dicalcium EDTA may be a more appropriate alternative (34).

The limitations of this study include the use of water as control rather than broth; the lack of nutrients may have lowered the number of colonies growing. However, water was used with all antibiotics, and there was still a difference in the colony count. In addition, the PFGE procedure has been shown to be limited in its ability to assess the clonality of VRE (23, 41). The study design is also limited by the small number of strains from a single medical center and by comparing infection isolates and stool isolates. Stool isolates could potentially cause CRB, limiting the ability to detect differences between the two groups. However, the fact that there was a heavier biofilm formation by the isolates causing CRB, despite this limitation, supports the hypothesis that this may be an important factor in the pathogenesis of VRE-CRB.

In conclusion, VREF-CRB organisms were more frequently associated with a particular DNA restriction pattern and produced heavier biofilm bioprosthetic colonization of silicone disks than did control VREF colonizing the gastrointestinal tract. The *esp* gene in VREF was not associated with catheter-related bacteremia or heavy biofilm colonization. All antibiotics active against VREF-CRB in suspension displayed reduced activity in biofilm. Daptomycin was the most active drug against VREF-CRB in biofilm, followed by minocycline. Linezolid was the least active.

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